ECOSYSTEM DYNAMICS AND TEMPORAL VARIATIONS IN A MICROBIALLY DOMINATED, COASTAL ANTARCTIC LAKE

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Ecology and Environmental Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 2009

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of a dissertation submitted by

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This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

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November 2009

ACKNOWLEDGEMENTS

I would like to thank Christine Foreman, my major advisor, for her guidance, support, trust and encouragement, and for enabling me to pursue my work in polar research. I am grateful for the valuable assistance of my committee members, John Priscu, Mark Greenwood, Brian McGlynn and Matthew Fields.

I am thankful for all the incredible people I was fortunate to meet and work with at Montana State University, McMurdo Station Antarctica, and colleagues in the Pony Lake and McMurdo LTER research teams. Thanks for your assistance and critical reviews on my research.

My Mom and Dad, Mathilde and Jürgen, who always supported me and believed in me. It would not have been possible without them. Thank you for opening the door to the world for me. My lady Sarah, for her friendship, advice and inspiration. Thank you for standing next to me for better or for worse. She is a tower of strength. My family and friends for their support and cheerfulness throughout all these years.

This research was funded by the NSF Office of Polar Programs and by the Montana Space Grant Consortium.

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ABSTRACT

This study investigated the microbial ecology of Pony Lake, Antarctica. The main objectives of this research were to 1) characterize physicochemical parameters in Pony Lake during the transition from ice covered to ice free, 2) highlight seasonal and inter-seasonal alterations in lake parameters, 3) relate the physicochemical conditions in the ice and water column to microbial processes and community structure, 4) characterize the effects of phototransformation of dissolved organic matter (DOM) on its bioavailability to bacteria, and 5) demonstrate the role of carotenoid pigments in protecting cells against environmental stresses. Also included are results from three McMurdo Dry Valley lakes on the survivability of microbes encapsulated within the lake ice covers.

The results of this study highlight the sensitivity of Antarctic environments and the close coupling of environmental conditions and biological processes. Inter-seasonal differences in weather conditions and snow accumulation strongly affected the physicochemistry of this lacustrine system. Biological processes were closely tied to the physicochemical lake conditions. As a consequence planktonic abundances, production rates, and community structure evolved distinctly in each year. Bacterial production was regulated by the quality of DOM. Whereas nutrient availability appeared to play a minor role in eutrophic Pony Lake water, it became more critical for microbial communities found within the ice column of Antarctic lakes. Especially in the ice cover of the McMurdo Dry Valley lakes, enhanced microbial diversity and survivability was associated with particles (nutrient enriched micro-zones) within an otherwise ultra-oligotrophic habitat. In Pony Lake ice, microbial activity and community structure differed with respect to different strata within the ice column, induced by freeze-concentration of solutes, bioavailability of DOM, and oxygen gradients. A comparison of carotenoid pigmented and non-pigmented heterotrophic bacteria indicated that pigmentation provides enhanced resistance to environmental stresses such as freeze-thaw cycles or solar radiation. Collectively, the Pony Lake data demonstrated that microorganisms that persist throughout the year were able to survive much more severe conditions while entrapped within the ice compared to those observed in the lake water during summer months. Further, this study contributes to a better understanding of the biogeochemical carbon cycle in a microbially dominated system.

CHAPTER 1

AN OVERVIEW OF MICROBIAL ADAPTATIONS TO THE COLD

Organization of the Dissertation

Chapter 1 is an introduction to microbially dominated ecosystems found in the continental Antarctic, provides a general background to the major physical constraints that limit life in a cold environment, describes physiological adaptation mechanisms evolved in microorganisms to withstand and survive the harsh Antarctic environment, and briefly characterizes the microbial diversity found in icy Antarctic environments. Also included in this chapter is a detailed description of hypotheses and objectives and the broader impacts of this work.

The main part of this dissertation focuses on the microbial ecology of a coastal, eutrophic, brackish lake in Antarctica (Pony Lake, Cape Royds). Chapters are presented as individual manuscripts that have either been submitted, or are in preparation for submission, to scientific journals (Chapter 2, 3, 5, 6, 7); thus repetitions in site description and methodology exist.

Pony Lake periodically transitions from a frozen solid lake during winter to an open water system for several weeks during the austral summer. Chapter 2 and 3 describe these two distinct phases of Pony Lake by addressing physicochemical parameters, microbial processes, and community structure in the ice and in the water column of Pony Lake during the transition from a frozen to open lake system. Chapter 4 includes a supplementary section in which seasonal and interseasonal differences in the

biogeochemistry of Pony Lake are analyzed. Chapter 5 presents the effects of phototransformation of the dissolved organic matter on the bioavailability to bacterioplankton in Pony Lake. The role of carotenoid pigments in heterotrophic bacteria as potential biomolecules to provide enhanced resistance to common Antarctic environmental stresses, such as freeze-thaw cycles and solar radiation exposure, is highlighted in Chapter 6. Pigmented and non-pigmented bacteria subjected to these treatments were isolated from Pony Lake and Cotton Glacier (Transantarctic Mountains north of Cape Roberts). Chapter 7 describes the detection of microorganisms found within the ice cover of the McMurdo Dry Valley lakes using propidium monoazide to differentiate between 'viable' cells and cells with compromised cell membranes. Chapter 8 summarizes the overall findings of this work.

The Appendix section includes additional experiments investigating the response of bacteria to freeze-thaw cycles and solar radiation exposure. Besides Antarctic isolates, the mesophilic strains *Escherichia coli* and *Serratia marcescens* were tested.

Introduction

"Don't forget that the flavors of wine and cheese depend upon
the types of infecting microorganisms."
- Martin H. Fischer

Have you ever asked yourself what would happen if microorganisms did not exist? Our world as we know it would cease. Microbes affect all life by being involved in the global cycle of key elements (hydrogen, carbon, nitrogen, phosphorus, sulfur, and oxygen). Without microorganisms the world would be a 'waste land', with accumulated organic tissue, toxic waste and a poisonous atmosphere. About 3.8 billon years ago the first ancestral microbes arose on this planet (Mojzsis et al., 1996) facing extreme and challenging environmental conditions. Now, billons of years later, certain microorganisms still endure the most extreme conditions imaginable (e.g. highs and lows in temperature, pH, salinity, pressure), yet have managed to adapt, diversify and proliferate. For instance, imagine surviving for 1.5 million years buried under hundreds of feet of Antarctic ice without heat, oxygen, water or sunlight as has been found for microbial assemblages beneath Taylor Glacier, Antarctica (Mikucki et al., 2009). The fact that microorganisms can survive these extreme environments ultimately raises questions about their physiology and diversity.

Most Antarctic ecology is restricted to marine environments and only a limited number of organisms are able to live in the terrestrial, ice free areas. Life becomes more challenging the further south it exists. The maritime Antarctic (Antarctic Peninsula, its offshore islands and the islands south of the maximum extent of the sea ice) is dominated

by cryptogams such as mosses. Also snow algae and macro-fungi are reasonably abundant (Smith, 1984; McGonigal, 2008). Invertebrates such as springtails and free living mites live in soil and among rocks and vegetation, together with nematodes and tardigrades (Block, 1984; McGonigal, 2008). In contrast, near the coast of continental Antarctica, mosses, lichens, algae, and bryophytes can be found, but are generally sparse. Invertebrates are represented by protozoans, platyhelminths, tartigrades, nematodes, rotifers, and crustaceans (McGonigal, 2008). The absence of land-based vertebrates, shrubs and trees and the poor occurrence of a terrestrial flora may lead one to believe that continental Antarctica is a vast, barren polar dessert. Sir Robert F. Scott (1903) wrote about the Dry Valleys, the largest ice free area on the Antarctic continent: "It is certainly a valley of the dead." However, appearances are often deceiving. What is not apparent at first glance is that microscopic life is teeming and diverse in this unique environment. Lakes, streambeds, soils, and ice are colonized and dominated by microbial communities. Microorganisms govern biogeochemical cycles and ecosystem functions. Realizing that the Antarctic continent hosts physiologically diverse and active microbial communities (e.g. Priscu et al., 1998; Glatz et al., 2006; Foreman et al., 2007; Niederberger et al., 2008) changed our perspective about this landscape and opened a new pristine 'laboratory' for studying species biology and dynamics, ecosystem processes and function, survival and adaptation mechanisms, and biogeochemical cycles.

Among the different environmental stresses to microbial communities, the most critical physical factors affecting survivability are water and temperature. Water availability in Antarctica is highly limited, as water mostly exists in a bound state. Except

for the deep, perennially ice covered McMurdo Dry Valley lakes or a few weeks during the austral summer, water is either frozen or bound to other molecules. Both temperature and water moisture levels fluctuate on a daily as well as seasonal basis, imposing physiological stresses on microorganisms year round.

"Adapt or perish, now as ever, is nature's inexorable imperative."

- Herbert G. Wells

Bacterial Adaptations to Water Stress

Physical and related chemical conditions shape microbial habitats and govern their key processes. Water stress can be provoked by water deprivation, water loss, or osmotic effects (a consequence of the presence of dissolved solutes, or due to the formation of ice in the extracellular milieu). Microorganisms possess the ability to respond to fluctuations in highly dynamic aquatic and chemical environments. For instance, the initial response of bacteria to hyper-osmotic stress occurs rapidly (e.g. accumulation of K^+ and glutamate); and allows the cells to maintain positive turgor pressure (Csonka, 1989). This initial response enables the cells to survive until more effective response mechanisms are activated. The second phase of osmo-regulation is triggered by the accumulation of compatible organic osmolytes. Microorganisms rely on osmo-protectant substances if they are available in the extracellular medium. If not, they synthesize metabolites such as trehalose (Csonka, 1989). A second strategy for successfully adapting to the variable and unpredictable hydration conditions in Antarctic environments may be the synthesis of extracellular polymeric substances (EPS). EPS

have a high affinity for water, and can hold up many times their weight in water (Ophir & Gutnick, 1994). EPS may therefore play an important role in maintaining an appropriate humid environment near the outer cell surface (Ophir & Gutnick, 1994). However, findings addressing EPS production as a positive adaptation to cope with alterations in the water content are contradictory. Whereas Ophir & Gutnick (1994) indicate that mucoid strains were significantly more resistant to desiccation than corresponding isogenic non-mucoid mutants, Hartel & Alexander (1986) demonstrated that the EPS production in mucoid strains did not provide increased protection against desiccation and that non-mucoid strains were more resistant. These conflicting results suggest that a variety of other mechanisms must play a role in protecting cells from decreasing external water concentrations.

The mechanisms prokaryotes use to tolerate desiccation are poorly understood (Potts, 1994). Many microbial communities in Antarctica survive anhydrobiotically (e.g. Hawes et al., 1992; Aislabie et al., 2006a; McKnight et al., 2006). Potts (1994) suggested that while some bacteria may survive only for seconds in an air-dried state, others can tolerate desiccation for thousands, perhaps millions, of years. For instance, cyanobacterial mat communities, commonly found in coastal Antarctic landscapes, are associated with sites where they are repeatedly exposed to desiccation. Although successful at colonizing glacier meltwater streams, these cyanobacterial mats are exposed to liquid water for only six to twelve weeks during the austral summer (McKnight et al., 2006). For the rest of the year they are freeze-dried. McKnight et al. (2006) observed that when reactivating a river channel that had not received substantial flow for approximately two decades,

cyanobacteria became abundant within a week. They concluded that these organisms were preserved in a cryobiotic stage. Most likely, due to low water availability and low humidity, bacteria isolated from arid Antarctic soils (Aislabie et al., 2006b), endolithic rock communities (Friedmann, 1982), or from ice cores (Christner et al., 2000) have had to endure and outlast periods of desiccation. Although there is limited knowledge of what happens in the cytoplasm of desiccated cells, it is believed that mechanisms for desiccation tolerance are distinct from those which preserve the integrity of osmotically, and freeze-thaw stressed cells (Potts, 1994). It appears that desiccation tolerance involves a complex array of interactions at the structural, physiological, and molecular levels (Potts, 1994), with disaccharides (e.g.trehalose and sucrose) synthesized to preserve the integrity of membranes and proteins (Potts, 1994).

Bacterial Adaptations to Temperature Stress

Colonizing permanently cold environments requires coping with numerous challenges: reduced enzyme activity; decreased membrane fluidity; altered transport of nutrients and waste products; decreased rates of transcription, translation and cell division; cold-denaturation of proteins; improper protein folding; and intracellular ice formation (D'Amico et al., 2006). Since low temperature has an adverse effect on the physical properties of membranes and enzymes, the maintenance of membrane fluidity and catalytic efficiency at low temperatures are vital requirements. Thus, modifications in membrane and protein structure in response to thermal shifts can be found in cold adapted organisms.

Decreasing temperature typically results in a reduction of membrane fluidity, triggering changes in the lipid composition in order to maintain physical properties and the function of membranes. Membrane modulations at lower growth temperatures are well documented in the literature and include for example, a higher concentration of unsaturated, polyunsaturated and methyl-branched fatty acids, the preferential synthesis of shorter chain length fatty acids, branched chain fatty acids, and/or a high proportion of cis-unsaturated double-bonds (Suutari & Laakso, 1994; Russell, 1997; Chintalapati et al., 2004). Besides changes in the lipid composition, Ray et al. (1994) proposed that psychrophilic microorganisms may have evolved a class of membrane proteins that may play a role in sensing external environmental temperatures due to temperature dependent phosphorylation and dephosphorylation of these membrane proteins. Another important component of membrane stabilization at cold temperatures has been attributed to carotenoid pigments (Chattopadhyay et al., 1997; Jagannadham et al., 2000; Fong et al., 2001). Although the function of these pigments in heterotrophic bacteria is largely unknown, increased carotenoid production was observed when bacteria were grown at low temperatures (Chattopadhyay et al., 1997; Fong et al., 2001). Further, carotenoids have been shown to be associated with membranes where they bind to membrane vesicles (Strand et al., 1997).

Enzymatic reaction rates drop exponentially with decreasing temperatures (D'Amico et al., 2006). An adaptation to cold temperatures requires enzymes that are active under low temperatures. Whereas thermal stability is typically associated with structural rigidity, in cold adaptation all known structural factors and interactions

involved in protein stability indicate that cold enzymes have a highly flexible structure (Feller & Gerday, 1997). This stability-flexibility relationship enhances their ability to undergo conformational changes during catalysis (Feller & Gerday, 1997). The flexibility in enzyme structure might also be responsible for the low stability generally observed in cold adapted proteins (D'Amico et al., 2003).

Other cold temperature adaptations to cellular building blocks can be found in antifreeze proteins (AFPs). Recently, a limited number of bacteria have been described in the literature that posses AFP activity, with the majority isolated from Antarctic environments (Mills, 1999; Yamashita et al 2002; Gilbert et al 2004). Bacterial AFPs are characterized by a lower thermal hysteresis (difference between melting point and freezing point); hence it is believed that the AFPs in bacteria work through freeze-tolerance (Chattopadhyay, 2006). This freeze-tolerance mechanism of AFPs limits the growth of ice crystals at sub-zero temperatures by adsorbtion to the ice surface. However, recent findings have disputed this mechanism. Gilbert et al. (2005) found a Ca^{2+}-dependent AFP in the Antarctic isolate *Marinomonas primoryensis*. This protein caused a freezing point depression over 2°C. Unlike many extracellular bacterial AFPs, the AFP activity for *M. primoryensis* was found in the concentrated supernatant of the cell lysate. Thus, *M. primoryensis* represents the first evidence of an effective freeze-avoidance strategy in a bacterium (Gilbert et al., 2005). Novel findings were revealed from the highly active, intracellular AFP found in the Antarctic strain *Flavobacterium xanthum* that provides both freeze-tolerance and freeze-avoidance (Kawahara et al., 2007). Ice-active substances (proteins and carbohydrates), which are considered to provide freeze-

tolerance, were observed in Antarctic cyanobacterial mat communities dominated by *Nostoc* and *Phormidium* species (Raymond & Fritsen, 2000; 2001). Although our knowledge about AFPs is restricted to a limited number of 'model' organisms, the discovery of antifreeze proteins highlights the complexity of cellular adaptation to cold growth temperatures.

"We don't know for sure how many species there are,
where they can be found or how fast they're disappearing.
It's like having astronomy without knowing where the stars are."
- Edward O. Wilson

Microbial Diversity

In addition to temperature and water stress, other challenges such as radiation, oxidative stress, and/or nutrient availability strongly affect the establishment of microbial communities in Antarctic environments. Despite the hostile environment, microbial life forms have been shown to thrive (e.g. Olson et al., 1998; Paerl & Priscu 1998; Foreman et al., 2007; Mikucki et al., 2009). Among the bacteria that have been detected in icy environments, more than 60% group into six main genera (Fig. 1.1.) (Priscu et al., 2008). Priscu et al. (2008) inferred that these commonly found bacterial genera arise from the selectivity of this harsh environment for specific microbes. In general, bacteria dominate in numbers and present a greater diversity over archaea in polar and other cold environments (Deming, 2002; D'Amico et al., 2006). Surveys of planktonic and mat

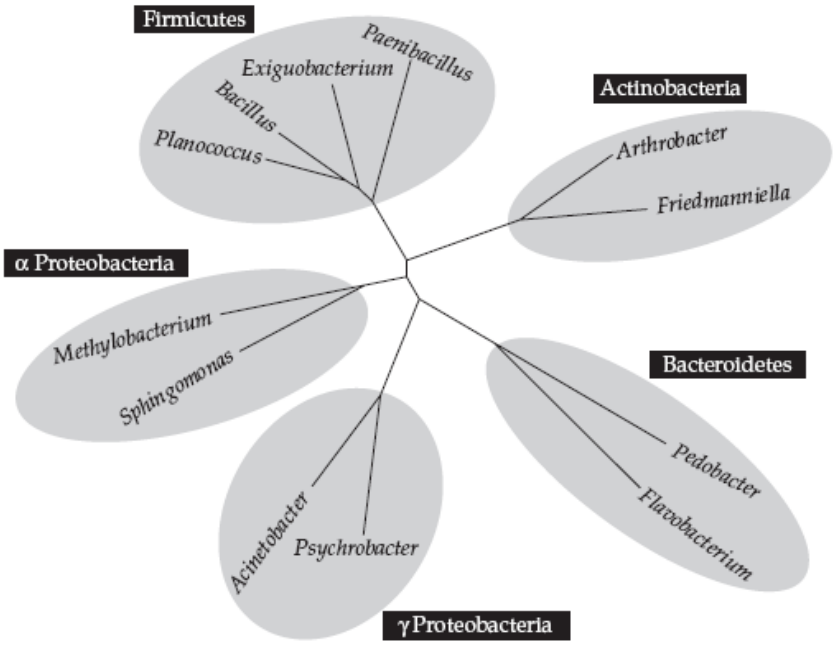

Figure 1.1.: The most frequently recovered genera from glacial ice and subglacial environments based on the phylogeny of the 16S rRNA gene (modified from Priscu et al., 2008).

cyanobacterial communities have identified *Nostoc*, *Phormidium*, and *Oscillatoria* as the dominant cyanobacterial genera in most Antarctic habitats (D'Amico et al., 2006).

Although microbial biomass can be abundant, and sometimes clearly visible in the form of algal blooms or cyanobacterial mat communities, diversity is generally lower than in temperate regions (Wynn-Williams, 1996). Although diversity may be constrained, the metabolic flexibility of these microbial assemblages may be high. Thus, in these environments 'biodiversity is the sum of biological potential' (Wynn-Williams, 1996). Microbial diversity or species richness in coastal, terrestrial Antarctic environments depends upon habitat availability, physicochemical conditions of the habitat (e.g. lakes systems can range from ultra-oligotrophic to hyper-eutrophic, from

freshwater to hyper-saline, from permanently ice covered to frozen solid), biogeographic isolation (speciation), dispersal , successful colonization, and adaptations to cryo-environments.

Microorganisms dominate many Antarctic ecosystems and are of fundamental importance to ecosystem function (Franzmann et al., 1997). Although often referred to as being relatively simple in structure, it has been demonstrated that these ecosystems harbor complex food chains and biogeochemical cycles (e.g. Paerl & Priscu 1998; Priscu et al., 1999). In this context, the autotrophic, microbial community forms the basis of the food web and the transformation of organic carbon by heterotrophic bacteria drives the biogeochemical reactions. Identification and characterization of the prokaryotic diversity is critical for understanding ecosystem processes and the interaction between microbial diversity and ecosystem function. By this means, biodiversity is influenced and controlled by the limiting factors imposed by the ecosystem, but contrarily, diversity itself also controls ecosystem function. Paerl & Priscu (1998) highlighted the importance of this closely linked interaction, spatially as well as temporally coordinated, between producers and consumers to maintain and optimize a life sustaining environment.

Nonetheless, biodiversity assays need to be conducted cautiously. Transitions in physicochemical conditions during the austral summer such as moisture content in soils, mixing events and melt water inputs in lakes may alter population structures significantly; hence, drawing conclusions from ‘snapshot’ taxonomic samples on the ecophysiology of an ecosystem may be misleading and samples collected at different time points may reveal significantly different community profiles.

All in all, species diversity in the Antarctic is regulated by isolation and environmental pressures. However, the increasing human activity in the Antarctic has already left its foot prints and introduced 'alien' species (Wynn-Williams, 1996). Especially we as scientists are obligated to safeguard this unique, isolated, and pristine Antarctic environment and it has to be our highest priority to minimize the risk of contamination.

Broader Impact of this Work

Antarctic environments provide ideal conditions to study the ecological role of microorganisms. The sensitive bottom-up controlled food web structure present in many Antarctic habitats stresses the life supporting role of primary producers and the essential role of heterotrophic bacteria in biogeochemical processes. However, the physiochemical constraints of the Antarctic climate also contribute to the structure and function of these environments. Seasonal and inter-seasonal differences in temperature, radiation, and precipitation can drastically influence the nature of these habitats causing unpredictable alterations in microbial productivity and community structure.

This study explores the seasonal and inter-seasonal variability of physical and chemical parameters in Pony Lake, Antarctica, and relates these changes to biological processes. The alterations in the amount and nature of the organic material are especially of great importance to characterize ecosystem processes. Thus, major changes in organic matter quantity and quality in the lake result from shifts in production, consumption or bioavailability. Dissolved organic matter is a significant component of aquatic systems; it

acts as a critical carbon source for microorganisms, absorbs harmful radiation in sunlight, plays a role in metal complexation, and can participate in important biogeochemical redox reactions. However, little is known about the biogeochemical fate of microbially derived dissolved organic matter, making Pony Lake the ideal site to study the effect of photolytic transformations and microbial processes on the composition and character of the DOM pool. With increasing levels of UV radiation in the southern hemisphere it is crucial that we understand its effects on carbon cycling and on microbial systems that dominate in polar regions.

Further, this study contributes to a better understanding of microbial life in ice and increases our knowledge about possible adaptation mechanisms of microorganisms to environmental stresses.

Hypotheses and Objectives

The overarching hypothesis of this research is that microorganisms inhabiting ice and lake ecosystems in Antarctica are able to survive, thrive, and adapt to permanently changing, extreme environmental conditions. A series of hypotheses are listed below and provide detailed, testable and falsifiable predictions.

H0 1: Microorganisms are unable to persist throughout the year in the periodically frozen Pony Lake due to constant alterations in environmental factors.

Rationale: Coastal ponds and lakes are a pervasive feature in the ice free margins of the Antarctic continent. A distinctive characteristic of these shallow aquatic systems is that they freeze solid to their base during winter. Microorganisms inhabiting these ecosystems are exposed to periodic alterations in osmotic pressure, pH, temperature, light conditions, and nutrient availability. In this context, they face warmest water temperatures, lowest salt concentrations and highest nutrient availability during summer and extreme physicochemical stressors due to freeze concentration processes of dissolved salts during winter freeze-up. If microorganisms that proliferate during the open water period in summer are in fact released from the melting ice column, consequently recolonizing Pony Lake, these microorganisms must be able to endure the hostile environment of being encaptured in ice.

Objectives addressing H1:

- Determine physiochemical conditions present in the ice column
- Characterize microbial community structure using the prokaryotic 16S rRNA gene
- Measure biological parameters such as abundances and productivity of microorganisms in the ice

H0 2: Microbial processes and community structure are unaffected by the physicochemical progression of Pony Lake over the course of the austral summer.

Rationale: Pony Lake is a brackish, eutrophic lake at Cape Royds, Antarctica. During summer months when the lake is partially or completely ice free the lake evolves chemically affected by solar radiation, wind, evaporation, and precipitation. Typically productivity increases following increased solar radiation, temperature and nutrient availability. Acclimatization of microorganisms to this physicochemical progression may cause a shift in the microbial community composition and alter biogeochemical processes.

Objectives addressing H2:

- Investigate physical and chemical parameters of Pony Lake over the course of two austral summer seasons
- Relate biological processes to physicochemical changes

- Phylogenetically characterize the microbial community structure during the transition from a frozen to an ice free lake system using the prokaryotic 16S rRNA gene

H0 3: Photolysis of microbially-derived dissolved organic matter (DOM) from Pony Lake does not change its bioavailability for microbial uptake.

Rationale: Photolytic reactions of organic material in aquatic ecosystems render fractions that are either more bioavailable or more recalcitrant, based on the source and chemical composition. It is believed that autochthonous dissolved organic matter becomes more refractory while allochthonous organic substances are transferred into more labile compounds of lower molecular weight after phototransformation. The organic matter in Pony Lake is algal derived (autochthonous) with microorganisms playing the major role in carbon cycling in this lacustrine system. Thus, changes in the chemical characteristic of Pony Lake due to photolysis may have an enormous effect on the bioavailability of DOM, community structure and carbon cycling.

Objectives addressing H3:

- Demonstrate direct effects of solar radiation on bacterioplankton
- Investigate bioavailability of photochemically altered dissolved organic matter and fulvic acids to bacterioplankton
- Characterize shifts in the microbial community structure related to photochemically altered organic matter.

H0 4: The presence of carotenoid pigments in heterotrophic bacteria does not affect the ability of these organisms to survive freeze-thaw cycles or exposure to solar radiation.

Rationale: Microorganisms that exist in Antarctic environments must be able to withstand numerous environmental stressors such as desiccation, intense solar irradiation while at the surface, freezing, a long period of frozen dormancy, and eventual thawing. The high frequency of pigment production observed in heterotrophic bacteria isolated from cold environments suggests that pigmentation plays a role in adaptation to these extreme environmental conditions.

Objectives addressing H4:

- Demonstrate the effect of carotenoids in heterotrophic bacteria to withstand repeated freeze thaw cycles
- Evaluate the role of carotenoids in heterotrophic bacteria as radiation protectants

H0 5: Microorganisms that are captured within the permanent, ultra-oligotrophic ice cover of the McMurdo Dry Valley lakes are unable to survive.

Rationale: Whereas many Antarctic coastal ponds and lakes annually cycle between an open water period and complete freeze-up, the McMurdo Dry Valley lakes are perennially ice covered. Microorganisms that are found within the ice cover are believed to be of terrestrial origin, associated with soil particles. These soil particles can provide nutrients and since they absorb more heat, they can create seasonally liquid water pockets within the ice. Entrapped within the ice these organisms experience exposure to

high levels of solar radiation while at or near the surface, desiccation, freeze/thaw cycles, and nutrient limitation; all environmental stresses that affect survivability of microorganisms. Thus, the ultra-oligotrophic ice cover of the McMurdo Dry Valley lakes is a hostile habitat for life. If microenvironments of sediment that exist within the ice cover improve habitat suitability, it can be assumed that increased survivability will most likely be associated with these sediment particles.

Objectives addressing H5:

- Detect differences between viable and dead cells isolated from the ice cover of three McMurdo Dry Valley lakes using a novel molecular approach and the dye propidium monoazide
- Demonstrate that increased microbial diversity and survivability is associated with sediment particles

CHAPTER 2

WHEN A HABITAT FREEZES SOLID: MICROBES OVERWINTER WITHIN THE ICE COLUMN OF A COASTAL ANTARCTIC LAKE

Adapted from: Markus Dieser, Rose M. Cory, Diane M. McKnight, Christopher Jaros, Mark Greenwood, Johanna Laybourn-Parry, John T. Lisle, Penney L. Miller, Yu-Ping Chin and Christine M. Foreman (Draft for submission to FEMS Microbiology Ecology)

Abstract

A major impediment to understanding the biology of microorganisms inhabiting Antarctic environments is the logistical constraint of conducting field work only during the austral summer. However, organisms that persist throughout the year encounter severe environmental changes between the summer and winter seasons. In an attempt to bridge this gap we collected ice core samples from Pony Lake, Ross Island. Pony Lake is a small coastal lake that freezes solid during the winter, providing an archive for biological and chemical processes occurring during winter freeze up. In Pony Lake the ice exhibited extreme chemical stratification towards the base, with high concentrations of ions in the bottom cores (e.g. Na >10000 mg L^{-1} and Cl 6253 mg L^{-1}). The ice column was colonized by bacteria and virus like particles. Flagellated algae and ciliates over-wintered in the form of cysts. Bacterial assemblages were metabolically active in the ice melt water (bacterial production ranged from 1.8-37.9 µg C L^{-1} d^{-1}) stimulated by newly produced dissolved organic carbon underneath the ice cover prior to freeze-up. The microbial community composition was strongly affected by oxygen gradients within the

ice column. Our study demonstrates that microorganisms are capable of over-wintering in these extreme environments.

Introduction

Over 100 years have passed since the first scientists set foot on the Antarctic continent during the heroic age of early Antarctic exploration. What they experienced were vast polar deserts and icy landscapes. Sir Robert F. Scott (1903) wrote about the dry valleys, "It is certainly a valley of the dead; even the great glacier which once pushed through it has withered away." Between the early explorations and today, focused research has broadened our understanding about life in the cold. However, our knowledge about the biology of microorganisms is primarily confined to brief periods during the austral summer due to logistical constraints. One way of filling in some of the gaps in the seasonal cycles of Antarctic environments is to examine ice cores from frozen ponds or lakes. In these ice cores, processes that occur during the stages of refreezing are preserved and serve to represent the water column after freeze up, thus allowing for insight into the chemistry and biology of these systems during the winter.

Coastal ponds are commonly found on the margins of the Antarctic continent and are particularly abundant in the ice free areas of the McMurdo Sound region (Armitage & House, 1962; Torii et al., 1988; Broady, 1989). These systems exhibit a wide rage of salinities (from freshwater to saline), ionic compositions, and nutrient concentrations (from oligotrophic to eutrophic). A commonly held distinction between ponds and lakes in polar regions is that ponds freeze solid during winter, whereas lakes do not. The

process of freezing solid causes considerable stress on organisms. Freezing occurs from the top-down when temperatures drop in the fall. The gradual freezing process attenuates light penetration through the ice cover and causes a steady concentration of solutes and particulates in the remaining liquid water column (e.g. Schmidt et al., 1991). During the spring this cycle is reversed with warmer temperatures driving melting of the ice cover and subsequent changes in physicochemical parameters over the course of the austral summer (Healy et al., 2006; Wait et al., 2006). Along with these changes, productivity in Antarctic aquatic ecosystems typically increases throughout the austral summer following the seasonal cycle of increased solar radiation, nutrient availability and temperature (Goldman et al., 1972). Consequently, organisms that persist throughout the year in these systems must be capable of surviving extreme alterations in environmental factors (e.g. osmotic pressure, pH, temperature, etc.) during summer melt and winter freezing.

Previous studies on Antarctic lakes and ponds have primarily focused on plankton community structure or benthic mat communities (e.g. James et al., 1995; Vinocur & Pizarro, 2000; Van Trappen et al., 2002; Jungblut et al., 2005; Glatz et al., 2006); hence little is known about bacterial communities that over-winter in frozen ponds or lakes. However, ice is increasingly being recognized as a suitable habitat for life (Priscu et al., 1998) and complex microbial consortia have been found in a wide range of icy systems including glaciers (e.g. Abyzov, 1993; Christner et al., 2000; Zhang et al., 2001), subglacial environments (e.g. Skidmore et al., 2000; Christner et al., 2008; Lanoil et al., 2009; Mikucki et al., 2009), or super cooled cloud droplets (Sattler et al., 2001). Less than 100 miles from Pony Lake, nutrient enriched microzones embedded within the ice

covers of the McMurdo Dry Valley lakes have been shown to sustain communities that are capable of photosynthesis, nitrogen fixation and decomposition of organic matter (Fritsen & Priscu, 1998; Olson et al., 1998; Paerl & Priscu, 1998; Priscu et al., 1998). Cryoconite holes on dry valley glaciers contain abundant algal and bacterial communities (Porazinska et al., 2004; Foreman et al., 2007). In these icy habitats liquid water inclusions form in response to solar heating during the austral summer providing conditions for biological activity for several days to weeks. However, for most of the year these habitats are frozen solid.

The purpose of this study was to investigate the biogeochemistry of Pony Lake, Antarctica, in early spring 2004 when the lake was still frozen solid to its base. This sampling strategy allowed us to investigate chemical and biological processes that occurred during the period of refreezing in fall and winter, which were preserved within the ice column.

Materials and Methods

Sampling Location

Pony Lake is a small (~120 m long, 70 m wide, and 1-2 m deep), eutrophic lake located at Cape Royds (77° 33’ S, 166° E), Ross Island, Antarctica. The proximity of the lake to McMurdo Sound and the Ross Sea gives the lake its brackish character (5.5 ppt) (Brown et al., 2004). On the western shore of the lake lies an Adelie penguin rookery. Except for a few weeks during mid austral summer the lake is frozen solid to its base. As Pony Lake has no visible inflow, melting of the accumulated snowpack replaces water

lost by sublimation of surface ice and evaporation in mid-summer. The basin is not colonized by higher plants, however algal populations are very abundant (McKnight et al., 1994; Brown et al., 2004). While Pony Lake has been dubbed a lake, it more closely resembles other Antarctic ponds in that it is shallow (max. depth ~2 m) and freezes solid to its base during winter. In fact, the New Zealanders refer to it as Pony Pond, however we will follow the American nomenclature and refer to the system as Pony Lake.

Sampling

Eight ice core samples were collected along a 1.5 m long transect from the outer edge of the pond towards the center in November 2004 using a SIPRE ice auger (10 cm in diameter). Samples were stored frozen in darkened coolers and transported (within 4 hrs) to the Crary laboratory in McMurdo Station, Antarctica. All ice core preparation steps were carried out in a -20°C cold room. Core samples were divided into three sections (top, middle, and bottom), with each segment being ~40 cm long. Dividing the cores into layers provides more information on how chemical conditions change during winter freeze up in the lake and the response of the biological community to these changes. Individual ice core sections were mechanically cleaned by scraping off approximately 1 cm of the outer surface using sterile blades in an environmental chamber (-20°C). Cleaned core sections were transferred into acid rinsed and autoclaved nalgene containers and allowed to thaw at 4°C in the dark.

Water Quality Analyses

Samples were processed according to the protocols of the McMurdo Dry Valleys Long Term Ecological Research Group (Priscu & Wolf, 2000). Dissolved organic carbon (DOC) and total nitrogen (TN) were analyzed on a Shimadzu TOC-V and a Shimadzu TNM-1 analyzer, respectively. For both analyses water samples were filtered in the dark through 25 mm pre-combusted GF/F filters and acidified with 6 N HCl to pH 2 prior to analysis. The filters were used for chlorophyll-*a* analysis and stored frozen until extraction. Chlorophyll-*a* extraction was performed in a 1:1 solution of 90% acetone and DMSO for 12 hrs under dark conditions in the freezer, followed by spectrophotometric analysis using a Turner 10-AU fluorometer. Dissolved inorganic carbon (DIC) was measured using an infrared gas analyzer on chloroform acidified samples. 25 mm pre-combusted GF/F filters were used to filter ice-melt water for macronutrient analysis. The filtrate was stored frozen until the nutrients were detected on a Lachatt autoanalyzer. Anion and cation samples were pre-filtered through 0.4 µm 47 mm nucleopore filters and the filtrate was analyzed on a Dionex DX-300 ion chromatography system. The degree of dilution required for this analysis ranged from no dilution to 1:100 (for Na in the bottom ice segment melt water). Results have not been corrected for different densities of solutions that might have occurred due to the cryo-concentration. Ion concentrations in Pony Lake, especially in the bottom ice core sections, may therefore be underestimated.

DOM Characterization

Ice-core samples were filtered for dissolved organic matter (DOM) characterization immediately following thawing through pre-combusted glass fiber filters (0.7 μm GF/F, Whatman). From this filtered water, a split was removed for DOC concentration, which was stored in pre-combusted amber glass vials at 4ºC until analysis. Absorption measurements were made against Nanopure water blanks using a Shimadzu spectrofluorometer. Fluorescence spectroscopy has been used extensively as a tool to characterize the sources and processes controlling DOM chemistry in many systems (e.g. Cory & McKnight, 2005), and was thus used here to characterize the fluorescent fraction of DOM (FDOM) in Pony Lake ice cores. Excitation-emission matrices (EEMs) were measured on all filtered water samples with a Fluoromax-3 fluorometer (Horiba, Jobin-Yvon). EEM collection and post-processing were completed as described in detail previously (Fulton et al., 2004; Cory & McKnight, 2005). All thawed ice-core samples were diluted five to 20-fold prior to EEM analysis to an optical density of less than 0.05 (1 cm cell) at 240 nm (Lakowicz, 1999) in order to minimize the inner-filter error in the EEM analysis. The fluorescence index (FI; McKnight et al., 2001) was calculated from the corrected EEM as the ratio of emission intensity at 470 nm over the emission intensity at 520 nm at an excitation wavelength of 370 nm (Cory & McKnight, 2005). Standard deviations of FI values analyzed in triplicate for select samples on the Fluoromax-3 were 0.01 at most.

Ice core samples represented roughly 40% of a dataset of 266 EEMs collected from Pony Lake during the 2004/2005 season. The remainder of the EEM dataset

included Pony Lake water samples collected after melt occurred (50%), as well as EEMs of the isolated fulvic acid fraction of Pony Lake DOM (10%). The dataset of 266 EEMs was analyzed by parallel factor analysis (PARAFAC), a statistical modeling approach which separates a dataset of EEMs into mathematically and chemically independent components (each representing a single fluorophore or a group of strongly co-varying fluorophores; Stedmon et al., 2003), as part of a related study to understand processes controlling Pony Lake DOM (Geurard et al., in preparation). A four component PARAFAC model was generated and validated following the four-way split-half analysis procedures in Stedmon & Bro (2008) using the DOM Fluor Toolbox in Matlab V 7.0. The model explained 99.9% of the variation within the dataset, with variation explained by each component decreasing in order from component one to component four.

Productivity Measurements

Heterotrophic bacterial productivity (BP) was estimated by 3H-thymidine incorporation (20 nM final conc.) into DNA as outlined by Takacs & Priscu (1998). Five ^{3}H-thymidine assays and triplicate formalin killed controls (5% final concentration, 30 min prior to ^{3}H-thymidine addition) for each ice core section were incubated with the radioactive compound at 4°C for 20 hrs in the dark. Disintegrations were detected in a liquid scintillation counter (Beckman LS 7200). Conversion factors of 2.0 x 10^{18} cells mol^{-1} TdR (Duckow & Carlson, 1992) and a cell-to-carbon conversion factor of 11 fg C $cell^{-1}$ (Kepner et al., 1998) were used to convert the thymidine incorporation rates into bacterial production rates.

Primary production (PPR) was measured by ^{14}C-carbonate/bicarbonate incorporation (114.4 µCi ml^{-1}, pH ~9.5; ICN/MP Biodmedicals) using the protocol of Lizotte et al. (1996). 100 ml meltwater aliquots were dispensed into clear quartz bottles (x4) for the light assays and into amber bottles (x2) wrapped with aluminum foil for the dark controls for each core section. Bottles were incubated at 4°C in a light incubator for 24 hrs. After incubation, samples were filtered through pre-combusted 25 mm GF/F filters in the dark. The filters were then transferred into 20 ml scintillation vials, acidified with 500 µl 3M HCl, and dried prior to analysis using a liquid scintillation counter (Beckman LS 7200).

Community Structure Analyses

Abundance: Ice core melt water from each section was preserved with formalin (2% final concentration). Bacteria were filtered onto 25 mm 0.2 µm black polycarbonate filters with a 0.45 µm nitrocellulose backing filter under gentle vacuum and stained with 500 µl of a 25X SYBR Gold solution for 15 min as described by Lisle & Priscu (2004). Glassware was baked (450°C) and solutions were filter sterilized (0.2 µm) prior to usage to reduce possible contamination. More than 400 cells per sample were counted in randomly selected fields using a Zeiss Axioskop epifluorescence microscope with a final magnification of 1000X.

Sterile 125 ml screw cap flasks were filled with ice melt water for the analysis of bacteriophage or virus like particles (VLP). Samples were flash frozen in liquid nitrogen and stored at -80°C until further processing, as recommended by Wen et al. (2004).

Following this protocol samples were thawed in the dark at room temperature overnight and then pre-filtered through 0.20 µm filters in order to remove bacteria. The filtrate was then filtered through a 0.02 µm filter to collect the VLP, and stained with SYBR Gold as detailed in Lisle & Priscu (2004). VLP were enumerated using an Olympus BX51 epifluorescent microscope.

1 L samples for algal and protozoan counts were fixed with Lugol's iodine (10 ml) and concentrated by settling for one week. Concentrated samples were transferred into 60 ml amber Nalgene bottles and shipped to the United Kingdom. Subsamples from the concentrated stock were collected and algal and protozoan abundances enumerated. Counts were conducted in a Sedgewick-Rafter counting chamber using phase microscopy with a magnification of 320X (Laybourn-Parry & Marshall, 2003).

Environmental DNA Extraction and DGGE: Ice meltwater samples (70-100 ml) for DNA extraction and downstream phylogenetic analysis were collected on 47 mm Supor®-200 0.2 µm pore size, sterile membrane filters under low pressure (<7 psi). Filters were transferred to cryovials containing TES buffer (100 mM Tris, 100 mM EDTA and 2% SDS), flash-frozen in liquid nitrogen, and stored at -80°C.

DNA was extracted from the filters using an Ultra Clean Soil DNA Kit (MoBio). Primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 534R (5'-AATACCGCGGCTGCTGG-3') were used to amplify a portion of the prokaryotic 16S rRNA gene (Muyzer et al., 1996). A 40 base pair GC clamp was added to the 5' end of the 341F primer (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG).

The amplification protocol included a hot start (94°C for 4 min) and a touchdown program. The touchdown program began with an initial annealing temperature of 65°C and decreased by 1°C every cycle for 8 cycles, followed by 17 cycles at 58°C, and a final elongation step for 10 min at 72°C. Each 50 µl PCR reaction contained 1.5 µl of environmental DNA extract, $MgCl_2$ buffer (final conc. 1X), Taq Master (final conc. 1X), PCR nucleotide mix (final conc. 800 µM), and Taq DNA polymerase (final conc. 0.025 u μl^{-1}) (all components from 5 Prime, Eppendorf), upstream and downstream primer (final conc. 0.5 µM), and nuclease free water (Promega). An automated thermal cycler (Mastercycler ep, Eppendorf) was used for PCR amplifications. Denaturing gradient gel electrophoresis (DGGE) was carried out on a BioRad D CodeTM system as outlined by Murray et al. (1996). PCR amplicons were loaded onto 8-12 % polyacrylamide gels exhibiting a 40-70% denaturing gradient. The gels were placed in a 1X TAE buffer at 60°C and ran for 17 hrs at 60 V. After staining with SYBR Gold (Invitrogen) for 15 min the gels were imaged with an Alpha Innotech FluorChemTM 8800 system.

<u>16S rRNA Clone Library:</u> The two universal 16S rRNA primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used for amplification of the 16S rRNA gene (Stackebrandt & Liesack, 1993) from the individual ice core sections [top (TC); middle (MC); and bottom (BC)]. PCR products were cloned into pCR®2.1-TOPO vectors (TOPO TA cloning kit, Invitrogen). A total of 70 white colonies for each core section were transferred onto LB agar plates and LB broth both containing 50 µg ml^{-1} kanamycin and incubated at 37°C overnight. Clones

were screened with DGGE (Burr et al., 2006) using primers 341F plus GC clamp and 534R. Clones were sent to Functional Bioscience, Inc. on LB agar plates for high throughput DNA preparation and DNA sequencing using primer M13F (20). Nucleotide sequences were edited using Sequencher 4.5 (Gene Code Corporation). The nucleotide sequences were compared to the NCBI nucleotide database using the BLAST search tool (BLASTN 2.2.21, ncbi.nlm.nih.gov/BLAST/, Zhang et al., 2000).

Statistical Analysis

A Bray-Curtis distance measure (Czekanowski, 1913; Bray & Curtis, 1957) was applied to assess differences in the patterns of presence/absence of clones between the different core sections by dividing the sum of unshared clones present by the total number of clones present. Considering the 3 different ice core sections, two possibilities (present or absent) and excluding the clones that were not present at any depth, 2^3-1=7 different patterns for each core section were possible. To compare the patterns of the presence/absence of clones a cluster analysis across the cores was performed. The results from a complete linkage amalgamation were used to join the clusters, which is based on the greatest distance between any two objects.

Results and Discussion

Water Quality Characterization

Chemical parameters and major ion analyses for Pony Lake ice core sections are shown in Table 2.1. The most abundant ions in the ice cores were Na, Cl, and SO_4. The top of the ice columns were dominated by Cl ions, although Na and SO_4 concentrations were also high. Towards the base of the lake chemical stratification was evident and major ion concentrations increased more than 10 fold. Samples for Na analysis were 100-fold diluted and still exceeded the upper detection limit (Na conc. <10000 mg L^{-1}). The bottom of the ice column was dominated by Na ions, followed by nearly equal concentrations of Cl and SO_4. Concentrations of Ca, Mg, and K were consistently low relative to other ions, but increased with depth in the ice profile. Although Pony Lake ice cores demonstrated chemical stratification as a function of depth with highly saline Na and Cl concentrations, a liquid basal brine was not observed. However, the dark yellow color of the bottom ice core section suggests that the brine was incorporated into the ice during the last stage of freezing. In contrast, the surface ice was glass-like and colorless. The bottom ice cores smelled strongly of H_2S, which indicates that reducing conditions were present prior to complete freeze-up.

Inorganic nitrogen and phosphorus concentrations were high in the ice cores (Table 2.1.), representative of the highly eutrophic state of Pony Lake. Total nitrogen (TN) consisted primarily of NH_4 with concentrations increasing with depth (NH_4 conc. top to bottom: 19.81-185.23 mg L^{-1}). Most chemical constituents, including dissolved organic carbon and dissolved inorganic carbon, increased down the cores, with highest

Table 2.1.: Chemical parameters measured in ice cores sections (Top, Middle, Bottom) from Pony Lake in November 2004. Dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), total nitrogen (TN), chlorophyll-*a* (Chla).

	Top	**Middle**	**Bottom**
DOC [mg L^{-1}]	21.84	28.50	135.90
DIC [mg L^{-1}]	7.06	18.60	101.78
TN [mg L^{-1}]	20.44	46.49	185.23
Chla [µg L^{-1}]	59.1	39.7	295.8
Nutrients [mg L^{-1}]			
NH_4^+-N	19.81	46.14	185.23
NO_3^-N	0.63	0.35	<D.L.
NO_2^-N	<D.L.	<D.L.	<D.L.
PO_4^3	5.0	7.2	3.5
Major ions [mg L^{-1}]			
Cl^-	437	1345	6253
SO_4^{2-}	299	817	4643
Na^+	344	-	>D.L.
K^+	28.3	-	39.0
Mg^{2+}	27.3	-	32.9
Ca^{2+}	14.2	-	110.0

(-) not analyzed
(D.L.) detection limit

concentrations, 135.9 mg L^{-1} and 101.78 mg L^{-1} respectively, found in the bottom sections. In contrast, NO_3 concentrations decreased with depth and in the bottom 40 cm NO_3 was below the lowest detection limit (<3 µg L^{-1}). As in summer melt waters (Chapter 3) NO_2 was generally below the detection limit (<3 µg L^{-1}) throughout the entire ice core. Chlorophyll-*a* values were ~6 times higher in the bottom section compared to the top 40 cm of ice.

The ionic composition of the ice cores was dominated by Na, Cl, and SO_4 ions, and reflects the input of marine salts due to the close proximity of Pony Lake to McMurdo Sound. The predominance of these three ions has also been found in other coastal ponds (Schmidt et al., 1991; Wait et al., 2006) or inland ponds (Healy et al.,

2006). In many of these ponds, basal brines have been reported to be present during the final stage of freezing and early melt in late winter (Schmidt et al., 1991; Healy et al., 2006; Wait et al., 2006). When water freezes, ice crystallizes as a more pure matrix, leaving dissolved salts and other minerals in the water column, resulting in a higher salinity brine. Consequently, suspended particles, algae, and dissolved solids are concentrated towards the bottom of the lake. Schmidt et al. (1991) studied two ponds located 100 m from the coast of Cape Evans, which is 7 miles up the coast from Pony Lake at Cape Royds. They reported that the gradual refreezing process was accompanied by a steady concentration of solutes and particulates, which in turn depressed the freezing point of the remaining water. Basal water temperatures of -13°C (Schmidt et al., 1991), -16 to -20°C (Wait et al., 2006), and -15 to -22°C (Healy et al., 2006) were reported. In contrast, Hawes et al. (1999) found little evidence of freezing point depression due to cryo-concentration of solutes. Basal freezing occurred at -4°C. However, conductivity in Brack Pond and Fresh Pond on the McMurdo Ice Shelf (Hawes et al., 1999) did not exceed 60 mS cm^{-1}, which was considerably lower than those reported by Schmidt et al. (1991) in coastal ponds at Cape Evans and Wait et al. (2006) in meltwater ponds on the McMurdo Ice Shelf near Bratina Island, reaching >150 mS cm^{-1} and 101-112 mS cm^{-1}, respectively. These differences in conductivity imply that the ionic concentration of the basal brine plays an important role during freezing. When temperatures dropped below the freezing point of this brine solution, the brine was incorporated into the ice (Healy et al., 2006, Wait et al., 2006) rather than salts being excluded as might be expected.

It is still unclear what triggers the initial melting of the ice in spring. It has been shown that in some ponds although adequate melting points were reached, melting did not necessarily occur (Hawes et al., 1999). One would expect that in ponds with the highest conductivities and decreased freezing points in bottom brine solutions prior to complete freeze up, melt would also be initiated at lower temperature. However, findings are contradictory. Hawes et al. (1999) observed melt in ponds by 16 December (conductivity: <60 mS cm^{-1}), which coincides with findings from Schmidt et al. (1991); however, Schmidt et al. (1991) reported conductivity values >150 mS cm^{-1}. Brines appeared in several other continental and coastal ponds in early October (Healy et al., 2006; Wait et al., 2006). Conductivities in these ponds ranged between 114-148 mS cm^{-1} and 101-112 mS cm^{-1}, respectively. In contrast, Wait et al. (2006) also discovered solid frozen lakes with bottom core conductivities <50 mS cm^{-1} in October near Bratina Island, McMurdo Ice Shelf. In general, Schmidt et al. (1999) suggested that the origin of summer melt water might be derived from melting ice on the edge of the pond draining down the sides into the center. We assume that independent from the conductivity range measured in basal ice cores or brines, year to year differences in air temperature may control the initial melt to a much higher degree. We observed that during the austral summer 2005/06 nearly the entire ice cover of Pony Lake remained throughout the summer, whereas in 2004/05 approximately 80% of the lake became ice free. Since 2005/06 was a colder and more snowy season, the snow cover and the ice column may have insulated and buffered the lake against air temperature changes.

Following the chemical stratification of Cl and Na ions during freeze-up, SO_4 concentration increased more than 15-fold towards the base of Pony Lake (Table 2.1.). Further, the odor of H_2S suggests reduced conditions and has also been noted in ponds where the basal brine was incorporated into the ice and the lake was frozen solid (Wait et al., 2006). In contrast to our findings in Pony Lake, others reported decreasing SO_4 concentrations in basal brines or ice cores in the first few centimeters above the base of the ponds (Schmidt et al., 1991; Healy et al., 2006; Wait et al., 2006). Since we divided Pony Lake ice cores into 40 cm sections, these small scale SO_4 removal processes that may have been evident only at the bottom of the cores would have been missed due to pooling of the bottom 40 cm of ice. Aside from the activity of sulfate reducing bacteria (Schmidt et al., 1991; Wait et al., 2006), precipitation of SO_4-bearing salts is believed to be the major process of SO_4 removal during the final stage of freezing (Marion and Farren, 1999), particularly in ponds where H_2S formation was not detected (Healy et al., 2006). Considering biological processes and precipitation effects that may occur in the few centimeters above the sediment, SO_4 concentrations at the base of the Pony Lake ice cores would have been lower than the average concentration over the entire bottom core segment.

The above mentioned indicators of reducing conditions in the bottom core section could also explain the elevated NH_4 concentrations and decreasing NO_3 levels towards the base of Pony Lake (Table 2.1.). Prior to the formation of a reduced basal water body, high levels of microbial decomposition could have also contributed to the increased NH_4 concentrations. In general, an anoxic regime allows for the upward diffusion of NH_4 from

the sediments (Ellis-Evans, 1990). Further, microbial processes such as denitrification and dissimilatory nitrate reduction have to be considered. Denitrification can occur when oxygen is depleted and bacteria respire nitrate as a substitute terminal electron acceptor. On the other hand, dissimilatory nitrate reduction reduces nitrate to ammonium. Under microoxic and anaerobic conditions both processes could explain the depleted NO_3 concentrations in the ice core section compared to lake water samples (Chapter 3) and the latter as well as microbial decomposition of organic material in an oxygenated environment could contribute to increased NH_4 levels.

K and Mg concentrations (Table. 2.1.) were two-fold lower in all ice core sections compared to concentrations measured in Pony Lake melt water during early spring 2004 (Chapter 3). Ca concentrations were slightly enriched relative to spring melt waters (Chapter 3), which is likely a consequence of cryo-concentration. Ca and Mg both depend upon pH and bicarbonate/carbonate concentrations and should follow similar precipitation characteristics. If HCO_3 is readily available, Ca and Mg will precipitate as calcite, dolomite or magnesite (Hardie & Eugster, 1970). In mid summer Pony Lake is alkaline (pH of 8.07-9.77), thus it can be assumed that most of the dissolved inorganic carbon is present as bicarbonate or carbonate at this pH. However, dissolved inorganic carbon concentrations in Pony Lake were much lower compared to HCO_3 concentrations (one particular fraction of DIC) in other Antarctic ponds (Wait et al., 2006) and precipitation of carbonate salts was not observed in these ponds. Consequently, if precipitation would have occurred in Pony Lake it would most likely be in the form of SO_4 minerals, which in turn should lead to a higher degree of SO_4 removal. However,

FREZCHEM model simulations conducted for several Antarctic melt water ponds predict that Ca containing SO_4 minerals such as gypsum ($CaSO_4 \cdot 2H_2O$) would be expected to precipitate earlier during freezing compared to Mg containing SO_4 minerals such as magnesium sulfate ($MgSO_4 \cdot 12H_2O$) (Healy et al., 2006; Wait et al., 2006). A possible explanation of the decreased Mg concentrations in ice cores compared to spring melt water in Pony Lake could be a response to evaporation or ablation during the summer, prior to freezing. The formation of magnesium bearing salts has been observed in concentric zones in ephemeral (dry) pond basins in the McMurdo Dry Valleys (Healy et al., 2006). Therefore, once the ice starts melting in spring precipitated salts at the margin of the pond could re-dissolve, thereby increasing ionic concentrations in the spring melt waters. Similar assumptions can be made for decreased NO_3 concentrations in relation to spring melt water. Aside from the reducing conditions that are believed to exist due to the formation of H_2S, which would also contribute to the decrease in NO_3 and the increase in NH_4 concentrations, NO_3 could have been precipitated in the form of sodium bearing salts at the margin of Pony Lake by evaporation or ablation of pond ice (Healy et al., 2006). Although salt formations on the margin of the lake were noted, salt minerals were not identified.

The characterization of fluorescent dissolved organic matter (DOM) in the ice demonstrates the importance of autochthonous material in the Pony Lake DOM. Fluorescence index (FI, McKnight et al., 2001) values in the core samples ranged from 1.52 to 1.75. These values overlapped well with the FI ranges observed in the McMurdo Dry Valley lakes (Fulton et al., 2004). In general, the fluorescence index of the DOM is

an indicator of the relative importance of allochthonous vs. autochthonous inputs to the bulk DOM pool and varies between <1.30 for strongly terrestrially-influenced DOM to >1.40 when autochthonous inputs into the DOM pool are high (McKnight et al., 2001; Cory & McKnight, 2005).

PARAFAC analysis of the Pony Lake DOM identified four components that are commonly found in freshwater and marine systems studied to date (Table 2.2.): a ubiquitously observed humic component (C1), a component strongly associated with recent microbial production stimulated by DOM substrates (C2), an additional commonly observed humic component (C3) and an amino acid-like component (C4). The humic component C1 accounted for the highest variation in Pony Lake fluorescent DOM, which is a result that has been consistently observed for other sources of DOM across different systems (e.g. Fulton et al., 2004; Murphy et al., 2008). As such, examining the relationship between other components relative to C1 is a useful tool to discriminate between sources and rates of processes controlling DOM components (Stedmon & Markager, 2005a; Murphy et al., 2008). The linear relationship between C2 relative to C1 shows that ice core DOM is distinguishable from lake water and from the isolated fulvic acid fraction of the DOM (Fig. 2.1.A). All samples plotted near the 1:1 line except for the fulvic acid fractions, which fell well below the 1:1 line being significantly enriched in the humic/fulvic component C1 relative to the C2 component, which is associated with recent microbial production. Top, middle and bottom core sections were distinguishable based on C1 and C2 concentrations. The highest amounts of C1 and C2 were found in the

Table 2.2.: Fluorescence characteristics of components identified in Pony Lake EEM dataset. Primary and (secondary) excitation (λ Ex) and emission (λ Em) maxima.

Comp. #	Ex/Em (nm/nm)	EEM Region (Coble et al., 1998)	Description & likely sources
1	**240**(370)/480	Peak A	Humic/fulvic DOM, ubiquitously observed[1-7]
2	**240**(300)/390	Peak M	DOM associated with recent microbial production stimulated by DOM substrates [1-4,6-7]
3	(245)**355**/430	Peak C	Humic/fulvic DOM, ubiquitously observed[1-7]
4	**275**/326	Peak B	Amino acid, tyrosine-like[1-7]

[1]Coble et al., 1998; [2]Stedmon et al., 2003; [3]Stedmon & Markager, 2005a, b; [4]Fulton et al., 2004; [5]Cory & McKnight, 2005; [6]Murphy et al., 2008; [7]Yamashita et al., (in press).

bottom section, whereas the lowest amounts of these compounds were evident in the top core section overlapping with levels of C1 and C2 found in Pony Lake water DOM.

It is interesting to note that a subset of the bottom ice core samples were most similar to the fulvic acid fraction of the DOM, showing enrichment of humic/fulvic component C1, while in contrast a subset of the middle ice core samples showed enrichment in the component C2 associated with recent microbial production. The linear near 1:1 relationship between C2 and C1 for most Pony Lake ice core samples suggests similar rates of production and removal processes controlling the amounts of each component in a given sample.

Linear relationships were also observed for C3 relative to C1 (Fig. 2.1.B). However, for the C3:C1 relationships, the mean slope of the regression was 0.57 ± 0.05, much lower than a slope of 1.0, indicating that processes controlling the production and removal of C1 occurred at rates greater than production and removal processes controlling amounts of C3. A linear relationship was also observed between C4 and C1

(Fig. 2.1.C), with a slope significantly lower than 1.0 (p-value <0.05) for all samples except the mid core section. Similar to the C2:C1 relationship the bottom ice core section showed the highest amount of these components and plotted closest to the fulvic acid fraction. For the middle ice core samples, there was no relationship between the amino acid-like component C4 and the humic/fulvic component C1, suggesting that processes controlling the amounts of these components were decoupled in the middle ice core section. Amino acid components have been shown to result from algal exudation or from subsequent bacterial processing of algae-derived DOM (Stedmon & Markager, 2005b). As such, a decoupling of C4:C1 in the middle ice core section, along with high rates of bacterial production (Table 2.3.), suggest that the DOM signature of the middle ice core section is influenced by bacterial processes, to a larger degree than in the bottom or top ice core sections.

Productivity Measurements and Abundance of Organisms

Bacterial and virus like particles (VLP) enumerated via epifluorescence microscopy (Fig. 2.2.) and microbial activity measurements detected via the incorporation of radiolabelled compounds are summarized in Table 2.3. Bacterial numbers ranged from 1.10×10^6 to 2.97×10^7 cells ml^{-1}. With the exception of the mid core section, VLP numbers were lower than bacterial counts and ranged between 2.05×10^4 and 1.89×10^6 cells ml^{-1}. However abundances increased with depth for both bacteria and VLP. Cyanobacteria were detected in Lugol's preserved samples from the middle core section only (6.93×10^3 cells L^{-1}) (Fig. 2.2.); however it should be noted that

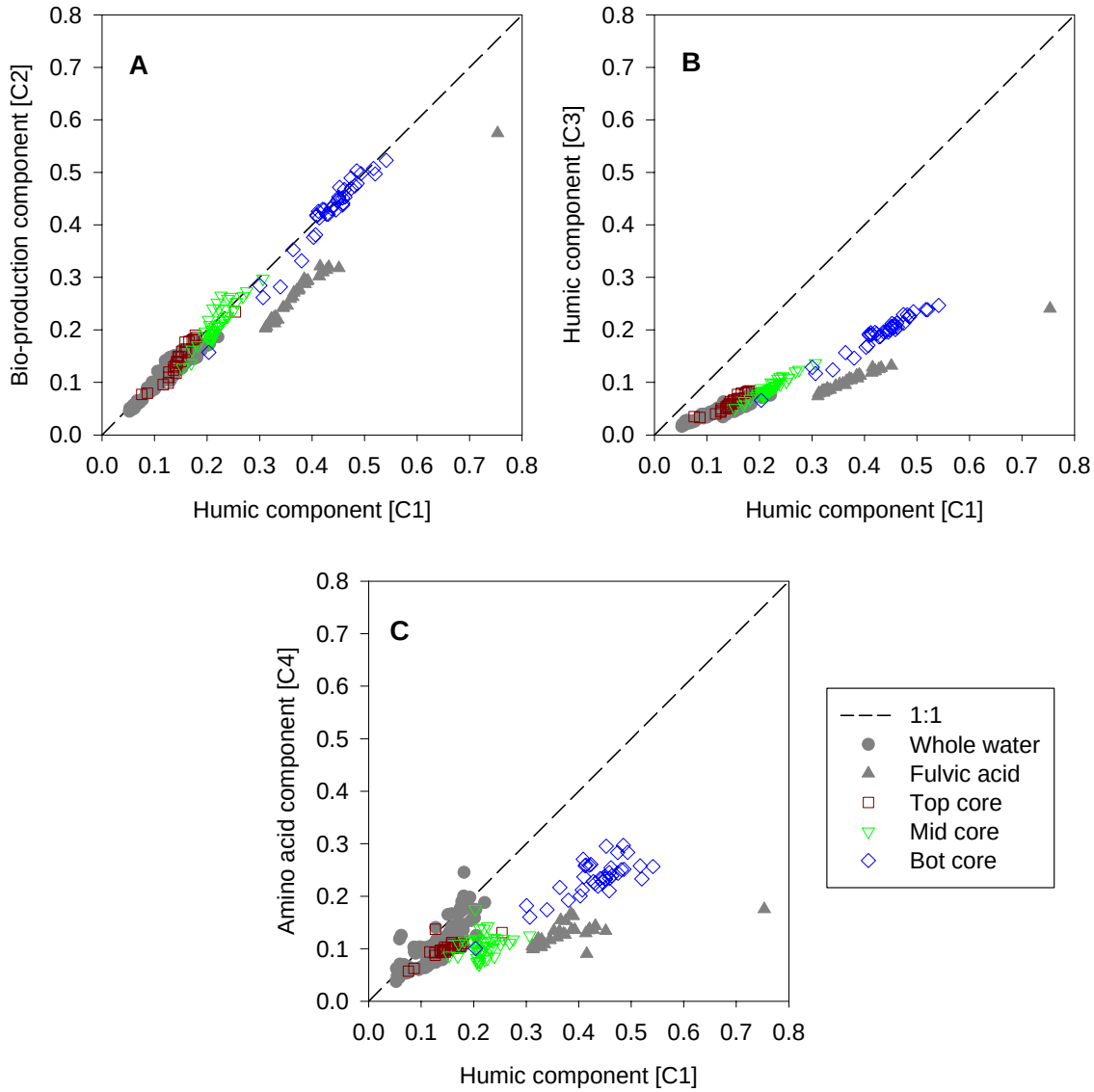

Figure 2.1.: (A) Co-variation of the fluorescence of components C1 and C2 indexed by DOM sample type. (B) Relationship between the fluorescence of components C1 and C3 for indexed by sample type. (C) Same as A and B, except relationship between the fluorescence of component C4 vs. component C1.

high concentrations of organic matter may have complicated the enumeration of preserved samples. The mechanical decontamination of the outer ice core surfaces may also have removed benthic mat organisms attached to the base of the bottom core section. Consequently, the absence of cyanobacteria in the bottom ice core section may not reflect their actual distribution.

Bacterial production was highest in the middle core section (37.9 µg C L^{-1} d^{-1}). This contrasts with the bacterial abundance and DOC concentrations, where highest cell numbers and concentrations were found in the bottom of the ice cores. This lack of correspondence was also seen between primary production and chlorophyll-*a* concentrations. While highest chlorophyll-*a* concentrations were detected in the bottom core section, PPR was highest in the upper 40 cm of the ice cores (931 µg C L^{-1} d^{-1}). Algae and ciliates were present in all ice core sections (Fig. 2.2.); however, active forms were not observed and algae and ciliates appeared to over-winter in the ice in the form of spores or cysts. These encapsulated forms were found in the entire ice core with average values of 2.28 x 10^5 cells L^{-1} for ciliated cysts and 3.06 x 10^5 cells L^{-1} for flagellated spores. Higher abundances were enumerated in the top and middle core sections than in the bottom section. Here again high concentrations of organic matter in the ice cores may have obscured some species from detection.

Our study provides evidence that bacteria, virus like particles, algae and ciliates (all dominant organisms found in Pony Lake's phytoplankton during summer, Chapter 3) could persist throughout the year within the lake ice. To the best of our knowledge this is the first time that microbial processes and community structure analyses were determined

Table 2.3.: Activity measurements of bacteria (bacterial production, BP) and primary producers (primary production, PPR) in 40 cm ice core sections (Top, Middle, Bottom) from Pony Lake on 15 Nov 2004

	Top	**Middle**	**Bottom**
BP [µg C L^{-1} d^{-1}]	6.69	37.9	1.80
PPR [µg C L^{-1} d^{-1}]	931	308	31

in ice core samples from an Antarctic lake that transitions from open water to completely frozen. Little information exists in the literature about the response of microbial mats during transitions between open water and complete freeze up in Antarctic ponds (Schmidt et al., 1991; Hawes et al., 1999). Ice core samples represent the entire water column of a lake, capturing chemical alterations during freezing and the cryo-concentration of solutes and particulates towards the base of the lake, the formation of a basal brine and accompanied biological processes.

VLP to bacteria ratios ranged between 0.01 and 1.41 which is within the range reported from the Pony Lake water column (Chapter 3). Bacterial production in Pony Lake upper and lower ice core melt waters are comparable to those found in the oligotrophic Dry Valley lakes (Takacs & Priscu, 1999), in saline lakes in the Vestfold Hills (Laybourn-Parry, 2002) or in the bottom sediment containing layers of cryoconites in the Taylor Valley (Foreman et al., 2007). Exceptionally high BP rates were found in the middle ice core sections (37.9 µg C L^{-1} d^{-1}) of Pony Lake. Nonetheless, BP rates in Pony Lake ice core melt waters were generally low relative to those from nutrient enriched or eutrophic lakes in the Vestfold Hills, Antarctica during the summer months (Laybourn-Parry, 2002). Since nutrients and DOC concentrations were elevated compared to other Antarctic lake or cryoconite ecosystems with similar production rates

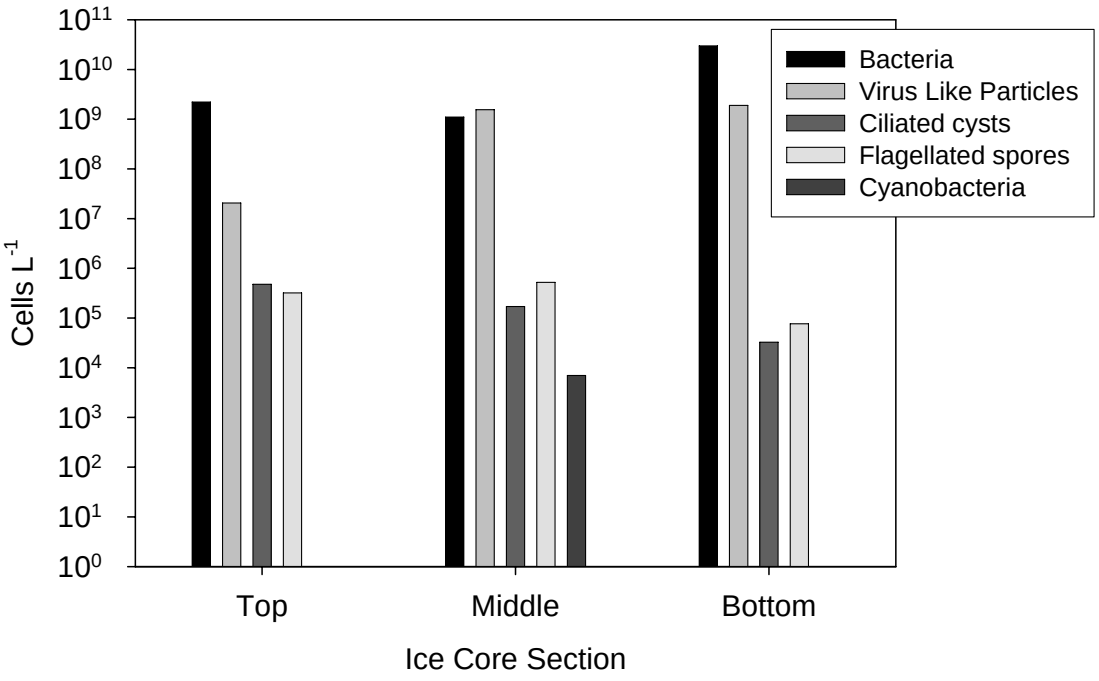

Figure 2.2.: Abundance of organisms in Pony Lake ice core sections collected in November 2004. The ice column of Pony Lake is colonized by bacteria, virus like particles, and to a small extent by cyanobacteria. Inactive forms of flagellates and ciliates are found as well.

our findings suggest that BP rates in Pony Lake may have been affected by different factors. It has been shown that the photochemically induced transformations of algal derived DOM may render it more refractory and hence less bioavailable for microbial uptake (Benner & Biddanda, 1998; Obernosterer et al.; 1999). Consequently, photochemically induced changes in Pony Lake DOM during summer would have negatively affected bacterial production in ice core melt waters. Spectrofluorometric analyses of Pony Lake DOM showed that the top core DOM closely resembles the DOM characteristic of Pony Lake water. Further, the bottom core section was enriched with the humic/fulvic acid component, a fraction the DOM pool which is less readily

biodegradable. Conversely, ongoing algal productivity underneath the ice cover during later summer and early winter produced fresh, new DOM as indicated the dynamics of the fluorescent component C2 and contributed positively to the higher BP rates in the middle core section. Hawes et al. (1999) monitored Antarctic ponds over an entire year from January 1997 to January 1998. They recorded a slow refreezing process towards the base in these ponds over ~three months. Ice began to form in shallower areas of the pond in late February. Temperature sensors installed in 'Fresh' pond indicated that the freezing point progression on 9 March was at a depth of 0.45 m, at 0.75 m on 4 April, and at 1.75 m on 10 June (Hawes et al., 1999). Accompanying the gradual freeze up, light levels declined. Photosynthetically active radiation (PAR) fell to zero on 7 April at a depth of 0.5 m (Hawes et al., 1999). Their findings showed that algal mats maintained a positive rate of net photosynthesis under low light regimes as long as they were exposed to liquid water. Mixotrophic behaviour of phytoplankton has been shown to be an important process during the dark winter season in Antarctic lakes (e.g. McKnight 2000). Consequently, phytoplankton may have remained active although light conditions would not have been sufficient to support photosynthesis. Both processes suggest that new DOM could have been produced underneath the ice cover, unaltered by photochemical reactions. During the ongoing freeze-up in Pony Lake this labile DOM was then captured in the middle ice core section and may have contributed to the higher BP. Higher bacterial production rates were also detected at the beginning of the melt period in December 2004 in Pony Lake water compared to mid-January, supporting the argument that newly produced DOM was more bioavailable than photochemically altered DOM

later in the season. It seems likely that cryo-concentration of the newly produced DOM towards the base of Pony Lake would have occurred. However BP rates in the bottom section were much lower compared to the middle section suggesting that remaining planktonic bacteria were exposed to extreme environmental conditions. For example, organisms living in basal brines would have been exposed to extremely cold temperatures, changes in osmotic pressure, pH, and a substantial increase in salt and nutrient concentrations (Schmidt et al., 1999; Healy et al., 2006; Wait et al., 2006). These extreme conditions may cause cellular damage or death and would have limited bacterial production. Moreover, assuming anoxic conditions, anaerobic bacteria would have faced limited growth conditions in the aerated ice core melt water.

Although no active forms of algae and ciliates were found, a large number of spores and cysts were present throughout the ice cores. The organisms were most likely forced to sporulate or encyst in response to freezing, darkness (for algae), desiccation, osmotic pressure, and/or anoxia (Mataloni et al., 1998; Bell & Laybourn-Parry, 1999; Roberts et al., 2000). These wintering spores from phototrophic flagellates may have germinated in response to our experimental manipulations (increased melting and light exposure) as indicated by the relatively high primary productivity rates (Table. 2.3.). Regardless, primary production rates were 30 times higher in the top of the core as compared to the bottom of the lake, where less than favorable conditions may have limited production as opposed to the lack of liquid water and light intensity. Chlorophyll-*a* concentrations were inversely related to the primary production rates. Highest concentrations were detected in the bottom ice core sections of Pony Lake where visibly

dark green layers were present at the base of the ice. Aside from the cryo-concentration of the lake algal consortia, benthic microbial mats may have contributed to the higher chlorophyll-*a* concentrations. Benthic microbial mats are ubiquitous in Antarctic melt water environments (e.g. Wharton et al., 1983; Vinocur & Pizarro, 2000) with cyanobacterial mats commonly found as the dominant form of vegetation in extremely cold environments (e.g. Vincent & Howard-Williams, 1986; Haves et al., 1993; Jungblut et al., 2005). Various types of epilithic algal mats have also been reported in Antarctic lakes (Vinocur & Pizarro, 2000). Regardless, cyanobacteria were not found in the bottom ice core sections of Pony Lake and flagellated spore counts were less abundant in the bottom core section compared to the upper core. Either the core sampler did not penetrate the frozen sediments sufficiently deep enough to remove microbial mat samples from the base of the lake or the deeper layers of Pony Lake are extremely light limited. During the summer Pony Lake is a very turbid, dark green-brownish color. Reduced light penetration can limit the establishment of a phytobenthic community as has been observed in other Antarctic nutrient enriched ponds. Hawes (1990) reported differences in benthic and planktonic communities in fresh water ponds of different trophic state on Signy Island, Antarctica. Eutrophic ponds were dominated by phytoplankton communities whereas oligotrophic lakes had little phytoplankton, but a well developed phytobenthos. No benthic vegetation was found on the floor of eutrophic Heywood Lake or in The Wallows.

Aside from cryo-concentration, chlorophyll-*a* may have simply been preserved in Pony Lake. Simmons et al. (1993) found abundant chlorophyll-*a* in several Lake Hoare

samples with no detectable ATP, nor any living organisms. They argue that several environmental conditions in the McMurdo Dry Valley lakes (low light, no arthropod herbivores and low temperatures) led to this preservation. Howard-Williams et al. (1989) also suggested that chlorophyll-a may be found in an inactive, preserved state in ponds on the McMurdo ice-shelf. Low temperatures have also been show to significantly suppress chlorophyll-*a* degradation (Vincent & Howard-Williams, 1986).

DGGE Fingerprinting Analysis

We used denaturing gradient gel electrophoresis (DGGE) as a molecular fingerprinting tool to characterize the microbial community structure found in the different ice core sections. The DGGE profiles mirror the cryo-concentration of solutes with an increasing number of bands found from the top to the bottom of the ice cores (Fig. 2.3.). The higher number of DGGE bands in the deeper core segments may imply that the bottom waters function as a refuge for microbial life during freezing and that these layers serve as nutrient rich zones supporting a more complex microbial consortium. It seems likely that the different layers within the ice cores support diverse populations of microbes. The gradual concentration of solutes and particles towards the lake bed during freezing alters the temperature, osmotic pressure, pH, oxygen concentration, salt and nutrient composition within the ice, providing environmental conditions which might favor more specialized microbial assemblages. Alternatively, depth specific differences in community composition may represent an aeolian origin of the organisms. Deposition of small soil particles during winter storms may have provided an influx of microorganisms to the upper portions of the ice core as has been found in ice

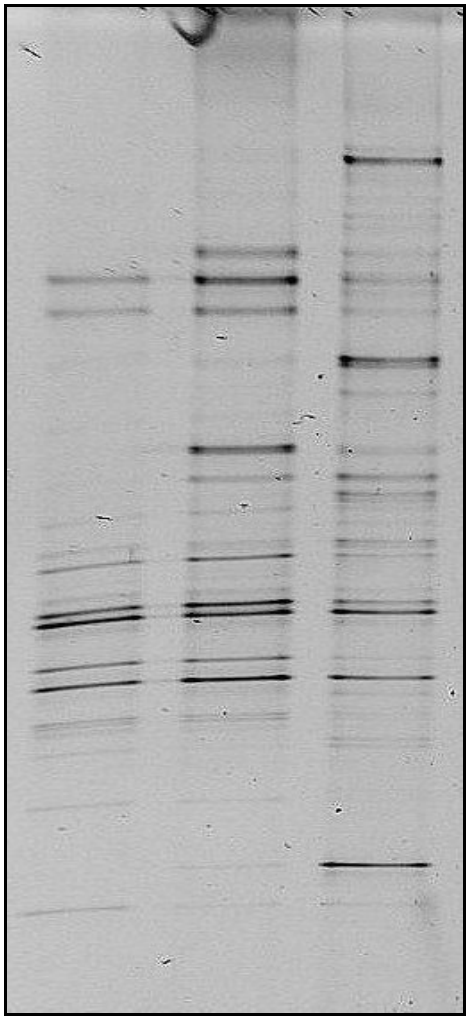

Figure 2.3.: Differences in DGGE banding patterns between microbial communities in ice core sections from Pony Lake in November 2004 based on the amplification of the 16S rRNA gene. From left to right: top, middle, and bottom 40 cm. Image colors were inverted on the camera, but not manipulated.

covers of the McMurdo Dry Valley lakes. Microbial assemblages in the perennial ice cover of these lakes were similar to those found in the surrounding terrestrial environment (Gordon et al., 2000).

16S rRNA Sequences

In the Pony Lake ice core sections 41 phylotypes were identified, which included the following bacterial linkages: *Bacteroidetes*, *Firmicutes*, *β*-, *γ*-, *δ*-, *ε-proteobacteria*, *Spirochaetes*, *Verrucomicrobia*, and several uncultured bacterial strains. Figure 2.4. shows the relative distribution of the groups within the clone libraries from the three ice core sections. The phylogenetic relationship of Pony Lake clones to their closest neighbors is summarized in Table 2.4. The number of different bacterial groups as well as the number of phylotypes present in each group varied greatly between the three ice core sections. The top core section contained 20 phylotypes. These clones belonged to four major phyla, and included *β-proteobacteria* (40%), *Bacteroidetes* (30%), *ε-proteobacteria* (15%), and *γ-proteobacteria* (5%). The remainder of the diversity was most closely related to uncultured bacteria. In the middle ice cores 12 different phylotypes were identified representing three bacterial phyla: *β-proteobacteria* (41.7%), *ε-proteobacteria* (41.7%) and *Bacteroidetes* (16.6%). The bottom core section showed the highest degree of diversity, 22 phylotypes were identified that associated with eight different phyla. The dominant phyla came from the *ε-proteobacteria* (27.3%), *β-proteobacteria* (22.7%), *Firmicutes* (18.2%), and *Bacteroidetes* (9.1%) lineages. All other phyla in the bottom core sections were described by only one sequence type.

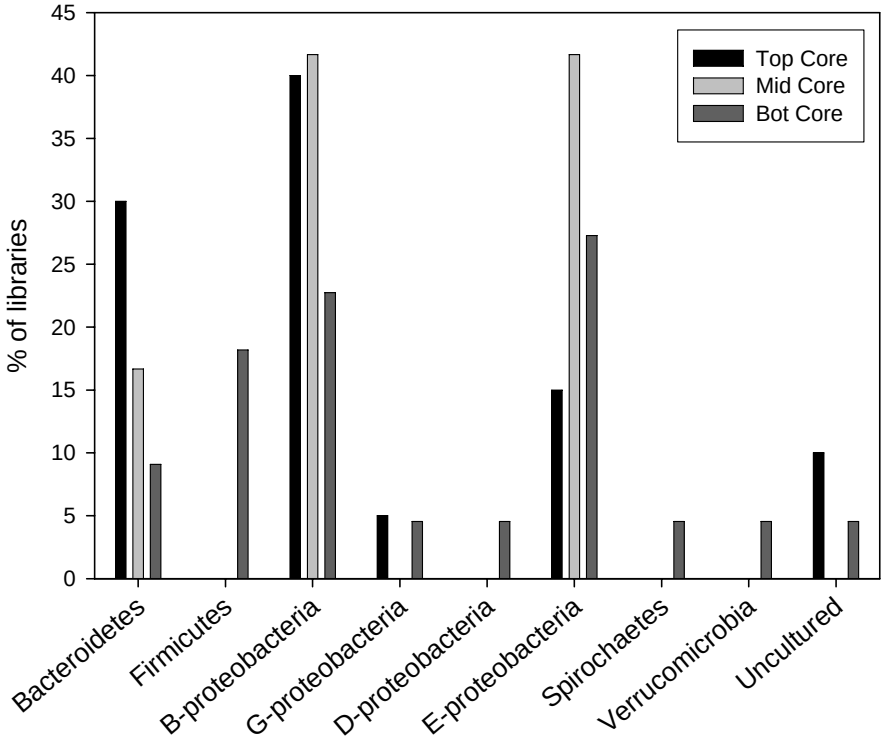

Figure 2.4.: Distribution of taxonomic classes within clone libraries from Pony Lake ice core sections. *Bacteroidetes*, *β*- and *ε-proteobacteria* are the dominant classes. Differences occur in the number of taxonomic classes in each core section.

Members of the *δ-proteobacteria*, *Spirochaetes*, *Verrucomicrobia* and *Firmicutes* lineages were exclusively identified in the bottom core sections. *γ-proteobacteria* were present in both the top and bottom cores with one similar sequence type. Cyanobacteria, which were detected in Lugol's preserved samples (~7 cells ml^{-1}) in the middle core fraction, were not captured in the clone library.

In an attempt to link the phylogenetic data to Pony lake biogeochemistry, the most informative relationships can be found within the *δ*- and *ε-proteobacteria*. The Pony Lake bottom core clone ANTPL_BC19 was most closely related to *Desulfuromonas*

svalbardensis 60, an Fe(III)-reducing bacteria isolated from Arctic sediments, Svalbard (Vandieken et al., 2006) and belonging to a group of sulfur reducing bacteria. Pony Lake ice cores had a strong H_2S odor, and the presence of such a phylotype indicates that in situ sulphur reduction may be occurring at the base of Pony Lake during winter. ANTPL_TC07, found within the top of the ice cores, showed close sequence identity with *Sulfurimonas denitrificans* DSM 1251, a sulfur oxidizing, chemolithoautotroph *ε-proteobacterium* (Sievert et al., 2008). *Sulfurimonas denitrificans* is involved in the transformation of sulfur through the process of sulfur oxidation, and turns nitrate to dinitrogen gas via denitrification. The process of denitrification could contribute to the decreased NO_3 concentrations measured in the ice cores (Table 2.1.) compared to lake water concentrations (Chapter 3). Therefore, it may be assumed that the two phylotypes play an important role in the sulfur cycle and the latter in the nitrogen cycle of the lake ecosystem.

Overall, ~69% of the phylotypes were related to bacteria reported from other Antarctic lake and marine environments. However, many of these phylogenetic neighbors were reported to be uncultured bacterial strains and clones. To provide a higher level of identification we assigned isolated bacterial strains from moderate temperature environments to Pony Lake clones (Table 2.4.). These uncultured bacterial strains and clones were related to members of *Bacteroidetes* (Prabagaran et al., 2007), *β-proteobacteria* (Van Trappen et al., 2002; Pearce et al., 2005) and *ε-proteobacteria* (Bowman & McGuaig, 2003), uncultured bacterial clones from penguin dropping

Table 2.4.: Affiliation of 16S rRNA gene phylotypes in Pony Lake ice core sections. Pony Lake clones with relatives reported from other Antarctic environments are labeled with an asterisk. Many of these close neighbors from Antarctic environments were unidentified bacteria or clones. To provide a higher level of identification well described relatives from non-Antarctic habitats were selected. These clones are labeled with (†).

Taxonomic phylum	Taxonomic class	16S rRNA identification (closest neighbor)	GenBank no. (closest neighbor)	Core Section: Top		Middle		Bottom	
				Clone	% id.	Clone	% id.	Clone	% id.
Bacteroidetes	*Flavobacteria*	*Cellulophaga algicola* S3-1	AY771718			ANTPL_MC77†	98		
		Flavobacterium frigidarium	AF162266	ANTPL_TC15*	98				
		Gelidibacter algens ACAM 536T	U62914	ANTPL_TC02*	99				
		Gelidibacter algens ACAM 551T	U62916	ANTPL_TC75†	98				
	Sphingobacteria	*Algoriphagus* sp. ZS3-3	FJ196000			ANTPL_MC48*	95		
		Uncult. *Cytophaga* sp. JTB220	AB015266	ANTPL_TC57	99				
	Uncultured	Uncult. *Bacteroidetes* bacterium MT054	AM157468	ANTPL_TC19	97				
		Uncult. *Bacteroidetes* bacterium F4C06	AY794184	ANTPL_TC21*	98				
		Uncult. *Bacteroidetes* bacterium 1D5	AJ627991					ANTPL_BC10	98
		Uncult. *Bacteroidetes* bacterium CF61	AY274859					ANTPL_BC73	97
Firmicutes	*Clostridia*	*Clostridiales* bacterium 12-2A	EU523731					ANTPL_BC06†	95
		Clostridium sp. D3RC-3r	FJ527031					ANTPL_BC67	97
		Uncult. *Clostridiales* bacterium LEO_13	EU158818					ANTPL_BC50	93
	Uncultured	Uncult. *Firmicutes* bacterium D25_36	EU266909					ANTPL_BC04	96
Proteobacteria	*β-proteobacteria*	*Bordetella trematum* DSM 11334 (T)	AJ277798	ANTPL_TC04†	99	ANTPL_MC39†	99	ANTPL_BC49†	98
		Hydrogenophaga atypica BSB 41.8T	AJ585992	ANTPL_TC01†	97	ANTPL_MC07†	97	ANTPL_BC42†	97
						ANTPL_MC58†	97		
		Hydrogenophaga taeniospiralis SE57	AY771764	ANTPL_TC03†	99	ANTPL_MC06†	99	ANTPL_BC30†	99
				ANTPL_TP13†	98				
								ANTPL_BC01†	100
		Hydrogenophaga taeniospiralis	AF078768	ANTPL_TC35†	97				
				ANTPL_TC73†	98				
		Rhodoferax antarcticus Fryx1	AY609198	ANTPL_TC46*	99			ANTPL_BC26*	99
	Uncultured	Uncult. *Achromobacter* sp. 2SN	EU887771	ANTPL_TC12†	97	ANTPL_MC03†	98		
	γ-proteobacteria	*Glaciecola polaris* LMG 21857	AJ293820	ANTPL_TC14†	97			ANTPL_BC51†	97
	δ-proteobacteria	*Desulfuromonas svalbardensis* 60	AY835390					ANTPL_BC19	99
	ε-proteobacteria	*Arcobacter* sp., R-28214	AM084124			ANTPL_MC02	98	ANTPL_BC34	98
		Arcobacter cibarius LMG 21997	AJ607392			ANTPL_MC12	98		
		Arcobacter sp. BSs20195	DQ514311					ANTPL_BC02†	95
		Sulfurimonas denitrificans DSM 1251	CP000153	ANTPL_TC07	98				
	Uncultured	Uncult. *epsilon proteobacterium* MERTZ_2CM_162	AF424297	ANTPL_TC06*	97	ANTPL_MC11*	97	ANTPL_BC03*	97
		Uncult. *Campylobacterales* bacterium DS057	DQ234141	ANTPL_TC20†	98	ANTPL_MC01†	98	ANTPL_BC05†	98
		Uncult. *epsilon proteobacterium* 131631	AY922199			ANTPL_MC51	97		
		Uncult. *epsilon proteobacterium* 131631	AY922199					ANTPL_BC33	96
		Uncult. *epsilon proteobacterium* D004025D06	EU721824					ANTPL_BC46	96

Table 2.4.: Continued

Taxonomic phylum	Taxonomic class	16S rRNA identification (closest neighbor)	GenBank no. (closest neighbor)	Core Section					
				Top		Middle		Bottom	
				Clone	% id.	Clone	% id.	Clone	% id.
Spirochaetes	*Spirochaetales*	*Sphaerochaeta* sp. TQ1	DQ833400					ANTPL_BC14	97
Verrucomicrobia	*Verrucomicrobiae*	Uncultured bacterium CLEAR-26	AF146249					ANTPL_BC45*	97
Unidentified	Unidentified	Uncultured bacterium KD3-68	AY218614					ANTPL_BC75*	98
		Uncultured bacterium KD6-47	AY218761	ANTPL_TC70*	95				
		Uncultured bacterium CARB_ER2_7	AY239579	ANTPL_TC63	97				

sediments, and uncultured bacteria from Heywood Lake, Antarctic (Pearce et al., 2005). The closest neighbor to a *Verrucomicrobia* organism was found in anoxic sediment from marine and coastal meromictic lakes in the Vestfold Hills, Antarctica (Bowman et al., 2000). Cultured isolates related to our *Bacteroidetes* clones were isolated from Antarctic sea ice habitats and marine sediments (Bowman et al., 1997; Humphry et al., 2001). *Rhodoferax* was associated with purple nonsulfur bacteria isolated from microbial mats from Lake Fryxell (Jung et al., 2004). The close similarity of Pony Lake clones to bacterial strains described in many different Antarctic environments implies a high degree of phylogenetic and ecophysiological consensus. One might see Antarctica as an ideal place to find evolutionary, endemic species; however, the close relationship of the same Pony Lake clones to bacteria from moderate temperature habitats suggests that bacteria found in Antarctica have adapted to the cold and harsh conditions rather than being restricted to this continent. Nonetheless, the occurrence of numerous Antarctic bacterial strains and clones related to Pony Lake clones suggests a certain level of geographic speciation in Antarctica.

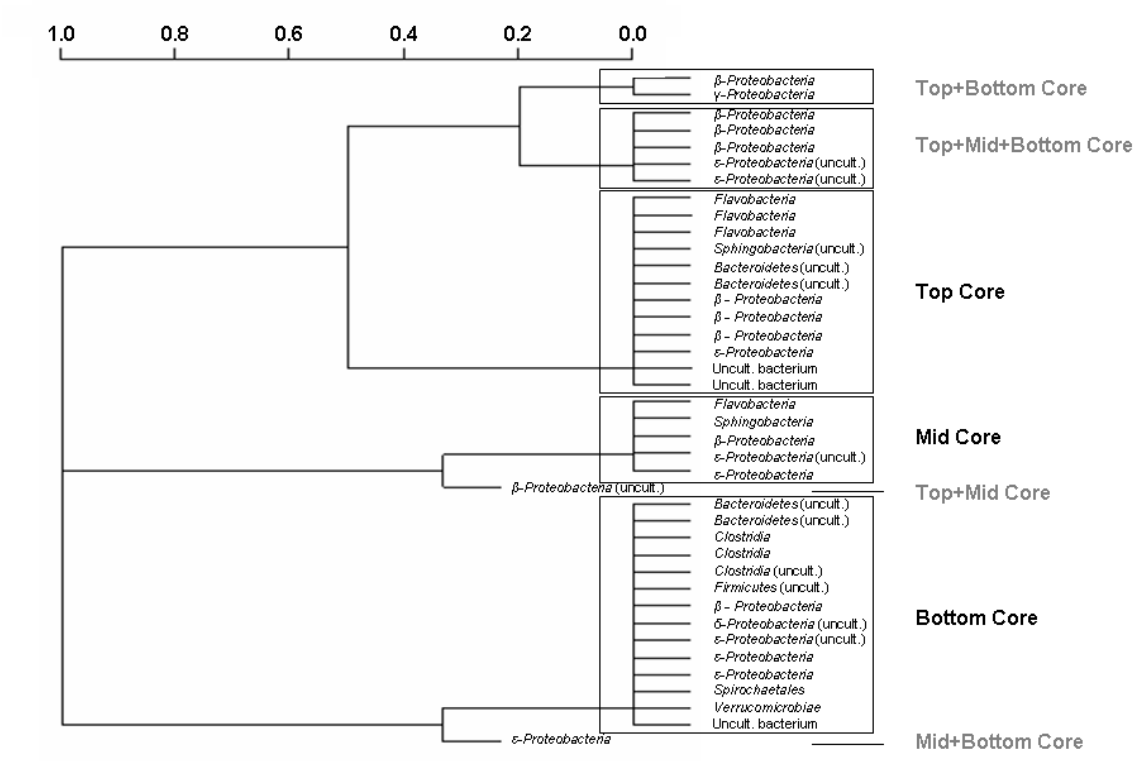

Figure 2.5.: Cluster analysis of closely related taxonomic classes or phyla to distinct clones found within Pony Lake ice cores sections. The dendrogram shows clusters of Pony Lake clones found in the individual core section and highlights clone library overlap between the core sections. The large clusters in clone libraries from the top and bottom section show the formation of distinct microbial communities within different layers of the ice column of Pony Lake. The horizontal axis describes the loss of information, in percentage, at each level of agglomeration.

Both the DGGE profiles and the clone libraries reflect differences in microbial community structure between the three ice core sections. Besides differences in the total number of phylotypes present (TC: 20; MC: 12; and BC: 22) and the number of phyla (TC: 4; MC: 3; and BC: 8) in each core fraction, the degree of sequence types which were present in only one of the three core segments was high. 60% of the phylotypes in the top core, 41.7% in the middle core and 63.6% of the bottom core sequence types were restricted to only that core section. Five sequence types were found in all three core sections related to *Bordetella trematum*, *Hydrogenophaga atypical*, *Hydrogenophaga taeniospiralis*, an uncultured *ε-proteobacterium*, and an uncultured *Campylobacterales* bacterium. Cluster analysis underscores the small degree of overlap between ice core sections and confirmed the pronounced variation between the top, middle, and bottom core clone libraries (Fig. 2.5.). The extreme chemical gradients, increased osmotic pressure and reduced temperatures may account for the variation in community structure; however, based on the phylogenetic information we infer that oxygen levels may have been the key regulator, dividing the ice column into an oxygenated upper section and micro-oxic or anoxic bottom section. Clones related to sulphate reducing bacteria, *Clostridium*, *Verrucomicrobiae*, or *Spirochaetales* were exclusively found in the bottom of the ice core. The closest relatives are obligate anaerobes or anaerobes, respectively and provide additional evidence for anoxic conditions.

Summary

The present study describes the chemical and biological nature of the ice column of a solid frozen lake in Antarctica. Cryo-concentration leads to a major increase in ion chemistry towards the base of the lake and the dark yellow coloration of the bottom ice core suggests the formation of a residual brine solution prior to complete freeze up. We demonstrate that the bacterial assemblages were not only able to withstand numerous physical and chemical stressors while enclosed within the ice column over the course of the Antarctic winter, but that they were highly metabolically active in the ice melt water. Further, physical stressors and ionic composition seem to directly influence the microbial community composition. As a result, the majority of phylotypes were restricted to distinct strata within the ice column of Pony Lake. A comparative study of Pony Lake water samples (Chapter 3) has shown that the ice column chemistry and community structure differ from that found in the summer lake water.

CHAPTER 3

PHYSICOCHEMICAL AND BIOLOGICAL TRANSITIONS IN PONY LAKE, ANTARCTICA

Adapted from: Markus Dieser, Diane M. McKnight, Christopher Jaros, John T. Lisle, Mark Greenwood, Johanna Laybourn-Parry, Penney L. Miller, Yu-Ping Chin and Christine M. Foreman (Draft for submission to FEMS Microbiology Ecology)

Abstract

Pony Lake, located at Cape Royds, Antarctica, is a shallow, eutrophic, coastal lake that freezes solid in the winter. Changes in physicochemical parameters and the microbial community in Pony Lake were studied as it transitioned from ice covered to open water. During the austral summer, from November to January, distinct changes evolved in the physical and chemical environment of Pony Lake, which affected the microbial community composition. Temperature, pH, conductivity, nutrients and major ion concentrations reach their maximum in January. Pony Lake was colonized by bacteria, virus like particles, algae and a small population of ciliates. Secondary production was highest in mid December (30.5 µg C L^{-1} d^{-1}). Photochemically induced alterations in the dissolved organic matter (DOM) composition affected its bioavailability and were reflected in bacterial utilization. Microbial clone libraries were dominated by members of the *β*- and *γ-proteobacteria* lineages. A transition in the community composition was evident as the lake moved from a frozen to open water system. Our data demonstrate that biological community dynamics in Pony Lake are closely linked to their physical and chemical environment. As a result, temporal changes in physicochemical

parameters during the summer month mediated a shift in the species composition of the bacterioplankton.

Introduction

Saline lakes are common features among Antarctic ecosystems in ice free areas of the Antarctic continent (Wright & Burton, 1981). These lakes are often marine derived ecosystems and are dominated by chloride ions (Laybourn-Parry et al., 2002). The combined effects of sublimation of the ice surface and evaporation have led to a concentration of salts in many of these lakes, resulting in waters ranging from slightly brackish to hypersaline (Perriss & Laybourn-Parry, 1997). The microbial assemblages in these lakes are exposed to repeated freezing and thawing, severe alterations in solar radiation intensities (~6 months of light and 6 months of darkness), changes in nutrient concentrations during brief summer melt periods and changes in salinity associated with winter freeze-up. Productivity typically increases throughout the austral summer following increased solar radiation, temperature and nutrient availability. The planktonic community in these lakes is dominated by microbial loop organisms, including bacteria, phytoplankton and protozoa, with little or no metazoans typically present (Perriss & Laybourn-Parry, 1997; Bell & Laybourn-Parry, 1999; Hodgson et al., 2001; Laybourn-Parry et al., 2002). Organisms that persist throughout the course of the year in these lakes must be capable of surviving more severe osmotic, pH, and temperature conditions during the winter months than typically exist during the summer (Schmidt et al., 1991). Nonetheless, due to limited logistical support for year-round research temporal patterns

of bacterioplankton in Antarctic lakes are not well documented. In general, limnological studies have focused primarily on phytoplankton and protozooplankton community structure (Laybourn-Parry & Bayliss, 1996; Laybourn-Parry et al., 1996; Butler, 1999; Butler et al., 2000) with lesser attention paid to the bacterioplankton assemblage. The current investigation describes the biological, physical, and chemical parameters of Pony Lake at Cape Royds, Antarctica, during the annual transition from an ice covered to an open lake system.

Pony Lake is a coastal, eutrophic lake located on Cape Royds (77° 33' S, 166° E), Ross Island, Antarctica (Fig.3.1.). The lake is ~120 m long, 70 m wide, and 1-2 m deep. Except for mid-summer, when warmer temperatures melt the ice-cover and strong winds cause thorough mixing of the water column, the lake is frozen solid. Ice melt typically begins in mid-December; however, during years with heavy snow fall (e.g. 2005-2006) the ice cover may persist throughout the summer season. The source of water to the lake is the accumulated snowpack, while water is lost by both sublimation and evaporation in mid-summer. As a result of its proximity to the sea Pony Lake is brackish (5.5 ppt). Previous studies have shown that Pony Lake may support very high dissolved organic carbon (DOC) concentrations (up to 100 mg C L^{-1} in some seasons) (McKnight et al. 1994; Brown et al., 2004). There are no higher plants in the basin, but algal populations in the lake are abundant. *Chlamydononas intermedia* Chodat has been reported as being the dominant chlorophytes algal species (McKnight et al., 1994), the same as found during Sir Ernest Shackelton's 1908 Expedition (West & West, 1911).

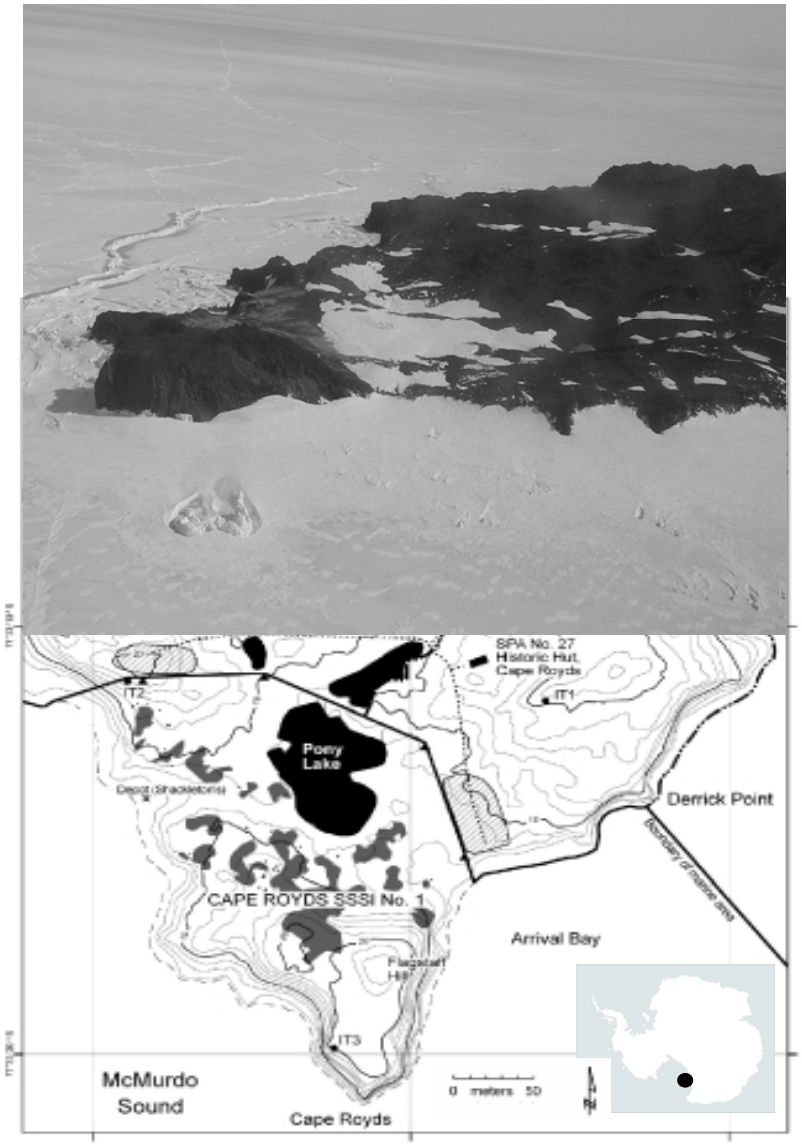

Figure 3.1.: Map of Cape Royds showing the location of Pony Lake (www.scar.org). The inset image was taken mid-December 2005 and shows an ice covered Pony Lake.

Brown et al. (2004) reported that during the transition from ice covered to ice free conditions the chlorophyte bloom in the lake was displaced by a cryptophyte bloom.

The organic matter in the lake is primarily autochthonous, from microbially (algae, cyanobacteria, bacteria and viruses) derived sources (Brown et al., 2004); however, on the western shore of the lake lies an Adelie penguin rookery. Runoff from the rookery into Pony Lake is believed to be minimal due to the distance from the lake and evaporitic conditions; therefore, Pony Lake is considered to lack significant terrestrial carbon inputs into the watershed. In a lacustrine system such as Pony Lake, which lacks protozooplankton and metazooplankton trophic levels, bacterioplankton play a significant role in the flow of energy and nutrients, as a result of their abundance, efficient nutrient uptake and large growth potential (Azam et al., 1983).

Materials and Methods

Sample Collection

Water samples were collected during the austral summer season of 2004/05. Four time points were chosen to measure basic limnological parameters (biological, physical, and chemical) in Pony Lake during the transition from an ice-covered to ice-free lake. During our studies melting began along the edges of the lake in early December and continued into January. Strong winds allow for frequent mixing in this shallow system. Water samples were collected in acid washed, DIW rinsed (x6) Nalgene bottles and stored in coolers for transport back to Crary laboratory in McMurdo Station. Samples were processed within 4 hrs of collection.

Physical Parameters and Chemical Analyses

Because strong winds fully mixed the shallow water column during the summer of 2004/05 one representative depth was chosen after preliminary analyses, and instrument data and water samples were subsequently collected ~30 cm beneath the water surface. Conductivity, salinity, and temperature were measured with a portable multi-meter (Hydrolab minisonde). The underwater photosynthetically active radiation penetration (PAR, 400-700 nm) was determined using a Licor 4π spherical quantum sensor (LI-193). Dissolved oxygen (YSI) and pH (Beckman) were recorded for each sample.

Samples were prepared for chemical analysis according to the protocols of the McMurdo Dry Valleys Long Term Ecological Research Group (Priscu & Wolf, 2000). Samples for dissolved organic carbon (DOC) and total nitrogen (TN) analyses were filtered under low vacuum (<7 psi) in the dark through 25 mm pre-combusted GF/F filters, acidified with 6 N HCl to pH 2 and analyzed on a Shimadzu TOC-V and Shimadzu TNM-1 analyzer, respectively. Filters from the above analyses were wrapped in aluminum foil and kept frozen until extraction for chlorophyll-*a* analysis. Chlorophyll-*a* was extracted in a 1:1 solution (90% acetone and DMSO) for 12 hrs under dark conditions at -20°C. Extracted chlorophyll-*a* was analyzed on a Turner 10-AU fluorometer. Samples for macronutrients were filtered through 25 mm pre-combusted GF/F filters and stored frozen until analyses (within a month of collection) on a Lachatt autoanalyzer. Samples for anion and cation determination were filtered through 0.4 µm 47 mm nucleopore filters. DI water was used as a filtration bank and samples were analyzed on a Dionex DX-300 ion chromatography system. Dissolved inorganic carbon

(DIC) was measured using infrared absorption following acidification and sparging of the sample with high purity N_2 gas. Peak areas were integrated and converted to mg L^{-1} using a standard curve based upon a freshly prepared standard of $NaHCO_3$.

Productivity Measurements

Bacterial productivity (BP) was measured via ^{3}H-thymidine incorporation (20 nM final conc.) as described in Takacs & Priscu (1998). Five ^{3}H-thymidine assays and triplicate formalin killed controls (5 % final concentration, 30 min prior to ^{3}H-thymidine addition) were incubated at 4°C for 20 hrs. Samples were analyzed using a liquid scintillation counter (Beckman LS 7200). Thymidine incorporation rates were converted to bacterial production rates using a conversion factor of 2.0 x $l0^{18}$ cells mol^{-1} TdR (Duckow & Carlson, 1992) and a cell-to-carbon conversion factor of 11 fg C $cell^{-1}$ (Kepner et al., 1998) as outlined by Takacs & Priscu (1998).

Primary production (PPR) was measured via ^{14}C-carbonate/bicarbonate incorporation (114.4 μCi ml^{-1}, pH ~9.5; ICN/MP Biomedicals) as described in (Lizotte et al., 1996). Quadruple light assays and duplicate dark controls were incubated at 4°C in an illuminated chamber for 24 hrs. Following incubation, samples were filtered through pre-combusted 25 mm GF/F filters in the dark. Filters were transferred into 20 ml scintillation vials, acidified with 500 μl 3M HCL and dried before liquid scintillation counting (Beckman LS 7200).

Plankton Analyses

Water samples for the determination of bacterial abundances were fixed with formalin (2% final concentration) and stained with a 25X solution of the fluorochrome SYBR Gold (Invitrogen Inc.) for 15 min following Lisle & Priscu (2004). Samples were filtered onto 25 mm 0.2 µm black polycarbonate filters with a 0.45 µm nitrocellulose backing filter under gentle vacuum. To reduce the possibility of contamination, filter towers were pre-combusted and all reagents used for the staining were passed through 0.2 µm sterile filters to remove extraneous particles and cells. Bacterial cells were enumerated using a Zeiss Axioscop epifluorescence microscope with a final magnification of 1000X.

Samples for bacteriophage or virus like particle (VLP) enumeration were collected in sterile 125 ml screw cap flasks. All flasks were immediately flash frozen in liquid nitrogen and stored at -80°C as recommended by Wen et al. (2004). Prior to sample processing all flasks were removed from -80°C storage and allowed to thaw in the dark at room temperature overnight. Samples were pre-filtered through a 0.20 µm pore size filter to remove bacteria. Filtrate from each sample was aseptically collected and filtered through a 25 mm diameter, 0.02 µm pore size filter to retain the VLP. VLP were stained with SYBR Gold as described by Lisle and Priscu (2004) and counted using an Olympus BX51 epifluorescent microscope.

Samples for phytoplankton (algae, diatoms, cyanobacteria) and protozooplankton (ciliates) analysis were fixed with Lugol's iodine (10 ml Lugol's to 1L of sample) and concentrated by settling for one week in amber Nalgene bottles. After settling, the upper

solution was gently siphoned off, leaving ~60 ml of sample, which was then transferred to a clean 60 ml Nalgene bottle for transport to the United Kingdom. Sub-samples were counted in a Sedgewick-Rafter counting chamber using phase microscopy at X320 magnification (Laybourn-Parry & Marshall, 2003).

Environmental DNA Extraction and DGGE

Water samples (70-100 ml) were filtered onto 47 mm Supor®-200 0.2 µm pore size, sterile membrane filters under low pressure (<7 psi). Filters were placed into 5 ml cryovials filled with TES (100 mM Tris, 100 mM EDTA and 2% SDS) buffer, flash-frozen in liquid nitrogen, and stored at -80°C.

DNA was extracted from the Supor®-200 membrane filters using an Ultra Clean Soil DNA Kit (MoBio). Denaturing gradient gel electrophoresis (DGGE) was used as a molecular fingerprinting tool to characterize the microbial population structure and diversity. A portion of the 16S rRNA gene was amplified with primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 534R (5'-AATACCGCGGCTGCTGG-3') (Muyzer et al., 1996). A 40 base pair GC clamp was added to the 5' end of the 341F primer (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG). The amplification protocol included a hot start (94°C for 4 min) and a touchdown program that consisted of an initial annealing temperature of 65°C followed by a 1°C decrease for 8 cycles down to 58°C, and 17 cycles with an annealing temperature of 58°C. A final elongation step occurred for 10 min at 72°C. Each 50 µl reaction mixture contained 1.5 µl of environmental DNA extract, $MgCl_2$ buffer (final conc. 1X), Taq Master (final conc. 1X), PCR nucleotide mix (final conc. 800 µM), and Taq DNA polymerase (final

conc. 0.025 u μl^{-1}) (all components from 5 Prime, Eppendorf), upstream and downstream primers (final conc. 0.5 µM), and nuclease free water (Promega). PCR amplifications were carried out in an automated thermal cycler (Mastercycler ep, Eppendorf). DGGE was performed with a BioRad D Code™ system as described by Murray et al. (1996). PCR products were loaded onto 8-12 % polyacrylamide gels. The denaturing gradient contained 40-70 % denaturant. The gels ran in 1X TAE at 60 V for 17 hrs. Gels were stained with SYBR Gold (Invitrogen) for 15 min and viewed with an Alpha Innotech FluorChem™ 8800 system. Gel images were analyzed using GelComparII software (Applied Math).

16S rRNA Clone Library

We constructed clone libraries for 3 different dates [Early December-11 Dec 2004 (ED); Late December-29 Dec 2004 (LD); and Mid January-14 Jan 2005 (MJ)] during the transition period from ice covered to ice free conditions by amplifying the 16S rRNA gene with primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Stackebrandt & Liesack, 1993). PCR products were cloned into pCR®2.1-TOPO vectors (TOPO TA cloning kit, Invitrogen). From each sample 70 clones containing inserts were picked for further analyses. Single clones were transferred to LB agar plates and LB broth both containing 50 µg ml^{-1} kanamycin and incubated at 37°C overnight. Pooled clones from each sample date were amplified by PCR using primers 341F plus GC clamp and 534R. The clone pool DGGE profiles were then compared to the DGGE profile obtained from the environmental DNA samples to compare banding pattern similarities (Burr et al., 2006). Bacterial clones were sent to

Functional Bioscience, Inc. on LB agar plates for high throughput DNA preparation and DNA sequencing using primer M13F (20). Nucleotide sequences were edited using Sequencher 4.5 (Gene Code Corporation). For each sequence a NCBI BLAST search was performed in order to determine the closest relatives (BLASTN 2.2.21, ncbi.nlm.nih.gov/BLAST/, Zhang et al., 2000).

Statistical Analysis

To assess differences in the patterns of presence/absence of clones between the three sample dates, a Bray-Curtis (Czekanowski, 1913; Bray & Curtis, 1957) distance measure was employed. Bray-Curtis distance was calculated using the sum of unshared clones present divided by the total number of clones present. With 3 dates and two possibilities on each date, excluding the clones that were not present on any date, 2^3-1=7 different patterns for each sampling date were possible. Cluster analysis was used to visualize which clones shared the same patterns of presence/absence. Results from a complete linkage amalgamation, which joins clusters based on the greatest distance between any two objects, were identified.

Results and Discussion

Physical Parameters and Chemical Analyses

Pony Lake was frozen solid to its base until late November of 2004. By the end of November, peripheral moating occurred at the west end of the lake and by 14 Jan 2005 approximately 80% of the lake was ice free. The remaining 20% of the ice layer was multi- year ice, but did not impede mixing of the main water column. Surface inflow or

runoff from the surrounding hills was not observed. During the open water period the water column was fully mixed by strong winds, which was in contrast to other Antarctic melt water ponds that become chemically or thermally stratified during the austral summer (Hawes et al., 1999; Healy et al., 2006; Wait et al., 2006). Turbidity increased over the course of the open water period, following phytoplankton blooms.

Pony Lake was sampled on four different time points during the austral summer 2004/05, corresponding to changes in the lake ice cover (see Table 3.1.). During mid-December 2004 to mid-January 2005 Pony lake was supersaturated with dissolved oxygen (DO) due to mixing and photoautotrophic activities. The water temperature increased gradually throughout the sampling period from 1.3 to 7.5°C. Lake water was moderately alkaline, ranging from a pH of 8.07 to 9.77. At this pH we assume that most of the dissolved inorganic carbon (DIC) was present as bicarbonate or carbonate. DIC depletion also negatively correlated ($r = -0.59$) with the summer phytoplankton bloom. Thus, when HCO_3 is the predominant form of DIC, hydroxyl ions are released during photosynthesis, raising the pH as was observed in Pony Lake. DIC concentrations were generally low, but comparable to those found in other shallow Antarctic ponds (Hawes et al., 1993).

Conductivity and salinity increased over the course of the austral summer with highest concentrations occurring in mid-January. The brackish character of the melt water was indicative of evaporitic conditions and the presence of marine aerosols. Dissolution of soil salts may also have contributed to the increased ion concentrations in Pony Lake. Salt deposits were observed along the edge of the lake. Melting of accumulated snow can

raise the water level during summer, thereby re-dissolving soil salts. Pony Lake was Cl dominated and showed abundant concentrations of Na and SO_4 ions, which is commonly found in coastal Antarctic ponds (Torii et al., 1988; Schmidt et al., 1991). Similar to changes in ionic concentrations found by Brown et al. (2004), the suite of major ion concentrations in 2004/2005 increased 4-23% during the initial melt period, and 22-48% from the end of December to mid-January. Although Brown et al. (2004) attributed these changes to evaporative concentration processes, wind induced mixing of higher concentrated bottom waters may more likely determine the chemical composition of the water column. Although concentrations increased over the course of the 2004/2005 summer, major ions were considerably lower than compared to previously reported data (McKnight et al., 1994; Brown et al., 2004). Year to year differences in nutrient concentrations have been shown in many other McMurdo Ice Shelf ponds (Hawes et al., 1993); however, in Pony Lake even though ionic concentrations varied from year to year, the overall chemical composition of Pony Lake remained stable.

Dissolved organic carbon (DOC) concentrations (11.75-28.62 mg C L^{-1}) were lower on all four dates sampled during this study than had been previously reported for Pony Lake 95-110 mg C L^{-1} (McKnight et al., 1994) and 32.4-92.4 mg C L^{-1} (Brown et al., 2004). Phytoplankton counts were two to three orders of magnitude lower than detected by McKnight et al. (1994), which probably accounts for the lower DOC concentrations. Although lower than in past studies, DOC concentrations found in Pony Lake during the 2004/05 season were within the range for other Antarctic lakes. For example, DOC concentrations in the McMurdo Dry Valley lakes typically increase with

Table 3.1.: General site characteristics, dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), total nitrogen (TN), nutrient and major ion concentrations for Pony Lake measured during the austral summer 2004/05.

	11 Dec 04	**21 Dec 04**	**29 Dec 04**	**14 Jan 05**
Temp. [°C]	1.3	-	4.2	7.5
Sal. [ppt]	1.86	2.25	3.25	3.93
pH	8.07	8.66	9.71	9.77
Cond. [µS cm^{-1}]	3429	4134	5895	7058
DO [mg L^{-1}]	18.0	>20	18.0	>20
PAR [µmol sec^{-1} m^{-2}]	183	86	512	342
DOC [mg L^{-1}]	11.75	12.35	28.27	28.62
DIC [mg L^{-1}]	10.84	4.30	3.40	4.19
TN [mg L^{-1}]	19.02	14.3	17.23	25.91
Chl-a [µg L^{-1}]	28.0	43.8	94.5	140.1
Nutrients [mg L^{-1}]				
NH_4^+-N	16.64	11.58	13.76	23.53
NO_3-N	2.38	2.72	3.47	2.38
NO_2-N	<D.L.	<D.L.	<D.L.	<D.L.
PO_4^{3-}	2.99	2.70	2.93	4.36
Major ions [mg L^{-1}]				
Cl^-	863	-	-	-
SO_4^{2-}	254	-	-	-
Na^+	547	708	1049	-
K^+	58.9	67.4	92.6	-
Mg^{2+}	62.7	72.9	95.7	-
Ca^{2+}	35	36.8	45.1	-

(-) not analyzed
(D.L.) detection limit

depth, ranging from 20-30 mg C L^{-1} in the bottom waters (Takacs et al., 2001). In contrast to Brown et al. (2004), who reported a ~threefold decrease in DOC concentrations in Pony Lake between 04 Dec and 30 Dec 1997; the DOC concentration in this study increased more than twofold between 11 Dec and 29 Dec 2004. Several processes may contribute to a decrease in DOC concentrations such as changes in phytoplankton blooms, progressive exploitation of the carbon pool during the austral summer by bacterioplankton (Bell & Laybourn-Parry, 1999; Laybourn-Parry et al., 2002;

Brown et al., 2004) or a reduced rate of dissolved organic matter (DOM) production by the algal population (Brown et al., 2004). However, while Brown et al. (2004) observed a shift in phytoplankton composition that was associated with decreasing DOC concentrations during the transition from ice covered to ice free conditions in 1997, in this study both chlorophyte and cryptophyte numbers increased gradually during the observation period. Furthermore, DOC concentrations in Pony Lake during the summer of 2004/05 correlated with chlorophyll-*a* levels (r = 0.93) and phytoplankton numbers (r = 0.82). Nonetheless, the increase in DOC concentration was minimal between the end of December and mid-January while phytoplankton concentrations were more than twice as abundant, suggesting different DOC production rates by these phytoplankton species. As bacterial production rates were markedly reduced in January compared to early December, progressive exploitation of the DOC pool by bacteria is negligible and variations in DOC production rates become even more evident. Conversely, bacterial abundances reached their maximum in mid-January (Table 3.2.), suggesting that alterations in the DOM composition may have altered its availability to the bacteria. Inorganic nitrogen and phosphorous concentrations in Pony Lake were elevated compared to those found in other eutrophic Antarctica lakes (Bell & Laybourn-Parry, 1999; Butler, 1999; Mataloni et al., 1998; Matalioni et al., 2000) with the exception of nitrite, which was below the detection limit (3 $\mu g\ L^{-1}$) throughout this study. Decreasing NH_4–N concentrations during the summer suggest that ammonium was the preferred nitrogen source for phytoplankton, as previously shown by Hawes in several other Antarctic lakes (1983). Maximum primary production rates were detected on 21 Dec

2004 when ammonium concentrations dropped markedly (Table 3.1., Table 3.2.). In contrast, while primary production rates gradually decreased between 21 Dec 2004 and mid January, the NH_4-N concentration peaked during this same time period. It is possible that evaporation led to the increase of ammonium. Furthermore, decomposition of organic matter by heterotrophic bacteria or photolytic reactions (Moran & Zepp, 1997) may have mineralized dissolved organic matter thereby generating NH_4.

Productivity Measurements and Plankton Analyses

In common with other continental Antarctic lakes, Pony Lake is populated by bacteria, viruses, phytoflagellates and protozooplankton. The high levels of inorganic nutrients and DOC concentrations (Table 3.1.) available throughout the study period imply that these factors did not limit summer plankton growth and that other parameters such as temperature, ionic composition, and light intensity may be more important in triggering plankton blooms. Numbers of planktonic organisms enumerated on the four different sample dates are presented in Table 3.2.

Bacterioplankton and Virus Like Particles (VLP): Bacterial abundances ranged between 2.15×10^5-1.36×10^6 cells ml^{-1} with highest numbers found at the end of the sampling period in mid-January (Table 3.2.). Similar abundance ranges were reported from eutrophic maritime or saline Antarctic lakes (Bell & Laybourn-Parry, 1999; Butler 1999). The lower range of the cell numbers in Pony Lake are comparable to bacterial populations enumerated from oligotrophic, maritime lakes (Butler et al., 2000) or the oligotrophic, continental Dry Valley lakes (Takacs & Priscu, 1998). Bacterial

Table 3.2.: Planktonic community abundance and productivity in Pony Lake during the austral summer 2004/05. BP (bacterial production), VLP (virus like particles), PPL (Phytoplankton), PPR (primary production).

	Bacteria [x 10^5 cells ml^{-1}]	**BP** [µg C L^{-1} d^{-1}]	**VLP** [x 10^5 cells ml^{-1}]	**PPL** [x 10^3 cells ml^{-1}]	**PPR** [mg C L^{-1} d^{-1}]	**Ciliates** [x 10^3 cells ml^{-1}]
11 Dec 04	4.05	27.7	3.56	0.16	0.67	1.02*
21 Dec 04	2.15	30.5	2.33	0.75	2.66	3.22
29 Dec 04	3.41	19.7	4.50	1.38	1.49	0.80
14 Jan 05	13.6	20.3	0.37	3.24	1.23	76.00

* including cysts

productivity ranged from 19.7 to 30.5 µg C L^{-1} d^{-1} with highest production achieved in mid December. This differs from the bacterial abundance and DOC concentrations, which were highest in January. It is likely that photochemically induced transformations of the dissolved organic carbon rendered it more refractory and hence less bioavailable for microbial uptake (Benner & Biddanda, 1998; Obernosterer et al., 1999). Photolysis experiments on the bioavailability of photochemically altered DOM from Pony Lake confirm this assumption (Chapter 4). This may also explain why DOC concentrations increased over the course of the austral summer instead of being exploited by the bacterioplankton community. Mixing of the water column and high turbidity may have counteracted the intensity of photodegradation and photobleaching of DOC, thus the formation of refractory photolytic products accumulated slowly throughout the season. Nonetheless, bacterial production measured in Pony Lake was considerably higher than compared to other oligotrophic Antarctic lakes (Takacs & Priscu, 1998; Butler et al., 2000; Laybourn-Parry et al., 2001; Laybourn-Parry et al., 2002; Laybourn-Parry et al., 2004), but lower than reported in other saline or eutrophic Antarctic lakes (Laybourn-Parry et al., 2002). Since the DOC concentrations in these lakes (Laybourn-Parry et al.,

2002) were similar to Pony Lake DOC concentrations, the differences in bacterial production highlight the idea that the late summer DOC pool in Pony Lake was less bioavailable for microbial uptake.

VLP abundances ranged from 3.72 x 10^4-4.5 x 10^5 cell ml^{-1} with highest values occurring at the end of December (Table 3.2.). Bacteriophage have been studied in numerous polar inland waters. A comparison with other Antarctic lakes such as Lake Druzhby, Crooked Lake, and Beaver Lake in the Vestfold Hills or Lake Hoare and East Lobe Lake Bonney in the McMurdo Dry Valleys showed that VLP abundances reported from Pony Lake are in the range of these lakes (see Säwström et al., 2008 for a review). In contrast, VLP numbers in saline lakes in the Vestfold Hills and in oligotrophic Lake Fryxell were two orders of magnitude higher than those found in Pony Lake (Säwström et al., 2008). The virus to bacteria ratio (VBR) in polar inland lakes usually falls between 1 and 34, however exceptionally high ratios (>120) have also been reported for the saline lakes in the Vestfold Hills (Säwström et al., 2008). The fact that bacterial abundances are usually lower than VLP numbers can be attributed to the infectious interactions of VLP with the host cell causing viral induced cell lysis. Conversely, the VBR in Pony Lake ranged between 0.03 to 1.32. Low ratios have been explained in part by the autochthonous nature of carbon substrates in Antarctic lakes (Säwström et al., 2008). Further, low bacterial growth rates in polar water do not sustain high infection rates. On average, only four viruses are released from each bacterial cell (Laybourn-Parry, 2009). Viral processes and infectivity have also been related to a range of abiotic factors. Madan et al. (2005) reported a negative correlation between VLP and temperature, indicating

lower decay rates under lower temperatures. Nutrient limitations can reduce bacterial proliferation, thereby indirectly affecting VLP numbers (Weinbauer, 2004). Solar radiation causes damage to viruses and the loss of viral infectivity (e.g. Wilhelm et al., 1998). Although the VBR was low in Pony Lake, the interaction between viruses and bacteria may play an important role in carbon and nutrient cycles in aquatic systems. Lisle and Priscu (2004) reported the occurrence of ~26% of lysogenic bacterial cells in the trophogenic zone in Lake Bonney, Antarctica. These virus infected cells could release ~23% of the bacterioplankton carbon demand (Säwström et al., 2008). Thus, virus induced release of organic carbon from bacteria can provide a significant portion of the DOC pool and importantly short-circuit the carbon cycle before bacterial produced carbon is removed by protozoan grazing.

Phytoplankton: Within Pony Lake the phototrophic nanoflagellate (PNAN) assemblage consisted of chlorophytes and cryptophytes (Fig. 3.2.). These phyla were at a maximum of 2.40×10^3 and 7.60×10^2 cells ml^{-1}, respectively in mid-January. In contrast, heterotrophic nanoflagellates (HNAN) were not detected in Pony Lake. The dominance of chlorophytes and cryptophytes in Pony Lake was previously reported by McKnight et al. (1994) and Brown et al. (2004). In general, it has been observed that these phyla dominate numerous Antarctic lakes (Butler et al., 2000; Roberts et al., 2000; Roberts et al., 2004), while *Chlamydomonas* species are typically found in eutrophic Antarctic lakes (Hawes, 1990; Mataloni et al., 1998; Butler, 1999). Variations in species abundance and the timing of phytoplankton blooms have been documented in Pony Lake between the different years it has been sampled (McKnight et al., 1994; Brown et al.,

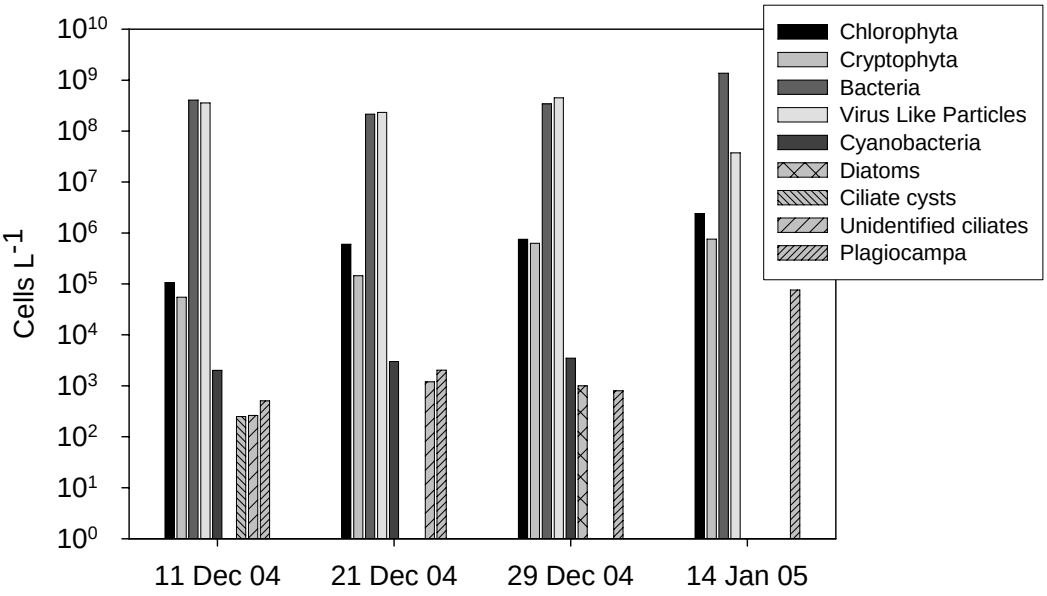

Figure 3.2.: Planktonic composition of Pony Lake during the austral summer 2004/05. Changes in the planktonic composition are displayed by the gradual increase of algal species (chlorophytes and cryptophytes), the increasing abundance of ciliates (e.g. *Plagiocampa*) and the short term appearance of cyanobacteria and diatoms during the observation period.

2004; and this study). Changes in abundances and algal blooms between years have also been observed by Mataloni et al. (1998) in the highly eutrophic Otreo Lake, Antarctica. Whereas McKnight et al. (1994) detected the dominance of chlorophytes in Pony Lake at the end of January 1994, the study by Brown et al. (2004) revealed that after the loss of the ice cover in mid-December 1997 the chlorophyte bloom was displaced by a bloom of crytophytes. Dominance of a particular phylum or a distinct algal bloom was not observed in the current study, where both chlorophytes and cryptophytes increased gradually during the transition from an ice covered to an ice free lake. In past studies, algal blooms also seemed to correlate with DOC concentrations in Pony Lake. McKnight

et al. (1994) reported high DOC concentrations during the chlorophyte bloom; and Brown et al. (2004) observed a decrease in DOC concentrations when the chlorophyte bloom was replaced by a cryptophyte bloom, underscoring the previous argument that different algal species exhibit different DOC production rates.

Primary production in Pony Lake ranged from 0.67 to 2.66 mg C L^{-1} d^{-1}, with highest rates measured in mid December. In general, primary productivity in Pony Lake was higher than found in other more oligotrophic Antarctic lakes (Priscu, 1995; Butler et al., 2000; Laybourn-Parry et al., 2001; Laybourn-Parry et al., 2002). However, similar or even larger primary productivity rates have been reported in several Antarctic lakes across trophic levels (Butler, 1999, Laybourn-Parry et al., 2002). Variations in the photosynthetic community structure between lakes may partially account for the differences in primary production (Laybourn-Parry et al., 2002). The December maximum was also observed in other Antarctic lakes (Laybourn-Parry et al., 2002). Similar to the findings of Laybourn-Parry et al. (2002) in several lakes in the Vestfold Hills, chlorophyll-*a* and phytoplankton numbers in Pony Lake did not follow the primary production peak. Chlorophyll-*a* concentrations in Pony Lake (Table 1) were highest in mid-January (140.1 µg L^{-1}) and correlated with the phytoplankton maxima ($r = 0.97$), but were weakly negatively correlated to primary production rates ($r = -0.12$). In many Antarctic lakes chlorophyll-*a* concentrations are typically <3 µg L^{-1} (Ellis-Evans et al., 1998; Vinocur & Pizarro, 2000). Chlorophyll-*a* concentrations >100 µg L^{-1} have only been reported from eutrophic Antarctic ponds or lakes (Butler, 1999; Vinocur & Pizarro, 2000).

Protozooplankton: Ciliated protozoa achieved their maximum concentration in mid January 2005. Concentrations were generally low, with only one dominant morphotype *Plagiocampa* sp. (Fig. 3.2.) identified. Similarly, in Lake Fryxell *Plagiocampa* accounted for >80% of the total ciliate biomass (Roberts et al., 2000). Although ~30% of the total number of ciliates remained unidentified (equivalent to ~1 cell ml^{-1}), ciliated protozoans were poorly represented in Pony Lake compared to other Antarctic lakes. 24 species have been found in Lake Hoare (Roberts et al., 2004), 25 species in Lake Fryxell (Roberts et al., 2000) and 23 species were recorded in Lake Heywood (Butler, 1999). Ciliate cysts were found in the water column of Pony Lake at the beginning of the transition period from ice covered to ice free. Because productivity is seasonally limited, ciliates form cysts as a strategy to survive unfavorable conditions during the winter (Mataloni et al., 1998; Bell & Laybourn-Parry, 1999; Roberts et al., 2000). Since cysts appeared in the water column at the beginning of the melt season they were probably released from the ice or re-suspended from the sediments.

Due to the absence of metazoan predators and the low abundance of raptorial ciliates (e.g. *Plagiocampa*) in Pony Lake that are capable of feeding on other organisms it seems unlikely that ciliates strongly controlled PNAN or bacteria by grazing. Cryptophytes may cause additional grazing pressure on bacteria as these photosynthetic flagellates are capable of mixotrophy (Roberts & Laybourn-Parry, 1999). However, cryptophytes in Lake Hoare were only able to graze ≤3% of bacterial biomass per day (Roberts & Laybourn-Parry, 1999). Consequently, there appears to be little top-down control in the food web, a general characteristic of lakes throughout Antarctica (Priscu et

al., 1999; Roberts et al., 2004). Nonetheless, top-down control has been demonstrated when metazoa (microcrustacean larvae) or large ciliates were more abundant (Laybourn-Parry et al., 1996; Mataloni et al., 2000).

Cyanobacteria and diatoms: Cyanobacteria and diatoms accounted for <0.5% of the total phytoplankton population in Pony Lake. Cyanobacteria were rare (~3 cell ml^{-1}) throughout December and undetectable in mid-January 2005 (Fig. 3.2.). The dominant cyanobacteria species found in Pony Lake belonged to members of the *Oscillatoria* and *Phormidium* genera. There was only one sampling date in which diatoms were present in the phytoplankton (29 Dec 2004). In general, the absence of a well-developed planktonic diatom flora is not unusual in Antarctic lakes, where most of the biological production is benthic (Jones, 1996). Low numbers of planktonic diatoms have been found in other continental Antarctic Lakes (Ellis-Evans et al., 1998; Butler, 1999). Typically the greatest accumulation of cyanobacterial biomass in Antarctic lakes and ponds occurs in benthic microbial mats (Vincent, 2000). Regardless, mixing events may have re-suspended benthic organisms in the water column (Mataloni et al., 2000). However, in this study phytobenthic samples were not collected, thus the presence of benthic algal mats can only be hypothesized.

DGGE Fingerprinting Analysis

DGGE banding patterns, analyzed with GelCompar II software, were used to compare the complex microbial community composition between samples dates (Fig. 3.3.). We further compared the DGGE patterns from these Pony Lake water samples to the DGGE patterns from Pony Lake ice core samples, which were collected earlier in November 2004 when the lake was frozen solid to its base. This analysis demonstrated that the bacterial community structure in Pony Lake changed during the transition from ice covered to ice free conditions. Three different clusters were distinguishable. The water sample collected on 11 Dec 2004 was most closely related to the ice core sample profile. This suggests that the microbial community found at the beginning of the transition period consisted of a bacterial assemblage which was seeded from the ice. Melt proceeded rapidly after 11 Dec 2004 over the following weeks and physicochemical changes occurred in the water column (Table 3.1.). Further, winds caused mixing of the shallow water column and algal blooms were observed. It can be surmised that these changing physicochemical parameters were responsible for changes in the community structure. Samples collected on 21 Dec and 29 Dec formed a second cluster. A third group could be distinguished in mid January 2005 when the warmest water temperatures, highest plankton abundances, and ionic concentrations were measured.

It has been reported that the DGGE method can only retrieve members of the dominant species within a community (Schaefer & Muyzer, 2001) and DGGE may have limited sensitivity when applied to complex communities. Traditional gel-based DGGE is estimated to detect only community members who represent at least 1-2% of the

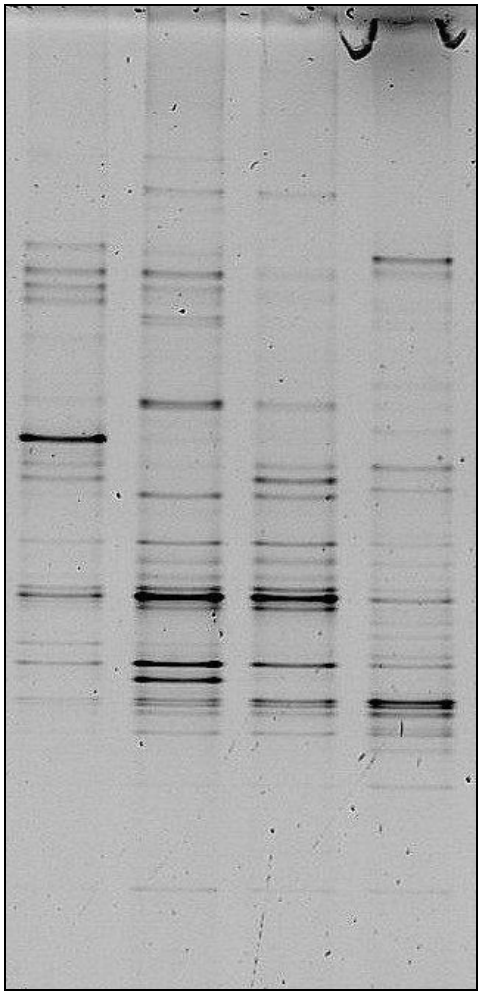

Figure 3.3.: DGGE profiles of microbial communities from different Pony Lake water samples collected between 11 Dec 2004 and 14 Jan 2005 show a shift in community structure. From left to right: 11 Dec 04, 21 Dec 04, 29 Dec 04, 14 Jan 05. Image colors were inverted on the camera, but not manipulated.

microbial population in an environmental sample (Nocker et al., 2007c). Therefore, absence of bands does not necessarily imply that an organism or a group of organisms is not present. Similarly, single species can produce a number of different bands (Pearce, 2003). Nevertheless, this profiling method is a useful technique for rapidly screening and comparing communities. However, in order to obtain more detailed information on the community structure, we constructed clone libraries.

16S rRNA Sequences

The relative distribution of major phylogenetic groups found within each clone library from the individual sampling dates is shown in Figure 3.4., while the phylogenetic relationship of Pony Lake clones to the closest neighbors, according to a BLAST search, is summarized in Table 3.3. A total of 37 clones were identified from the three sampling dates. *β-proteobacteria* represented the dominant fraction of clones for each date (47.1% of ED, 64.7% of LD, and 50.0% of MJ). This group also showed more compositional overlap between the three clone libraries than the other phylogenetic groups. For example, *Bordetella trematum*, *Hydrogenophaga taeniospiralis*, and *Hydrogenophaga atypical* were the closest reported relatives to clone sequences that occurred in all three libraries. In addition, many clones appeared on two of the three different sampling dates. Members of the *γ-proteobacteria* comprised the second largest group making up 29.4% of ED, 11.8% of LD, and 20.0% of the MJ clone libraries. *Actinobacteria, Bacteriodetes,*

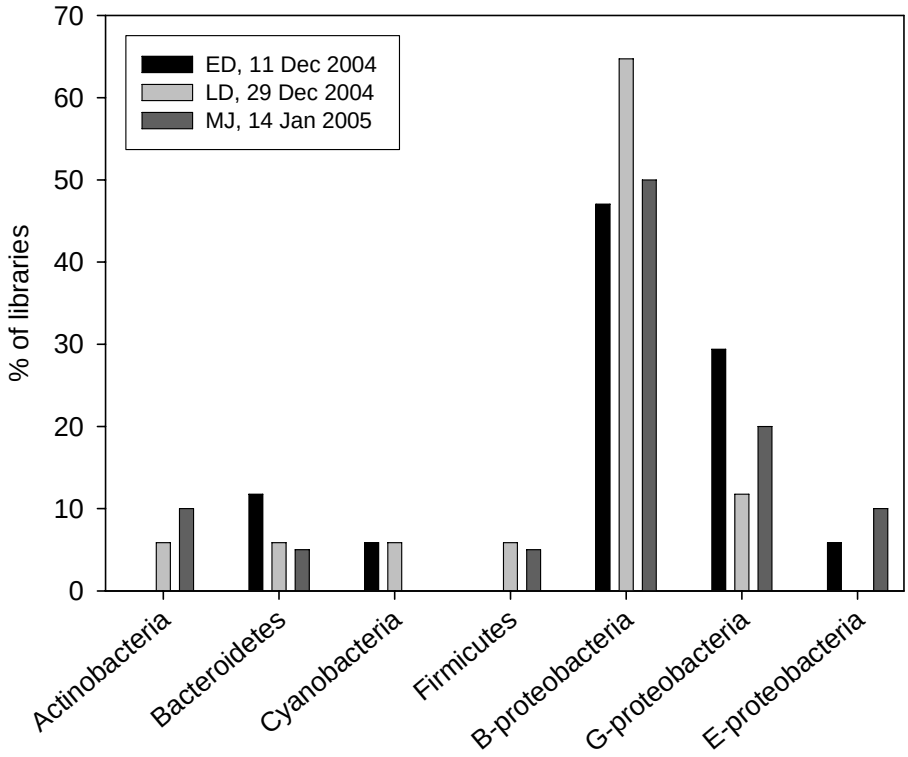

Figure 3.4.: Distribution of taxonomic classes within the Pony Lake water sample clone libraries from three sampling dates.

Table 3.3.: Affiliation of 16S rRNA gene phylotypes in Pony Lake lake water. Pony Lake clones with relatives reported from other Antarctic environments are labeled with an asterisk (*). Many of the close relatives from Antarctic environments were uncultured bacteria or clones. To allow for better comparison of Pony Lake clones, clearly identified closest neighbors from non Antarctic environments were assigned instead. These clones are labeled with (†).

Taxonomic phylum	Taxonomic class	16S rRNA dentification (closest neighbor)	GenBank no. (closest neighbor)	Sampling Date					
				11 Dec 2004		29 Dec 2004		14 Jan 2005	
				Clone	% id.	Clone	% id.	Clone	% id.
Actinobacteria	*Actinobacteridae*	*Candidatus aquiluna rubra* MWH-CanK2	AM999977			ANTPL_LD03†	98	ANTPL_MJ01†	98
		Salinibacterium xinjiangense 0543	DQ515964					ANTPL_MJ11†	98
Bacteroidetes	*Flavobacteria*	*Flavobacterium weaverense* AT1042	AY581114	ANTPL_ED02*	96				
		Cryomorphaceae bacterium Haldis-1	FJ424814			ANTPL_LD54	95		
	Uncultured	Uncult. *Flavobacteriaceae* bacterium F4C94	AY697925	ANTPL_ED16*	97				
		Uncult. *Bacteroidetes* bacterium 1D5	AJ627991					ANTPL_MJ40	97
Cyanobacteria	*Oscillatoriales*	*Phormidium autumnale* Ant-Ph68	DQ493874	ANTPL_ED03*	99	ANTPL_LD39*	99		
Firmicutes	*Clostridia*	*Clostridium acidiurici*	M59084			ANTPL_LD31			
	Lactobacillales	*Carnobacterium* sp. NJ-46	AM396913					ANTPL_MJ13*	98
Proteobacteria	*β-proteobacteria*	*Acidovorax* sp. G3DM-83	EU037287	ANTPL_ED57	99				
						ANTPL_LD27	99	ANTPL_MJ53	99
		Acidovorax sp. BSB42	*Y18617*			ANTPL_LD02	100		
		Acidovorax delafieldii DSM 50263	*AJ420323*			ANTPL_LD05	100		
		Acidovorax sp. UFZ-B530	AF235013					ANTPL_MJ30	99
		Achromobacter sp. EP24	AM403526					ANTPL_MJ10†	95
		Bordetella trematum DSM 11334 (T)	AJ277798	ANTPL_ED22†	98	ANTPL_LD42†	99	ANTPL_MJ28†	98
		Herminiimonas arsenicoxydans	CU207211					ANTPL_MJ59	99
		Hydrogenophaga taeniospiralis SE57	AY771764	ANTPL_ED04†	99			ANTPL_MJ08†	98
				ANTPL_ED49†	100	ANTPL_LD34†	100		
						ANTPL_LD08†	99	ANTPL_MJ09†	99
		Hydrogenophaga taeniospiralis	AF078768	ANTPL_ED06†	98	ANTPL_LD41†	98	ANTPL_MJ24†	98
		Hydrogenophaga atypical BSB 41.8T	AJ585992	ANTPL_ED32†	97	ANTPL_LD10†	97	ANTPL_MJ25†	97
		Rhodoferax antarcticus	AF084947	ANTPL_ED09*	98	ANTPL_LD15*	98		
						ANTPL_LD36*	96	ANTPL_MJ32*	96
		Rhodoferax antarcticus Fryx1	AY609198	ANTPL_ED66*	99				
		Rhodoferax ferrireducens T118	CP000267			ANTPL_LD07†	100		
	γ-proteobacteria	*Glaciecola polaris* LMG 21857	AJ293820	ANTPL_ED10†	97				
				ANTPL_ED68†	95				
		Glaciecola punicea ACAM 611T	U85853	ANTPL_ED30*	99	ANTPL_LD06*	99	ANTPL_MJ48*	99
		Glaciecola pallidula ACAM 615T	U85854					ANTPL_MJ02*	93
		Pseudomonas sp. An18	AJ551156					ANTPL_MJ27*	99
		Rheinheimera perlucida BA131T	AM183347	ANTPL_ED48	97				
		Rhodanobacter lindaniclasticus	L76222					ANTPL_MJ14†	95
		Shewanella baltica NCTC10735	AJ000214			ANTPL_LD26†	99		
	Uncultured	Uncult. *Glaciecola* McMurdo.541	AF277554	ANTPL_ED51*	99				
	ε-proteobacteria	*Arcobacter cibarius* LMG 21997	AJ607392	ANTPL_ED61	98			ANTPL_MJ35	98
		Arcobacter cryaerophilus	U25805					ANTPL_MJ23	92

cyanobacteria, *Firmicutes*, and *ε-proteobacteria* were described by only one or two sequences each. With the exception of *Bacteroidetes* for ED (11.8%), these sequence types made up less than 10% of each clone library. In concurrence with the planktonic compositional analysis of Pony Lake (Fig. 3.2.), cyanobacteria sequences were not found in the sample from 14 Jan 2005.

Overall, approximately 74% of the closely identified neighbors were reported from various Antarctic lake and marine environments. It should be noted that the closest relatives listed in Table 3.3. do not necessarily represent a linkage to bacteria from Antarctic environments. Since many of the closest phylogenetic neighbors from Antarctic habitats are documented in GenBank as unidentified or uncultured bacteria, clearly identified bacteria from non-Antarctic environments were assigned in the table instead. These unidentified or uncultured relatives were primarily related to *Flavobacteria* (Van Trappen et al., 2002; Glatz et al., 2006; Mosier et al., 2007), *Cytophaga* (Glatz et al., 2006), *β-proteobacteria* (Van Trappen et al., 2002; Pearce et al., 2005), *γ-proteobacteria* (Van Trappen et al., 2002), Gram-positive *Actinobacteria* (Van Trappen et al., 2002; Glatz et al., 2006; Mosier et al., 2007), uncultured bacterial clones from sediments and freshwater lakes (Pearce et al., 2005) and from penguin droppings. Cyanobacteria clones were related to uncultured clones from Lake Vida (Mosier et al., 2007) or the genus *Phormidium* (Comte et al., 2007). Although many of the Pony Lake clones were phylogenetically distinct from the nearest sequences in the database, the presence of related phylotypes from geographically diverse cold environments reveals a high level of similarity for microorganisms inhabiting these ecosystems.

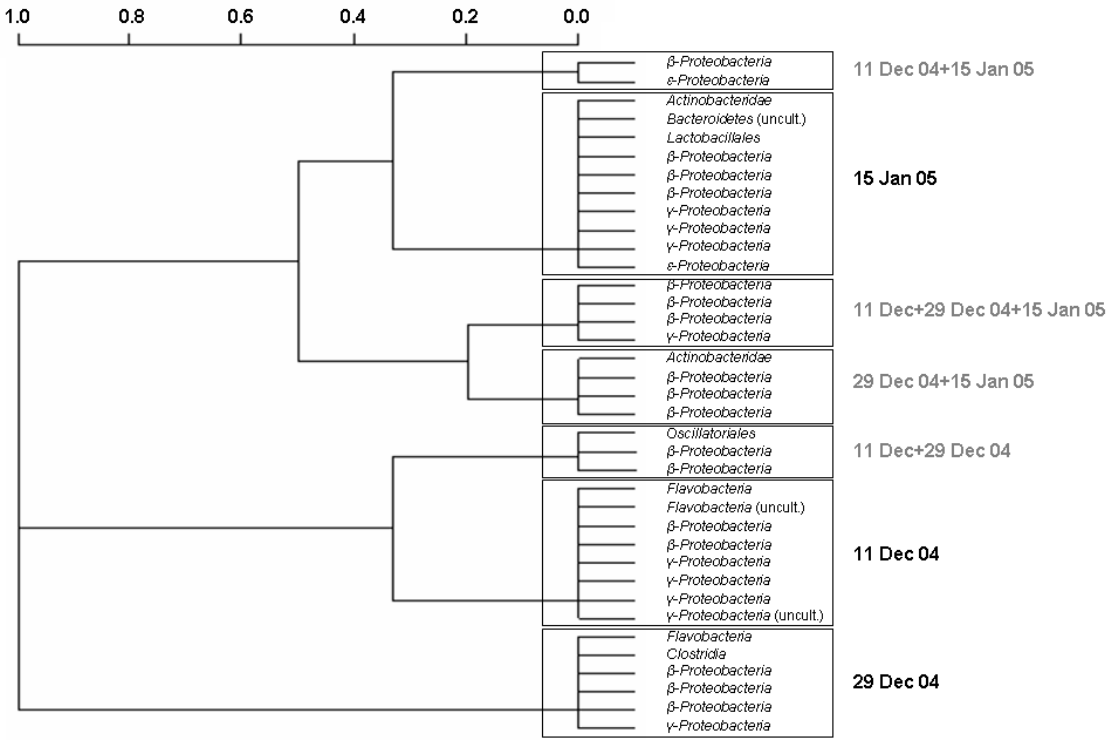

Figure 3.5.: Cluster analysis of closely related taxonomic classes or phyla to distinct clones found within Pony Lake water samples during the transition from ice-covered to ice-free conditions. The dendrogram shows distinct clusters of Pony Lake clones found in the water column on individual sampling dates and highlights clone library overlap between sampling dates. The horizontal axis describes the loss of information, in percentage, at each level of agglomeration (1 = 100%).

An important goal of the phylogenetic analysis was to characterize any changes in the microbial community composition over the course of the austral summer in Pony Lake. A comparison based on the presence or absence of sequence types was used to discriminate differences between clone libraries (Fig. 3.5.). Distinct clusters as well as overlap can be seen for each individual clone library. Several features of this analysis indicate that a shift in the bacterioplankton community did occur during the austral summer of 2004/05. Clone libraries from early-December and mid-January consisted of large numbers of clones (50% each) that were restricted to these individual sampling

dates. Compositional overlap between clone libraries was small and more likely to occur between consecutive sampling dates. Only four sequence types were apparent on all three sampling dates. It can be hypothesized that the shift in community composition was caused by seasonal changes in water quality, increasing water temperatures, interactions between community members, or most likely based upon the data, in the nature of the changing composition of the DOM pool. We demonstrated that the microbial community evolved differently when exposed to DOM from Pony Lake over a 12 day incubation period (Chapter 4).

A change in the community structure also appeared when the lake transitioned from a solid frozen system to an open water environment. 40 and 37 unique sequence types were assigned to the Pony Lake ice column (Chapter 2) and Pony Lake water (Table 3.3.), respectively. Interestingly, only 8 sequence types could be identified in both environments, with the highest degree of sequence overlap found among members of the *β-proteobacteria* lineage (5 sequence types). Representatives from the *δ-proteobacteria*, *Spirochaetes*, and *Verrucomicrobia* lineages were only present in the ice core samples (Chapter 2). In contrast, the phyla *Actinobacteria* and cyanobacteria were only found in the Pony Lake water samples. Clear differences in phylotype richness were observed within the different taxonomic classes. Within the most dominant classes, *Bacteroidetes*, *β*-, *γ*-, and *ε-proteobacteria*, the total numbers of sequence types diverged widely. In the Pony Lake ice column a total of 10, 10, 1 and 9 different sequence types were related to *Bacteroidetes, β*-, *γ*-, and *ε-proteobacteria* respectively (Chapter 2). In contrast, lake water samples revealed 4, 17, 9, and 2 different sequence types for these groups.

Summary

This study presents the biogeochemical progression of a brackish, eutrophic lake at Cape Royds, Antarctica, as it transitions from ice-covered to ice-free. The lake geochemistry reflects the influence of marine aerosols due to its close proximity to the Ross Sea. The lake was Cl dominated and showed abundant concentrations of Na and SO_4 ions. Strong winds and the shallow nature of Pony Lake impeded the formation of thermal or chemical stratification within the water column. Changes in the lake chemistry were driven by compositional shifts, the lack of inflow, and mixing of the water column over the course of the austral summer. The planktonic community consisted of bacteria, viruses, phytoflagellates and protozooplankton. 16S rRNA analysis of the bacterioplankton revealed a total of 37 unique sequence types. Importantly, approximately 74% of the clones were related to microorganisms from other Antarctic lake and marine environments. Further, our findings indicate that a shift occurred in the microbial community structure between early December and mid January most likely due to alterations in the bioavailability of DOM. This study was part of a comprehensive research program to understand the effects of physical, chemical, and microbial processes on the composition and character of the DOM pool in Pony Lake and provides new insight into seasonal changes of physicochemical and biological processes in coastal Antarctic lakes. Moreover, the "seasonal" data set highlights the idea that organisms that persist throughout the year in Pony Lake must be capable of adapting to rapidly changing conditions and surviving severe environmental factors while captured in the ice column (Chapter 2).

CHAPTER 4

TEMPORAL VARIABILITY AND STABILITY OF PHYSICOCHEMICAL PARAMETERS AND BACTERIOPLANKTON ASSEMBLAGES IN A COASTAL ANTARCTIC LAKE

(Supplement to Chapter 2 and Chapter 3)

Introduction

In addition to the extensive study of Pony Lake conducted during the summer season 2004/05, Pony Lake was investigated in the following season 2005/06. As described in Chapter 2 and Chapter 3 microbial dynamics and lake energetics strongly depend on the physical state of the lake. Planktonic communities thrive in the presents of liquid water during summer, but are limited within the ice column. It is typical for high latitude, Antarctic habitats to experience large seasonal fluctuations in climatic conditions. Thus, during the summer months one may expect Pony Lake to be partially or completely ice-free, water temperature will be highest and salinity will be low relative to brine solutions during complete freeze up. However, a comparison to previous reports (McKnight et al., 1994; Brown et al., 2004) also revealed year to year variations in the chemical and phytoplankton composition of Pony Lake. Since the time frame of the open water period of Pony Lake is controlled by air temperature, wind, and snow accumulation during the short Antarctic summer, annual variabilities in physicochemical and biological factors are not surprising. Figure 4.1. highlights differences in the appearance of Pony Lake between two consecutive seasons 2004/05 and 2005/06 when environmental

Figure 4.1.: Observed differences in the appearance of Pony Lake over the course of the austral summer 2004/05 and 2005/06. A+B: ice core collection in November (B shows the massive snow accumulation on Pony Lake), C+D: ice cores (D displays the difference in coloration the bottom section of an ice core collected in 2005). E: *In situ* measurements in late December 2004 (wind mixing). F: measurements early January 2006 (calm).

conditions led to distinct differences. Ice core samples collected in mid November in 2004 and 2005 were from ice of various ages. The cores collected in 2004 probably represented refrozen lake water from the previous austral summer (2003/04). Conversely, cores from 2005 are believed to be from an area that remained frozen for at least two years (Foreman, personal communication). Differences were observed in coloration of the ice cores. Cores from 2004 were clear near the surface, the middle section was slightly yellow and the bottom 30 cm were dark yellow to brown. On the other hand, ice cores from the second year were clear, colorless and only became slightly yellow towards the base.

During the season 2004/05 the transition period from a solid frozen lake to open water system started in early-December and the lake became mostly ice free by the end of December - beginning of January. Winds caused mixing of the shallow water column and algal blooms were observed. In contrast, 2005/06 was an unusually snow rich year and Pony Lake was buried under one meter of snow cover. Only at the end of December did a small area at the west shore of the lake become ice free. Moreover, on most days when water samples were collected the surface of the lake was calm and no mixing was observed. In conjunction with these observational differences in ice and lake characteristics, the physical, chemical and biological conditions exhibited seasonal variations as well between the two studied seasons (2004/05, 2005/06).

Table 4.1.: A comparison of productivity rates and bacterial abundances detected in ice cores from Pony Lake collected in November 2004 and 2005. Bacterial production (BP) and primary production (PPR) in ~40 cm ice core sections (top, middle, bottom)

	2004			**2005**		
	Top	**Middle**	**Bottom**	**Top**	**Middle**	**Bottom**
BP [µg C L^{-1} d^{-1}]	6.69	37.9	1.80	2.13	1.95	1.98
PPR [µg C L^{-1} d^{-1}]	931	308	31	31	15	40
Bacteria [cells ml^{-1}]	2.20×10^6	1.10×10^6	2.97×10^7	2.13×10^7	4.09×10^7	2.40×10^7

(-) not analyzed

The purpose of this chapter is to characterize the temporal (seasonal and interseasonal) variability or stability of physicochemical conditions in Pony Lake and relate these physicochemical factors to microbial processes and community structure. Focus is thereby directed on physical, chemical and microbial dynamics present within the ice column of Pony Lake as well as the summer melt water. The study uses existing data from Pony Lake documented in the literature (McKnight et al., 1994; Brown et al., 2004), results from 2004/05 and 2005/06 in an integrated, comparative survey to provide a better understanding of the temporal dynamics in a coastal Antarctic lake. Methods used for the physical, chemical and biological characterization of Pony Lake ice cores and water samples collected during the summer season 2005/06 were previously described in Chapter 2 and Chapter 3.

Pony Lake Ice

The area of Pony Lake that melts out during the austral summer varies from year to year, thus certain parts of the lake stay frozen for longer periods. Ice cores collected in November 2005 represent an area that had remained frozen for at least two years. Ice cores from 2004 and 2005 show interesting similarities as well as differences in the chemical composition and the behavior of the microbial community. Four distinct features can be identified in the ice cores from 2005 compared to cores from the previous year (Table 4.1., Table 4.2.): i: the ionic constituents in the basal ice cores were more diluted, with the exception of K and Mg. ii: dissolved organic and inorganic carbon, and total nitrogen concentration were two to sevenfold decreased in the bottom section, whereas dissolved organic carbon and chlorophyll-*a* were two to threefold increased in the middle core section. iii: the chemical composition of the top core was similar in both years. iv: production rates in the top and middle core segments were markedly lower.

Based on the 'Ringer-Nelson-Thompson' model, mirabilite (Na_2SO_4) precipitates during seawater freezing at -8.2°C, hydrohalite ($NaCl{\cdot}2H_2O$) at -22.9°C, sylvite (KCl) and $MgCl_2{\cdot}12H_2O$ at -36°C, and antarcticite ($CaCl_2{\cdot}6H_2O$) at-54°C (Marion & Farren, 1999). Healy et al. (2006) predicted precipitation of calcite ($CaCO_3$), dolomite ($CaMgCO_3$) or magnesite ($MgCO_3$) beginning at temperatures from 0 to -5°C for melt water ponds in the McMurdo Dry Valleys. However, the precipitation of salts is complex in nature. For instance, at -22.9°C hydrohalite begins precipitation thereby lowering the molarity of Na. This causes the dissolution of mirabilite and increases SO_4 molarity, which in turn initiates gypsum ($CaSO_4{\cdot}2H_2O$) precipitation (Marion & Farren, 1999).

Table 4.2.: Chemical parameters measured in ice cores collected from Pony Lake in November 2004 and 2005. Ice cores were divided into ~40 cm sections (top, middle, bottom). Dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), total nitrogen (TN), chlorophyll-*a* (Chla).

	2004			2005		
	Top	**Middle**	**Bottom**	**Top**	**Middle**	**Bottom**
DOC [mg L^{-1}]	21.84	28.50	135.90	21.2	51.57	57.02
DIC [mg L^{-1}]	7.06	18.60	101.78	6.46	13.87	32.31
TN [mg L^{-1}]	20.44	46.49	185.23	5.47	19.48	25.24
Chla [µg L^{-1}]	59	40	296	46	123	305
Major ions [mg L^{-1}]						
Cl^-	437	1345	6253	506	1710	3527
SO_4^{2-}	299	817	4643	237	1865	3432
Na^+	344	-	> D.L.	364	1673	3395
K^+	28.3	-	39.0	17.1	83.5	234
Mg^{2+}	27.3	-	32.9	35.4	98.2	214
Ca^{2+}	14.2	-	110.0	16.9	43.1	82.1

(-) not analyzed
(D.L.) detection limit

Temperatures in basal brines have been reported for numerous Antarctic ponds ranging from -13 to -22°C (Schmidt et al., 1991, Healy et al., 2006; Wait et al., 2006). Hawes et al. (1999) recorded temperatures in ponds of the McMurdo Ice Shelf down to -35°C. Although temperature has not been measured in Pony Lake during winter months within the ice or in the basal brine, it can be assumed that Pony Lake experienced temperatures low enough to cause salt precipitation. Therefore, temperature is not considered as the regulating factor to explain the differences between ionic concentrations in ice cores from 2004 and 2005. Solutions will require a certain degree of saturation before temperature induced precipitation of minerals is initiated (Marion & Farren, 1999). For instance, for a NaCl-H_2O system, the eutectic temperature is -21°C at a salinity of 230 ppt (Medjani, 1996). Due to the lower concentrations of Cl, Na, and SO_4 in Pony Lake bottom ice cores from 2005 it seems very unlikely that eutectic conditions were approached. Therefore, the diluted nature of the ionic constituents itself may explain

the elevated K and Mg concentration in cores from 2005 compared to 2004 since the precipitation of K and Mg bearing salts would not have occured.

Although ice cores from 2005 were collected from an area that stayed frozen for at least two summer periods there is evidence that this area was not always frozen solid during summer months, but rather was partially ice covered with an underlying water column. However, since no observations have been recorded about the physical state of Pony Lake between 1998 and 2004 further explanations are purely speculative. Schmidt et al., (1991) demonstrated that two ponds at Cape Evans, Ross Island, were frozen over from February to November with complete freeze up to base observed from late July until December. At no time were these ponds completely ice free. Even during the summer melt period from December to late January, an ice cover remained in the center of these ponds, but was reduced to 20 cm in thickness. Evidence for a similar scenario of a partially ice covered Pony Lake may be provided by the similarities in the chemical composition of the top core sections and in the differences found between the middle and bottom sections from both years (Table 4.2.). Chemical constituents in the middle and bottom core sections from 2005 especially point towards the presence of liquid water during previous summer months and the enhanced effect of microbial induced alterations in the concentration of these compounds due to production and consumption. Dissolved organic carbon and chlorophyll-*a* concentrations in the middle core section from 2005 were ~2 and 3 time higher compared to the middle core from 2004. On the other hand dissolved inorganic carbon and total nitrogen levels were decreased in this core section in 2005. Assuming the presence of liquid water, it appears that ambient light levels

underneath the ice cover were sufficient to sustain a viable phytoplankton community. Similarly, in the bottom core fragment from 2005 dissolved organic and inorganic carbon and total nitrogen were depleted compared to the bottom section in 2004. The concentration of these chemical constituents may be an indicator of active planktonic communities, but more importantly may highlight differences in metabolic efficiencies between bacterioplankton and phytoplankton. The data suggest that during periods where liquid water was present, bacterioplankton effectively exploited the dissolved organic carbon pool. Nonetheless, primary and secondary production rates were clearly reduced in core sections from 2005 with the exception of the bottom fragment (Table 4.1.). Ice cores collected in 2005 strongly smelled of H_2S, which implies that the bottom core sections were anaerobic. Consequently, aerated ice meltwater would not have provided a suitable growth environment for an anaerobic community, which may explain the low production rates measured in the bottom core section. In contrast, low production rates in the middle core segment were unexpected. Using the production measurements from the top section 2005 as a reference, the key may lie in the age of the ice. Under the assumption that over several austral summers Pony Lake was at least ice covered in that area, the microbial community in the top core remained entrapped within the ice enduring temperatures below zero, desiccation, starvation, solar radiation and periods of complete darkness. Thus, this entrapment may have severely compromised the physiology of the microbial assemblage over time. Further, although there was indication that active, microbial processes occurred beneath the ice cover, the low bacterial production rates

detected in 2005 in the middle core section suggest that the deeper layers of Pony Lake remained frozen for a longer period of time.

Pony Lake Water

Temperature and Dissolved Oxygen

Temperature varies greatly over seasonal and diurnal timescales in Pony Lake. Brown et al. (2004) reported water temperatures ranging between 0.6 °C on 04 December 1997 and 3.9°C on 19 January 1998. During 2004/05 lake water temperature increased gradually between 11 December 2004 and14 January 2005 from 1.3 to 7.5 °C. In 2006 several physical and chemical features including temperature were recorded daily in Pony Lake over a two week period from 07 January to 20 January (Fig. 4.2.). Maximum temperature was reached on 07 January (6.2°C). Diel fluctuations were large (up to 3°C) and at the end of the study period the water temperature dropped to 1.7°C. Accounting for diel changes in water temperature over the range of several degrees Celsius it seems justifiable to attach less value to seasonal variations in temperature since the available data represent only single measurements on chronologically different dates between seasons. However, focus should be directed on significant temperature volatilities between January 2005 and January 2006. Importantly, the highest temperature in 2006 was recorded one week earlier compared to the previous season and 1.3°C lower. Lower water temperatures may affect Pony Lake in several ways. Earlier temperature maxima followed by a successive decrease in water temperature would mean a shorter growth season for the planktonic community inhabiting this lake. Further, temperature inevitably

affects the interior of a cell. Exposure to lower temperatures will decrease membrane fluidity, enzyme-substrate affinity and reaction rates, which may lead to a lower overall growth rate. This in turn would cause a slower accumulation of biomass in the planktonic community.

Pony Lake was generally saturated or supersaturated in terms of oxygen concentration. Whereas strong winds mixed the water column during the summer 2004/05, calm weather conditions were noticed on most sampling days in January 2006. The two week oxygen profile obtained during this month provides an indication that Pony Lake was stratified in late summer 2006. Fig. 4.2. displays the oxygen content in the surface water column of Pony Lake. Diel variations were evident and oxygen concentrations typically ranged between 18.1 to 19.5 mg L^{-1}. However, on a few days abrupt changes in oxygen concentrations were detected (largest difference 5.3 mg L^{-1}). On these days, strong winds disturbed the otherwise calm surface water of Pony Lake. The synchronization of both events points towards an established stratification of the water column that became unstable by wind induced mixing. By this means, Pony Lake was divided into a smaller oxygen saturated top layer induced by water-air exchange of gases and biologically produced oxygen, and a large oxygen depleted bottom stratum, dominated by oxygen consuming processes. In the case of a mixing event the oxygen depleted bottom water would provoke a decrease in oxygen levels in the upper water layer. The predominance of oxygen consuming processes towards the base of the lake would be in clear contrast to the summer fluid geochemistry of many Antarctic ponds

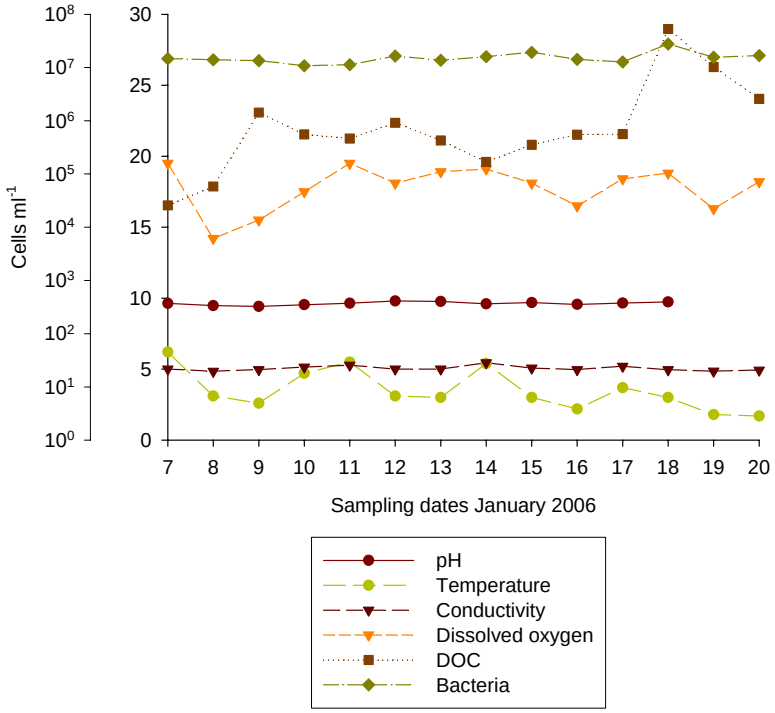

Figure 4.2.: Diel changes and stability of physicochemical parameters and bacterial numbers in Pony Lake between 07 January and 20 January 2006. Temperature [°C], conductivity [mS cm^{-1}], dissolved oxygen [mg L^{-1}], and dissolved organic carbon (DOC) [mg L^{-1}].

where increased oxygenated conditions with depth have been reported (Healy et al., 2006; Wait et al., 2006).

Within Season Stability of pH and Conductivity

Pony Lake is moderately alkaline (Brown et al., 2004, this study) with pH typically increasing during the austral summer. Brown et al. (2004) reported an increase in pH between early December 1997 and mid-January 1998 from 8.77 to 10.5. During the study period 2004/05 the pH in Pony Lake changed from 8.07 to 9.77 over a similar time frame. The same pattern applies to conductivity. Early December 1997 lake water

Table 4.3.: Year to year variation in dissolved organic matter (DOC) and major ion concentrations in Pony Lake.

	Date						
	01/28/92[1]	**12/04/97[2]**	**12/30/97[2]**	**01/19/98[2]**	**12/11/04**	**12/29/04**	**01/07/06**
DOC [mg L^{-1}]	95.0 / 110[*]	92.4	32.4	48.0	11.8	28.3	16.5 / 26.29[†]
Major ions [mg L^{-1}]							
Cl^-	6098	2375	2333	3701	863	-	1952
SO_4^{2-}	5470	1624	1537	2767	254	-	503
Na^+	5420	1938	2184	3593	547	1049	1134
K^+	-	129	125	215	58.9	92.6	88.9
Mg^{2+}	510	158	163	238	62.7	95.7	120
Ca^{2+}	160	64	60	88	35	45.1	36.8

(-) not analyzed
[1] McKnight el al. (1994), ([*]) from samples collected 01/27/94
[2] Brown et al. (2004)
(†) from samples collected 01/19/06

conductivity was 5.45 mS cm^{-1} (Brown et al., 2004). Although Brown et al. (2004) did not record conductivity on the other sampling dates it can be assumed that conductivity increased during summer in Pony Lake based upon ionic concentrations. Major ion constituents increased between 37 and 85% during the 1.5 months of observation (Table 4.3.). In 2004/05 conductivity was twice as high in mid January (7.06 mS cm^{-1}) compared to early summer (3.43 mS cm^{-1}). Since Pony Lake does not have any apparent inflows this increase was likely due to wind mixing of basal waters. As compared to previous seasons, surveys conducted in January 2006 convey a different picture of Pony Lake. Conductivity as well as pH remained stable over the two week study period (Fig. 4.2.), even during the mixing events. Many Antarctic meltwater ponds show stratification with respect to conductivity (Healy et al., 2006; Wait et al., 2006) and conductivity increases significantly in the basal water (bottom 20 cm) as a result of saline brines formed during winter freezing. Using this scenario for Pony Lake it appears that a bottom solution with higher salinity was not established, mixing was not extended

throughout the whole water column and thus the bottom brine was not disturbed, or lakes areas that contained basal brines did not become ice free.

Variabilities in pH can be related to phytoplankton activity. In a moderately alkaline milieu such as found in Pony Lake, free CO_2 is generally low and photosynthetic reaction can raise the pH by releasing hydroxyl ions during photosynthesis from the predominant inorganic carbon source, HCO_3. The stable pH observed during the season 2005/06 may therefore indicate that environmental conditions (e.g. lower temperature) in Pony Lake only allowed for a low rate of carbon fixation, which in turn would minimize the impact of the phytoplanktonic community on pH shifts.

Between Season Differences in Lake Chemistry

Data on dissolved organic carbon (DOC) and major ions in Pony Lake are available for four separate seasons. Pony Lake water is predominately of Cl, Na, and SO_4 composition, which can be attributed to the close proximity of Pony Lake to the Ross Sea and its input of marine aerosols. Cl is the most abundant ion. Although the overall character of Pony Lake has remained constant, major ion concentrations became strongly diluted between 1992 and 2006 (Table 4.3.). Concentrations of Cl, Na, and SO_4 measured on 07 January 2006 decreased by 68, 79, and 91% respectively compared to 28 January 1992. Some of the variability may be explained by temporal differences in sampling dates; however samples were collected at approximately the same times of year. Year to year fluctuations in the ionic composition are likely a result of differences in solar radiation, wind mixing, duration of the open water period, dilution effects from accumulated snow, and the amount of ice free lake area. Cryo-concentration during

winter freeze-up pushes a steady concentration of dissolved salts into the remaining water column, and leads to the formation of a basal brine in the deepest areas of the lake. Ice cores collected in 2004 showed that these basal brine solutions were also incorporated into the ice during the final stage of freeze-up (Chapter 2). The partial exclusion of dissolved salts during ice formation creates not only basal water of higher salinity, but also a more pure ice matrix for large areas of the lake. Consequently, if the deeper lake areas where basal brines were enclosed into the ice remain frozen during the next year's summer, the ion concentration in the lake water will decrease. The fact that during the summer 2005/06 only peripheral melting occurred and that the major ions were highly diluted compared to previous years (Table 4.3.) may provide supportive evidence for this assumption.

DOC concentrations were also markedly reduced in January 2006 as compared to previous seasons (Table 4.3.). The exudation of photosynthate as DOC may differ in relation to the photosynthetic community and different taxa may have different photosynthetic efficiencies (Laybourn-Parry et al., 2002). Laybourn-Parry et al. (2002) have shown higher primary productivity with increasing salinity from studies on brackish to hypersaline lakes in the Vestfold Hills, Antarctica. Unfortunately, simultaneous measurements for salinity, primary production or phytoplankton numbers are only available for the season 2004/05. Therefore it is difficult to demonstrate a correlation between salinity and phytoplankton characteristics or to infer a trend in the ~3 to 6-fold decrease in DOC concentration between January 1992 and 2006. Secondary production measurements from samples collected on 28 December 2005 and 06 January 2006

showed extremely low production rates of 2.62 and 2.49 µg C L^{-1} d^{-1}, respectively. These low rates are comparable to the bacterial productivity found in ice cores collected earlier in November 2005 (Table 4.1.) from a lake area that remained frozen for at least two years; however these rates are an order of magnitude lower than those reported in Pony Lake during 2004/05; hence, a massive exploitation by the bacterioplankton community can also be ruled out. Differences in productivity may be related to the composition of the DOC pool and its ratio of labile to recalcitrant compounds. Nonetheless, the decrease in DOC concentrations remains an open question. A partial explanation may be provided by the physiological stage of algae (McKnight et al., 1994). When Pony Lake was sampled in January 1992 more than 90% of the phytoplanktonic cells were in the palmella stage, a condition in which the cell secretes a mucilaginous mass, but continue to metabolize. McKnight et al. (1994) estimated that the mucilage around the cell walls contained ~18 mg C L^{-1} and clearly could be a large portion of the DOC. It appears that during the two consecutive field seasons 2004/05 and 2005/06 an abundant phytoplankton community was not established. Cell numbers for both *Chlorophytes* and *Cryptophytes*, the dominant species in Pony Lake, were between two and three orders of magnitude lower in 2004/05 compared to January 1997 (McKnight et al., 1994). Since Pony Lake is nutrient enriched and mixing events can enhance productivity by reducing light limitation (Hansson, 1992) further measurements would be required to resolve which process affected phytoplankton growth.

Between Season Differences and Stability in Bacterioplankton

Pony Lake is colonized by bacteria, virus like particles, algae, and a small population of ciliates. Historical documentation about algal populations in Pony Lake can be traced back to 1908 (West & West, 1911). The dominate species discovered at this time was *Chlamydomonas intermedia* Chodat, a finding which was affirmed 84 years later (McKnight et al., 1994). Brown et al. (2004) also reported an abundant population of *cryptophytes*. However, little attention has been paid to the bacterioplankton in Pony Lake. A detailed study on the entire microbial community during the austral summer 2004/05 was described previously in Chapter 3.

Denaturing gradient gel electrophoresis (DGGE) banding patterns were used to visualize differences in the community structure, followed by an analysis and comparison of electrophoresis patterns (GelCompar II software, Applied Maths). Bands were automatically detected and matched by the program and band assignment was confirmed manually. Cluster analysis was applied to determine the presence of groups of similar banding patterns in gels.

The molecular fingerprints from the DGGE analysis highlight differences in the bacterial community structure in ice core sections and water samples within each season, and between the two seasons (Fig. 4.3.). Variations in banding profiles are apparent in the ice core sections with an increasing number of bands towards the bottom of the core and also in a progressive shift in the community structure during the open water period 2004/05. During 2005/06 the transition in bacterioplankton was not observed in Pony Lake water samples.

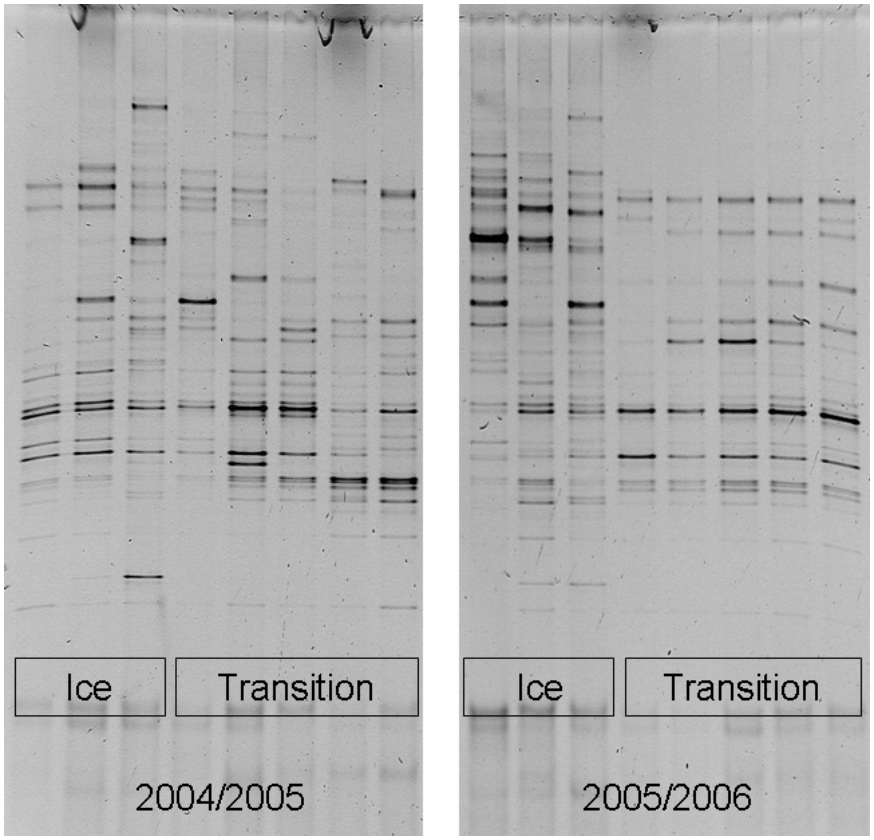

Figure 4.3.: Differences in DGGE profiles of 16S rRNA gene fragments from Pony Lake ice core and water samples collected from two consecutive field seasons. Ice cores were divided into ~40 cm section (top, middle, bottom). Left profile: samples from 2004/05. From left to right: Top, middle, and bottom core segments (ice core samples), water samples during the transition period from different dates: 12/11/04, 12/21/04, 12/29/04, 01/14/05, 01/15/05. Right profile: samples from 2005/06. From left to right: Top, middle, and bottom core segments (ice core samples), water samples during the transition period from different dates: 12/29/05, 01/14/06, 01/15/06, 01/24/06, 02/02/06.

Changes in DGGE profiles affiliated with deeper core segments possibly reflect processes accompanying freeze concentration. The formation of an ice cover in late summer - early winter may partially insulate the lake against air temperature changes and the gradual freeze concentration of salts will lower the freezing temperature with increasing salinity. Thus, liquid water can persist longer in the deeper zones of the lake allowing the microbial community to remain metabolically active. Ongoing microbial

processes in these physically isolated bottom waters would consume oxygen and deplete oxygen levels. Pony Lake ice cores had a noticeable smell of H_2S indicating reducing conditions. This gradient of an oxygenated top layer to an anoxic zone at the bottom of the lake will undoubtedly affect the microbial community structure. While these chemical gradients cause a shift in the microbial assemblages during freezing towards the base of the lake, aeolian transport and deposition of organisms onto the surface has to be considered as well. The seeding mechanism via soil particles is characteristic of icy habitats (Gordon et al., 2000; Foreman et al., 2007) and may introduce microorganisms into the upper portion of the ice during winter storms. Over the course of the summer these particles absorb more heat and slowly melt into the ice. Particularly in areas where the ice column or ice coverage extended over a number of years, small soil particles may accumulate in the ice column. Sediment grains were scattered throughout the top core sections in ice cores collected in 2005 from an area that was ice covered for multiple years. Assuming that these particles were of aeolian origin, the seeding of microorganisms may partially explain differences in the banding pattern within the top ice core sections from both seasons.

A pronounced difference between DGGE banding patterns was apparent in Pony Lake water samples from the two sampling years. During the prolonged open water period in Pony Lake a distinct change in DGGE profiles from mid-December to mid-January was observed in 2004/05; this trend was only marginally present during the second year from the end of December to the beginning of February (Fig. 4.3.). Physicochemical conditions in the water column may be responsible for these

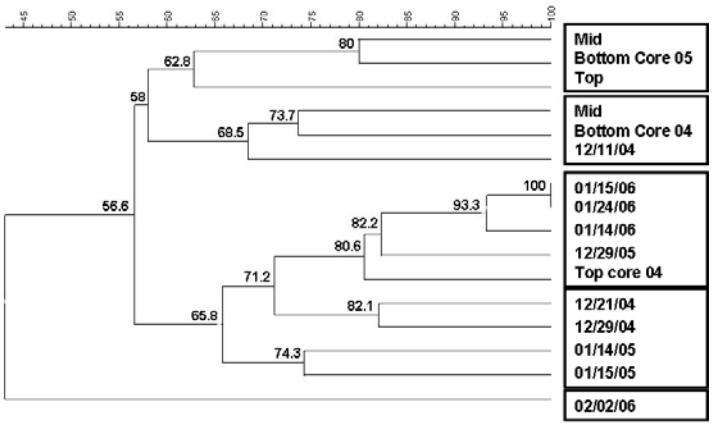

Figure 4.4.: Dendrogramm derived from GelCompar II analysis showing ice core and water samples collected during the austral summer 2004/05 and 2005/06. The horizontal axis denotes the linkage distance where samples are jointed together into a new cluster.

differences. Physical and chemical characteristic s of Pony Lake evolved during 2004/05. For instance, temperature, salinity, and pH increased gradually, with most chemical constituents becoming more concentrated. Simultaneously, bacteria and algae became more abundant. Changes in the community structure in 2004/05 were also attributed to alterations in the bioavailability in dissolved organic matter composition. Conversely, in 2006 the water column appeared stable. Conductivity and pH remained constant and no change in bacterial numbers was detected (Fig. 4.2.).

Cluster analysis via GelCompar II confirmed the presence of distinct groups between different seasons and sample types (Fig.4.4.). In general, ice and lake water samples formed segregated clusters with closely related DGGE profiles from the two consecutive sampling years. Interestingly, within each cluster samples collected from the individual seasons formed their own group. Nevertheless, three samples fell out of this pattern (Fig. 4.4.). An ice core segment (Top Core 04), a water sample collected during the early melt period in 2004 (PL 11.12.04), and a water sample from late summer 2006

(PL 02.02.06) was completely separated from any cluster. The latter was collected late in the season 2006 when temperatures dropped and the lake started to freeze over again. This required breaking through the ice cover to collect the water sample. The fact that the water sample collected in early December 2004 (PL 12/11/04) was more closely related to the middle and bottom ice core sections from 2004 suggests that melt was initiated on the bottom of the lake and progressed from bottom to top. The microbial community found at the beginning of the melt period therefore seems to consist of a bacterial assemblage seeded from the lower ice core sections. On the other hand, the similarities in DGGE banding patterns between the top ice core section from 2004 and Pony Lake water samples collected later in the season imply that the initial microbial community found in Pony Lake is replaced by the microbial assemblage released from the top ice layer.

There is little, if any, allochthonous input of nutrients to Pony lake (McKnight et al., 1994, Brown et al., 2004), thus nutrient availability depends upon consumption and recycling. However, Pony Lake is a eutrophic system and inorganic nutrients are plentiful throughout the summer open water period. Nonetheless, in the case of bacterioplankton, photochemically induced alterations of dissolved organic matter can reduce its bioavailability. Our data suggests that the microbial community in Pony Lake is most likely affected by physical parameters such as temperature, salinity, and light during summer, regulators which are even more pronounced during refreezing in fall and winter. Although microbes are capable of over-wintering in the ice column of Pony Lake, the duration of being trapped within the ice may affect their survivability.

CHAPTER 5

DIRECT AND INDIRECT EFFECT OF SOLAR RADIATION OF MICROBIALLY DERIVED DISSOLVED ORGANIC MATTER AND BACTERIOPLANKTON

Adapted from: Markus Dieser, Penney L. Miller, Ryan L. Fimmen, Jennifer J. Guerard, Rose M. Cory, Diane M. McKnight, Yo-Ping Chin and Christine M. Foreman (in preparation for Limnology and Oceanography)

Introduction

Dissolved organic matter (DOM) is a major reservoir of organic carbon in aquatic ecosystems and represents a vital source of organic substrates for microorganisms. Microbial processes are integrally linked to biogeochemical reactions of DOM and the bacterially mediated turnover of DOM plays an important role in carbon cycling. DOM is defined by chemically heterogeneous biomolecules of humic and non-humic substances and the proportional distribution of these two compounds affects bioavailability for microorganisms. In general, humic substances are of varying (moderate to high) molecular weight and are biologically recalcitrant (e.g. McKnight & Aiken, 1998). Concentrations of humic substances vary greatly among aquatic ecosystems with higher concentrations in freshwater environments compared to marine systems (Thurman, 1985). Humic substances are further divided into fulvic acids and humic acids, where fulvic acids account for the majority of humic compounds in aquatic environments, ranging from 25 to 90% of the dissolved organic carbon (DOC) (Thurman, 1985; Moran & Hodson, 1990). Although bioavailability and chemical reactivity of DOM depends upon its source and chemical composition, recalcitrant fractions of DOM can be

photochemically transferred into more labile molecules (Geller, 1986; Wetzel et al., 1995). Humic substances play an essential role in light absorbance and can have a substantial impact on the biogeochemistry of aquatic systems (Lindell et al., 1995; Bano et al., 1998). Photolytic reactions can alter humic substances, thereby enhancing or stimulating bacterial growth in several ways. A source of substrate can be provided by the cleavage of labile carbon biomolecules associated with humic compounds, such as dissolved free and combined amino acids, or total dissolved carbohydrates (Jorgensen et al., 1998). Further, photochemical reaction can increase the pool of limiting nutrients like nitrogen and phosphorus rich compounds (including NH_4^+ and PO_4^{3-}) (Moran & Zepp, 1997). Lastly, the breakdown of high molecular weight DOM into low molecular weight organic photoproducts such as carboxylic acids (Wetzel et al., 1995) and aldehydes (Mopper & Stahovec, 1986) can render humic substances biologically labile. Although photocleavage and photooxidation may transform recalcitrant DOM components into more labile and bioavailable compounds, there is also evidence showing the phototransformation of bioavailable substrates into more recalcitrant compounds (e.g. Brenner & Biddanda, 1998; Obernosterer et al., 1999; Kaiser & Sulzberger, 2004). The transformation of labile compounds may also lead to humification processes, thereby immobilizing nitrogen species by incorporating them into organic structures (Kieber et al., 1997). Other photolytic reactions may result in a loss of dissolved organic matter via the production of volatile organic compounds (Mopper et al., 1991), carbon gases (Mopper et al., 1991; Miller & Zepp, 1995) or may generate reactive oxygen species

(Fridovich, 1986). The latter have been shown to cause severe damage in the cellular structure and physiology of microorganisms (Fridovich, 1986).

Aquatic systems vary in their source of humic substances from terrestrially derived (allochthonous) material to microbial *in situ* production (autochthonous). Allochthonous sources are typically found in freshwater systems (Thurman, 1985). Also, estuarine and nearshore waters may be dominated by riverine inputs (Yan et al. 1991). In contrast, autochthonous humic substances prevail in seawater, highly productive lakes, or lakes with limited terrestrial inputs (McKnight et al., 1991; Hedges et al., 1992; McKnight et al., 1994). Prior to irradiation exposure, freshly produced autochthonous DOM is usually composed of labile, more bioavailable molecules, whereas terrigenous DOM is more refractory (Sun et al., 1997). Moran & Covert (2003) proposed a model where solar radiation reverses these characteristics rendering initially bioreactive DOM (autochthonous) biologically more recalcitrant, while phototransformation of initially low bioavailable DOM (allochthonous) would have a stimulating effect on bacterial processing.

Pony Lake is a shallow eutrophic lake located at Cape Royds, Antarctica. The lake is characterized by autochthonous, microbilly derived DOM (Brown et al., 2004; Schwede-Thomas et al., 2005). Previous studies revealed that the fulvic acid fraction in Pony Lake only accounts for 13% to 22% of the total DOC pool (McKnight et al., 1994; Brown et al., 2004). This percentage is low compared to the range of 30% to 60% usually found in temperate lakes (Thurman, 1984). Pony Lake fulvic acids are characterized by a low C:N ratio and low aromaticities, which are distinct features of algal-derived fulvic

acids (McKnight et al., 1994). Consequently, the nitrogen content of Pony Lake fulvic acids is greater than typically observed (Brown et al., 2004).

In this study we examined the ability of microorganisms to utilize autochthonously produced Pony Lake organic matter. The primary objective was to determine how the chemical nature and photolytic reactions affect the bioavailability of the DOM. Pony Lake whole water or selected DOC fractions (isolated fulvic acids) were subjected to photochemical bioassay experiments. To relate photolytic effects to the bioavailability of Pony Lake organic matter, chemical properties of DOM and fulvic acids as well as bacterial production rates were investigated. Additionally, differences in substrate turnover by bacteria between autochthonously (Pony Lake) and primarily terrestrially derived fulvic acids (Suwannee River) were tested.

Materials and Methods

Study Area and Sample Collection

Pony Lake is a small (~ 120 m long, 70 m wide, and 1-2 m deep), coastal lake located at Cape Royds (77° 33’ S, 166° E), Ross Island, Antarctica. Pony Lake is characterized as a brackish, eutrophic system colonized by bacteria, algae and a small population of ciliates. Its western shore is flanked by an Adelie penguin rookery. Except for a few weeks during the brief austral summer the lake is frozen solid to its base. Pony Lake is a closed basin with no visible inflows. Gains and losses of water are attributed to the melting of the accumulated snowpack and evaporation in mid-summer, respectively.

Exposure Experiments

During the summer seasons 2004/05 and 2005/06 two different experiments were conducted. To test the direct effect of solar radiation on bacterioplankton in 2004/05, 0.6 µm pre-filtered Pony Lake water samples were exposed in quartz tubes to *in situ* Antarctic temperature and solar radiation regimes on a flat roof on the laboratory facility in McMurdo Station for 24 hrs. Briefly, 125 ml of water samples were filled into duplicate quartz tubes. Tubes were sealed with rubber stoppers, which had been rinsed with 1N HCl and Milli-Q water prior to use. Duplicate dark control samples were prepared in amber PE (polyethylene) bottles. To ensure full 24 hrs light exposure the quartz tubes were mounted on a sample holder (~ 75° angle) on a revolving table (Fig. 5.1.). Throughout the 24 hrs exposure period, the position of the table was adjusted following the course of the sun. Dark controls were placed under the table in a box. During 2005/06 a fulvic acid sample, isolated from Pony Lake, was amended with a bacterial suspension (enrichment culture in marine broth from a frozen, concentrated Pony Lake stock culture). Triplicate 300 ml light and dark control samples were prepared as described above, followed by the 24 hrs solar radiation exposure.

In order to examine the bioavailability of photolytic alterations of labile or recalcitrant DOM, a series of bioassay experiments were conducted in a second experimental setup. During 2004/05 unaltered Pony Lake whole water samples (WW 04/05) were chosen for bioavailability studies. During the summer season 2005/06, Pony Lake whole water samples (WW 2005/06), fulvic acid isolates collected from Pony Lake water in 2004/05 (WW XAD 04/05) and 2005/06 (WW XAD 05/06), and a Suwannee

Figure 5.1.: Image of the glass tube holder mounted on a revolving table.

River fulvic acid sample (International Humic Substance Society, Minnesota) were subjected to photolysis treatments. Prior to exposure to natural sun light, water samples were filter sterilized by passing through a 0.2 µm membrane filter. The fulvic acid solutions were prepared as follows. Fulvic acid powder was reconstituted in Milli-Q water to form solutions of 10 mg C L^{-1} and pH was adjusted to 9-9.5. Duplicate 125 ml Pony Lake whole water samples were prepared in 2004/05 and in 2005/06 all solutions were incubated in triplicate 300 ml light and dark quartz tubes and amber PE bottles respectively, for 24 hrs as described above. Immediately after exposure to sun light, water from the quartz tubes were transferred to amber PE bottles and the irradiated and dark treatments were amended with bacterial inocula. While samples in 2004/05 were inoculated with a natural microbial community from Pony Lake (1:10 inoculum ratio) to

a final cell concentration of approximately 10^6 cells ml^{-1}, samples in 2005/06 were exclusively amended with enrichment cultures from the same frozen stock culture (enrichment culture in marine broth from a frozen, concentrated Pony Lake stock culture). Inoculated samples were then incubated in the refrigerator (~4°C) for 12 days. During the season 2005/06 only photolytic experiments conducted on Pony Lake whole water fulvic acid isolates from 2004/05 (WW XAD 04/05) were directly processed on site in the laboratory at McMurdo Station and inoculated with bacterial assemblages. Triplicate light and dark samples for Pony Lake whole water fulvic acid (WW XAD 05/06), unaltered Pony Lake water (WW 05/06), and Suwannee River fulvic acid (SRFA) were acidified to pH 2 after solar radiation exposure to avoid oxidation of fulvic substances and shipped to the laboratory at Montana State University. Samples were stored in a dark refrigerator (~4°C) until further processing.

Bacterial Production

Samples for bacterial production measurements were collected from the two different exposure experiments. In the first experimental setup (direct effect of solar radiation as described above), sub-samples were taken before and after the 24 hrs exposure experiment to natural solar radiation. In the second exposure experiment (bioavailability assay) bacterial production was investigated after inoculation with bacteria and after a 12 day incubation period. Heterotrophic bacterial productivity (BP) was measured by ^{3}H-thymidine incorporation (20 nM final conc.) as outlined by Takacs & Priscu (1998). Each photolytic replicate was incubated in quintuple ^{3}H-thymidine assays and triplicate formalin killed controls (5 % final concentration, 30 min prior to ^{3}H-

thymidine addition) for 18 hrs at 4°C. Disintegrations were detected in a liquid scintillation counter (Beckman LS 7200). A conversion factor of 2.0 x 10^{18} cells mol^{-1} TdR (Duckow & Carlson, 1992) was used to convert the thymidine incorporation rates into cell production rates.

Denaturing Gradient Gel Electrophoresis

Samples for DNA extraction and downstream phylogenetic fingerprint analysis via denaturing gradient gel electrophoresis (DGGE) were collected from each irradiated and dark replicate from the Pony Lake whole water samples (WW 04/05) after the 12 day incubation experiment in 2004/05 and at the beginning and the end of the 12 day bioavailability assay of photolyzed fulvic acid solutions (WW XAD 04/05) in 2005/06. Samples were concentrated onto 47 mm Supor®-200 0.2 µm pore size, sterile membrane filters under low pressure (<7 psi). Filters were placed into cryovials containing TES buffer (100 mM Tris, 100 mM EDTA and 2% SDS), flash-frozen in liquid nitrogen, and stored at -80°C.

Following DNA extraction (Ultra Clean Soil DNA Kit, MoBio) a portion of the prokaryotic 16S rRNA gene was amplified using primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 534R (5'-AATACCGCGGCTGCTGG-3') (Muyzer et al., 1996). A 40 base pair GC clamp was added to the 5' end of the 341F primer (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG). PCR reaction mixtures as well as the amplification parameter followed the protocol described in Chapter 3. DGGE analysis for PCR amplification products was performed on a BioRad D Code™ system as outlined by Murray et al. (1996). Optimal results were obtained in

denaturing gradient gels with a 40-70% denaturing gradient. Time of electrophoresis to resolve gene segments was 18 hrs. Gels were stained with SYBR Gold (Invitrogen) for 15 min and imaged with an Alpha Innotech FluorChem™ 8800 system.

Solar Radiation Measurements

Solar radiation measurements were obtained from the spectroradiometer system at McMurdo Station and downloaded from the biospherical NSF website (http://www.biospherical.com/nsf/mcmurdo/mcmurdo.asp).

Statistical Analysis

To test for an effect of solar radiation on the bioavailability of dissolved organic matter or isolated organic fractions (light exposure vs. dark control) a one-sided Wilcoxon rank sum test with continuity correction was applied using The software package R (R Development Core Team, 2008). It is important to mention that with the small sample size (n=3) it is impossible to obtain p-values below 0.04, which suggests that the statistical power of this test is limited. Since studies with similar smaller sample sizes have been reported (e.g. Obernosterer et al., 1999; Obernosterer & Herndl, 2000) we considered p-values close to 0.04 as indicators of significant difference between the two treatment groups.

Results and Discussion

The solar radiation dosages for UV-B (290-320nm), UV-A (320-400nm) and PAR (400-600nm) were measured at 15 min time intervals. The integrated values for the radiation regime over the 24 hrs exposure time for each photolytic experiment are summarized in Table 5.1. On the two exposure dates in 2004/05 the solar radiation doses for UV-B were 6.28 and 6.79 J cm^{-2}, and UV-A reached 162.26 and 182.35 J cm^{-2}. The solar radiation doses during exposure experiments conducted at the end of the second season (2005/06) in late January – beginning of February, 2006, were slightly lower and ranged between 3.77 to 5.23 J cm^{-2} for UV-B and 119.12 to 150.77 J cm^{-2} for UV-A. Highest doses were detected in mid-December 2005 where UV-B was found to be 8.17 J cm^{-2} and UV-A 207.73 J cm^{-2}.

Direct Effect of Solar Radiation on Bacterioplankton

Exposure of unaltered Pony Lake whole water samples (natural DOM plus bacteria) to 24 hrs solar radiation showed a negative effect on the bacterioplankton community and induced photochemical alterations in the dissolved organic matter (Fig. 5.2.) Compared to dark control samples, bacterial production was markedly reduced in the light exposed bioassays (Fig. 5.3.). Bacterial cell production rates per day calculated in both seasons, 2004/05 and 2005/06, were 73.5% and 97.5% reduced after irradiation exposure. It is well documented that the more energetic, shorter wavelengths can have

Table 5.1.: Integrated *in situ* Antarctic solar radiation doses [J cm^{-2}] for specific wave lengths over the 24 hrs exposure periods. The source describes the origin of the organic matter from Pony Lake used in the exposure experiments. Bulk dissolved organic matter from a whole water sample (WW), isolated fulvic acid fraction from Pony Lake whole water samples (WW XAD), and Suwannee River fulvic acid (SRFA).

Source	**Date**	**UV-B [290-320]**	**UV-A [320-400]**	**PAR [400-600]**
WW 04/05	30-31 Dec 04	6.79	182.35	929.04
WW 04/05	15-16 Dec 05	6.28	162.26	821.74
WW XAD 04/05	12-13 Dec 05	8.17	207.73	1090.09
WW 05/06	27-28 Jan 06	3.77	119.12	594.03
WW 05/06	28-29 Jan 06	4.91	143.94	724.39
WW 05/06	29-30 Jan 06	5.23	150.77	754.37
WW XAD 05/06	01-02 Feb 06	4.82	141.46	749.51
SRFA	02-03 Feb 06	4.78	138.40	724.79

negative effects on biological processes in aquatic ecosystems (Ekelund, 1992; Ross and Vincent, 1998; Cockell and Knowland, 1999) and that the occurrence of stratospheric ozone depletion (Schoeberl & Hartman, 1991) has caused an increase in ground level UV-B radiation (Madronich, 1992). Specifically UV-B radiation (280-320 nm) has a broad range of direct and indirect inhibitory effects on aquatic biota. These effects include lethal and mutagenic damage to DNA (Buma et al., 2001; Haeder and Sinha, 2005), inhibition of photosynthetic rates, or pigment bleaching in phytoplankton (Kim and Watanabe, 1993; Haeder et al., 1998), reduction in microbial motility (Ekelund, 1992; Donkor et al., 1993), and a decrease in microbial growth rates (Bothwell et al., 1994). UV-A radiation (320-400 nm) causes indirect damage to cellular DNA, proteins, and lipids by catalyzing the intracellular formation of chemical intermediates (Agogué et al., 2005) and is also detrimental to primary production (Karentz et al., 1994). Besides the

Figure 5.2.: Image of a photobleached Pony Lake whole water sample after exposure to 24 hrs of natural Antarctic sun light. Left vial: Photobleached sample. Right vial: Unaltered sample.

direct impact of shorter wavelengths on cellular structure and physiology of aquatic microorganisms, the production of reactive oxygen species (ROS) may have accounted for the decrease in bacterial activities. However, an assessment of the extent ROS affected the microbial community was beyond the scope of this study. When DOC absorbs ultra violet radiation (UVR), superoxide is formed, which dismutates to hydrogen peroxide (Cooper et al., 1994). Unlike most ROS, hydrogen peroxide is not charged and can pass freely through the cell membrane causing an acute stress on aquatic organisms (Kieber et al., 2002). However, compared to the intracellular production of ROS that imposes extreme challenges on biological systems, the effect of externally produced radicals on aquatic microorganisms is expected to be minimal (Kieber et al., 2002). The high reactivity of certain ROS species results in an extremely short lifetime, which minimizes the chance of cell surface interactions. Nonetheless, Xenopoulos & Bird (1997) reported that the addition of H_2O_2 concentrations as low as 0.1 μM to humic lake

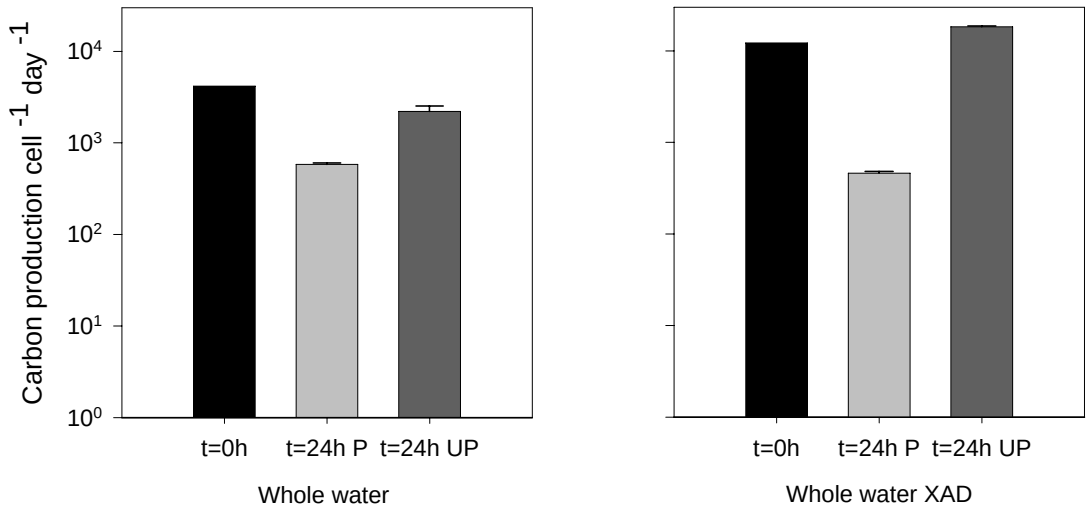

Figure 5.3.: Direct effects on the bacterioplanktonic community after 24 hrs exposure to natural Antarctic sun light. Production rates are corrected for bacterial abundances. Photolysed (P), unphotolysed (UP).

water can inhibit bacterial production by as much as 40%. Anesio et al. (2005) found a strong correlation between bacterial carbon production and photochemically formed H_2O_2 and suggested that H_2O_2 concentrations of ~2-3 µM were sufficient to inhibit bacterial production. A parallel laboratory study of H_2O_2 production in photolyzed Pony Lake water and in the fulvic acid fraction indicated that H_2O_2 production rates in Pony Lake can be high (Miller, personal communication). At near ambient pH, approximately 35 µM and 8 µM of H_2O_2 were produced during 6 hrs of light exposure in Pony Lake water and the fulvic acid fraction respectively (Miller, personal communication). Since the light intensity in the photoreactor (450 W Xenon Arc lamp) was approximately half of the intensity measured in Antarctica on a sunny day, H_2O_2 production would be expected to be even greater under natural Antarctic light conditions. Consequently, it is possible that inhibitory effects of H_2O_2 on the bacterial community may have contributed

to a decrease in bacterial production rates. Nonetheless, the physical regime of Pony Lake (low water temperature, alkaline pH) could also counteract the negative effect of reactive oxygen species since it has been shown that ROS production in natural waters is generally more favorable under low pH environments (Zepp et al., 1992) and low temperatures may have an inhibitory effect on the formation of hydrogen peroxide (Scully et al., 1996).

Exposure of unaltered Pony Lake water samples not only showed direct photoinhibition of the bacteria, but visible changes in coloration of the water samples due to photobleaching of DOM were consistently observed during both seasons (Fig. 5.2.). This clearly visible effect of DOM bleaching was somewhat surprising because the absorptivity of Pony Lake fulvic acids is three to five times lower compared to plant/soil derived fulvic acids (McKnight et al., 1994, Brown et al., 2004). Photobleaching of DOM is the result of absorption of solar radiation or may be induced by photoproduced ROS, and occurs more rapidly in alkaline waters (Reche et al., 1999). Since photobleaching of DOM is accompanied by a gradual loss in the absorbance capacity of exposed water samples, this photochemically induced alteration of DOM may change the radiation climate for the aquatic microbial community by controlling the penetration of photochemically active and UV damaging radiation into the water column (Morris & Hargreaves, 1997).

A parallel study on the photochemical behaviour of Pony Lake water samples and isolated fulvic acid fractions conducted during both summer seasons confirmed the occurrence of photobleaching and the effect of ROS on the photodegradation of organic

matter (Guerard et al., in preparation). Although the fulvic acid fraction comprised less than 20% of the total DOM in Pony Lake, photolysis of both fulvic acids and whole water DOM resulted in a gradual decrease in absorbance and total fluorescence with prolonged exposure time. ROS contributed approximately 30% to the photolytic decay of Pony Lake whole water DOM, and roughly 14% to the degradation of fulvic acid isolates (Guerard et al., in preparation). The change in absorbance was induced by the decay of chromophores. However, differences in photobleaching and the rate of photolytic decay between fulvic acids and whole water Pony Lake DOM suggested that the fulvic acids, which are typically the more chromophoric fractions of DOM, disguised the effect of photobleaching in Pony Lake whole water samples. Also, some chromophores were more susceptible to degradation than others. Conversely, fluorescence decay did not appear to be specific for particular fluorescent functional groups (Guerard et al., in preparation).

Effect of Solar Radiation on the Bioavailability of Pony Lake Bulk DOM and Fulvic Acids

After light/dark exposure to natural solar radiation, bulk DOM water samples from Pony Lake and re-suspended fulvic acid samples were amended with bacterial cultures and incubated for 12 days. Bacterial cell production was assayed at the beginning and at the end of the incubation period. Surprisingly, exposure of the different DOM fractions to ambient Antarctic light regimes over 24 hrs had both stimulatory and inhibitory effects on the bioavailability to bacterioplankton (Fig. 5.4. and 5.5.).

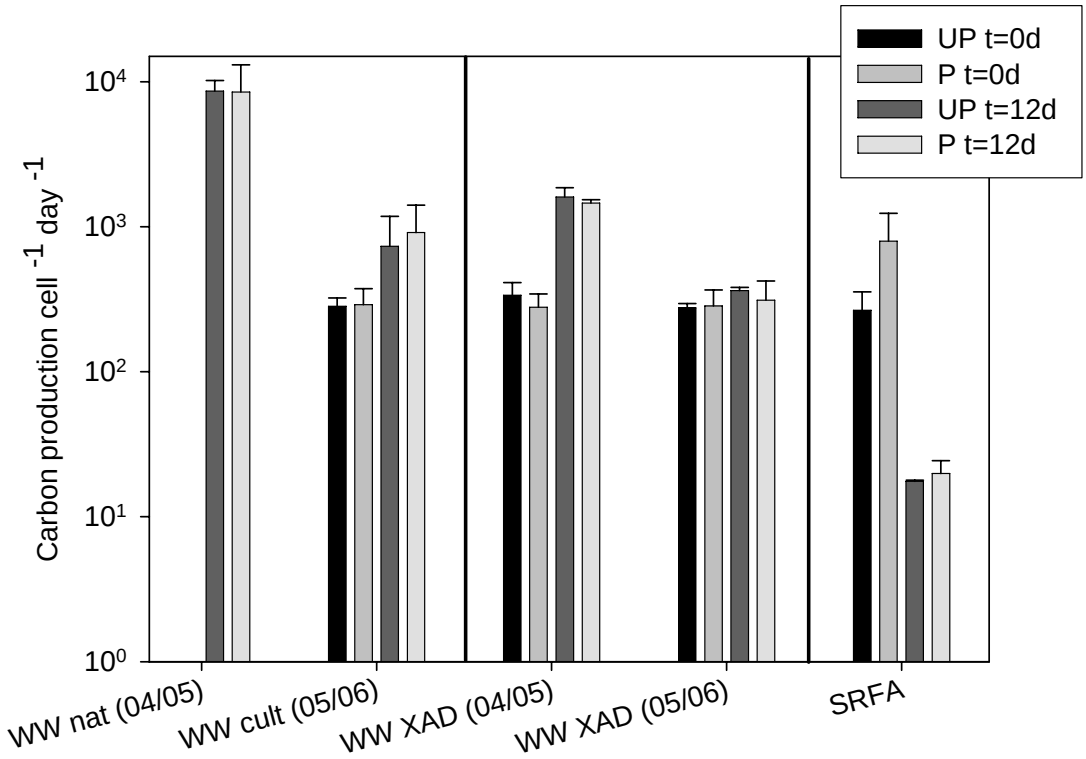

Figure 5.4.: Long term bioavailability assays on different photochemically altered organic matter samples isolated from Pony Lake. After exposure to ambient Antarctic light regimes for 24 hr, irradiated and dark control samples were amended with bacterial suspensions and incubated for 12 d. Bacterial production rates (corrected for cell numbers) were calculated in the initial inocula and at the end of the incubation period. Pony Lake whole water sample amended with a natural bacterial community (WW nat (04/05)), Pony Lake whole water sample amended with an enrichment culture (WW cult (05/06)), fulvic acid fraction isolated from Pony Lake water in 2004/05 (WW XAD (04/05)) and 2005/06 (WW XAD (05/06)), Suwannee River fulvic acid (SRFA). Photolyzed (P), unphotolyzed (UP).

Exposure of Pony Lake whole water (WW 04/05) showed a negligible effect on its bioavailability to the natural Pony Lake bacterial community. Bacterial cell production exhibited only a minor change, 1.6 % decrease, in the light exposed samples after the twelve day incubation period. Data for production rates after the initial inocula are not available.

In contrast to the exposure experiments in 2004/05, light and dark bioassays conducted in 2005/06 were amended with a bacterial enrichment culture from Pony Lake. Different to the previous year, bacterial production was slightly stimulated, 2.6% increase, in the irradiated Pony Lake whole water sample (WW 05/06) after the initial supplement of the bacterial culture. This stimulating effect became even more pronounced after the twelve days of incubation (Fig. 5.5.).

The response of the bacterial cultures to the various fulvic acid samples (WW XAD 04/05 and WW XAD 05/06) was different. Whereas bacterial production was reduced by 17.5% in the light exposed WW XAD 04/05 sample, an increase of 2.9% was observed in the irradiated WW XAD 05/06 fulvic acid sample at the beginning of the bioassay compared to the dark controls. This stimulation effect in WW XAD 05/06 became reversed after the long term incubation and the photolysed fulvic acid was less bioavailable to the bacteria (Fig. 5.5.). Conversely, the difference in bacterial production rates between the photochemically altered WW XAD 04/05 isolate and its dark control were less pronounced at the end of the incubation period.

Despite the differences in bioavailability of various photolytically altered organic substrates from Pony Lake, no evidence of a significant increase in bacterial production

rates in the dark control samples based on the Wilcoxon rank test were found in any incubation assay immediately after inoculation (one-sided p-values: WW 05/06 p-value = 0.81; WW XAD 04/05 p-value = 0.19; WW XAD 05/06 p-value = 0.33) or after the 12 day incubation period (WW 04/05 p-value = 0.65; WW 05/06 p-value = 0.67; WW XAD 04/05 p-value = 0.33; WW XAD 05/06 p-value = 0.33).

A comparison between the bulk DOM sample from Pony Lake (WW 05/06) and the fulvic acid fraction isolated from the same source (WW XAD 05/06) revealed that the fulvic acid component in Pony Lake may account for 50% of the cellular production rates in the whole water sample. Since the fulvic acid fraction in Pony Lake comprises ~20% of the total DOC (McKnight et al., 1994; Brown et al., 2004; Guerard et al., in preparation) this finding implies that the quality of the remaining 80% of the DOC in Pony Lake as a substrate for microbial growth is comparably poor and indicates that the humic fraction is a more relevant substrate for heterotrophic bacteria in Pony Lake.

In contrast to the lacustrine DOM sample and the Pony Lake fulvic acid isolates, exposure of terrestrially derived fulvic acids from Suwannee River (SRFA) increased the initial bioavailability of DOC to the bacterioplankton by 200% (Fig. 5.5.) indicating the release or production of labile photoproducts of lower molecular weight. However, this stimulating effect was only temporary. Bacterial cell production rates in the initially more bioavailable light exposed samples were reduced by 93.4% after the 12 days of incubation. A similar decrease by 97.5% in the cell production rates were observed in the dark controls. No significant difference in bioavailability was detected after 12 days (one-sided p-value = 0.50).

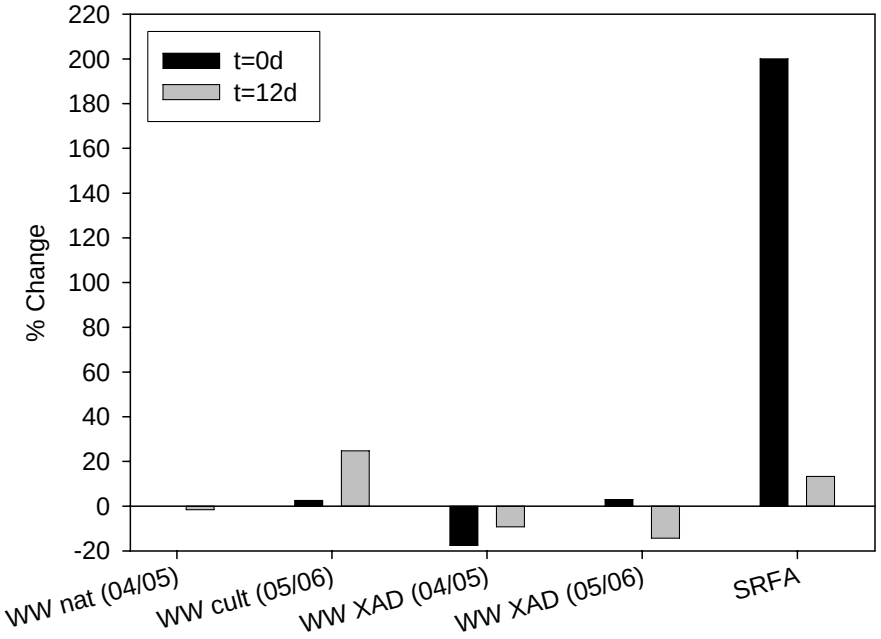

Figure 5.5.: Percentage of solar radiation induced inhibition or stimulation on the bioavailability of organic matter samples isolated from Pony Lake in the twelve day incubation bioassays. Percentages are displayed for the initial bacterial inocula to 24 hr light exposed and dark controls and at the end of long term incubation experiment. Pony Lake whole water sample amended with a natural bacterial community (WW nat (04/05)), Pony Lake whole water sample amended with an enrichment culture (WW cult (05/06)), fulvic acid fraction isolated from Pony Lake water in 2004/05 (WW XAD (04/05)) and 2005/06 (WW XAD (05/06)), Suwannee River fulvic acid (SRFA).

It has been shown that photolytic reactions of organic material in aquatic ecosystems render fractions that are either more bioavailable (e.g. Geller, 1986, Lindell et al., 1995, Wetzel et al., 1995) or more recalcitrant (e.g. Brenner & Biddanda, 1998; Obernosterer et al., 1999; Kaiser & Sulzberger, 2004). In general, these findings resulted from short term bioassays (several hours up to several days) on the bioavailability of photochemically altered DOM fractions (e.g. Brenner & Biddanda, 1998; Obernosterer et al., 1999; Kaiser & Sulzberger, 2004). Conversely, long term incubation experiments

(>2 weeks) on the bioavailability of phototransferred DOM revealed that the initial difference observed in substrate bioavailability among treatments (light exposed vs. dark) levelled off over time (Bertilsson et al., 2004; Judd et al., 2007). The findings in this study differ in that stimulatory or inhibitory effects in the initial inocula were relatively small. Brenner & Biddanda (1998) and Obernosterer et al. (1999) found a 75% and 50% reduction in bacterial production respectively in irradiated samples collected from oceanic surface waters. Focusing on the ecological relevance of the phototransformation on Pony Lake bulk DOM amended with a natural bacterial Pony Lake community (WW 04/05), solar radiation had a negligible effect on the bioavailability of Pony Lake whole water DOM to bacterioplankton. This is even more surprising as substantial photobleaching and decay of DOM by ROS were observed in irradiated Pony Lake bulk DOM samples (Guerard et al., in preparation). Thus, it appears as if photochemically induced DOM alterations are decoupled from substrate availability. A possible explanation can be found in the chemical composition of photolytically altered Pony Lake organic material. Parallel experiments addressing the photochemical nature of the organic matter revealed that although diminished in fluorescence, the relative distribution of fluorophores remained relatively unchanged during exposure to natural Antarctic sun light (Guerard et al., in preparation). The same holds true for the fluorescence index (FI; McKnight at al., 2001), an indicator of the source of the DOM (microbial vs. terrestrial derived) (e.g. for fulvic acids: observed change in FI from 1.45 to 1.43 after 24 hrs of photolysis). Furthermore, the chormophores did not significantly change in molecular weight (MW) during photolysis (Guerard et al., in preparation). Guerard et al. (in

preparation) concluded that photodegradation seems to have a minor effect on the overall quality of the organic matter in Pony Lake.

Nonetheless, the reason why photolytic reactions of Pony Lake whole water DOM exert little to no effects on the bioavailability to bacterioplankton remains poorly understood. One can argue that the initial quality of DOM plays an important function in regulating phototransformation processes. Thomas & Lara (1995) exposed aged autochthonous DOC obtained from an Antarctic marine diatom to simulated, ambient solar radiation. Analysis of humic substances showed no changes after irradiation (Thomas & Lara, 1995). Further, Jorgensen et al. (1998) demonstrated that dissolved, free amino acids which have been reported as labile photoproducts, did not change significantly during light exposure. Thomas & Lara (1995) suggested that the pool of autochthonous marine DOM may be relatively resistant against natural UV radiation. Previous studies on the DOC concentrations in Pony Lake reported a DOC concentration up to 100 mg C L^{-1} (McKnight et al., 1994; Brown et al., 2004), whereas the DOC concentrations over both seasons in this study only reached ~28 mg C L^{-1}. This decrease in DOC concentrations (between 1992 and 2004) was also accompanied by a decrease in phytoplankton abundances over several orders of magnitude (Chapter 3). Algal derived DOC (e.g. algal exudates) is considered to be of young age, freshly produced, which can be rapidly hydrolysed or remineralized by bacterioplankton. However, when these biomolecules undergo polymerization and condensation reactions induced by solar radiation exposure they may become less susceptible to enzymatic cleavage (Harvey et al., 1983). The several fold decrease in DOC concentration observed in Pony Lake during

the presented investigation period compared to earlier studies may be an indicator of less productive seasons during previous years. Therefore, the more labile DOM fractions may have been preferentially consumed while the remainder was altered into more recalcitrant forms. In combination with direct inhibitory effects of solar radiation on bacterioplankton this process may have lead to an accumulation of photochemically altered DOM over time. Mao et al. (2007) demonstrated that the fulvic acid fraction in Pony Lake has undergone significant humification processes from its algal precursor material that generated highly cross-linked structures. Thus, it is hypothesized that the organic matter in this study was 'aged', which made it less prone to abiotic transformations. The minimal effect of solar radiation on the overall quality of the organic matter in Pony Lake (Guerard et al., in preparation), and minor effects of photochemical reactions of Pony Lake bulk DOM on the bioavailability to natural bacterial assemblages support this idea. Additionally, the observed differences between photolysis experiments on Pony Lake whole water DOM conducted in 2004/05 and 2005/06 may indicate changes in the chemical characteristics of Pony Lake DOM. However, a simple explanation may lie in the difference of the bacterial community amended to the treatment samples. While in 2004/05 a natural bacterial sample from Pony Lake served as an inoculum, an enrichment culture was used in 2005/06. An enrichment medium more selectively favors bacterial strains that are capable of growing under the provided conditions and alters the community structure found in an environmental sample. Thus, the stimulated effect in light exposed Pony Lake whole water samples in 2005/06 may be related to compositional changes in the enrichment culture. Nonetheless, a more detailed

characterisation of the chemical composition of Pony Lake whole water DOM and of the physiology of bacteria would be required to better understand the photolytic-induced trophic linkage between DOM and microorganisms.

A further explanation why the observed differences in substrate availability between light and dark controls were less pronounced in Pony Lake compared to other aquatic systems with autochthonous derived organic matter (Brenner & Biddanda, 1998; Obernosterer et al., 1999) may be found in the physical environment of these samples. Since the samples were incubated at low growth temperatures (4°C) in this study, temperature may have be of significant importance for the metabolic rate of microorganisms. Nedwell & Rutter (1994) demonstrated that heterotrophic bacteria show a reduced substrate affinity at low temperatures. Further, high substrate concentrations are required for bacteria to remain active and to overcome the effect of limited substrate affinity in energy limited environments (Wiebe et al., 1992). Consequently, one could assume that more temperate incubation temperatures would have enhanced metabolic rates and the difference between treatments would have become more pronounced.

In contrast to Pony Lake bulk DOM, a stronger decrease in bioavailability was observed in the fulvic acid fraction (WW XAD 04/05). Solar radiation dosages in the UV-B and UV-A range was 21.1% and 4.8% higher compared to the exposure date in 2004/05 and on average exceeded the radiation dose by 41.8% and 32.5% respectively compared to the dosage during the series of experiments conducted between 01/27/06 and 02/03/06 (Table 5.1.). However, the initial inhibitory effects of photoproducts on bacterial production were alleviated after 12 days of incubation. Since H_2O_2 production is

highly correlated with the absorption coefficient at 310 nm (Scully et al., 1996), we assume that the higher solar radiation doses in the UV range caused an enhanced ROS production. As described previously, reactive oxygen species may be responsible for inhibitory effects on bacterial activity during these photolytic bioavailability assays. However, also for the most slowly decaying ROS, H_2O_2, half-life varies between 1-8 hr in natural waters (Cooper & Zepp, 1990; Tranvik & Kokalj, 1998). Thus, we assume that the stronger suppression of the initial bacterial activity in the light exposed fulvic acid sample was related to ROS production. Consequently, the loss of ROS during the bacterial incubation would explain the decrease in production differences between the irradiated and dark control samples. Anesio et al. (2005) showed that the bacterial carbon production in irradiated humic substances was highly correlated with H_2O_2 photoproduction. Following the decay of H_2O_2 nearly all irradiated samples showed enhanced bacterial activity after 43 hrs.

In contrast to the WW XAD 04/05 experiment, the initial bacterial production rates in light exposed WW XAD 05/06 isolates were slightly stimulated, whereas long term incubations resulted in a suppression of bacterial activity. The observed decrease in bioavailability of fulvic acids goes against our current understanding that photochemical breakdown of humic substances generates more labile substrates of lower molecular weight (e.g. Geller, 1986; Lindell et al., 1995; Bano et al., 1998). This inconsistency with previous findings is even more inexplicable since a degradation of the chromophoric structures and a slight degrease in the average molecular weight of fulvic acids were detected in the light exposed samples (Guerard et al., in preparation).

Effects on Community Structure

Denaturing gradient gel electrophoresis (DGGE) was used to highlight possible changes in the development of the bacterioplankton community under the various treatment conditions. One can assume that the long term experiment (12 days) would provide sufficient time for the microbial community to adapt to photochemically induced alterations in the substrate composition by synthesizing new enzymes for photoproduct degradation or that the microbial community may evolve differently in light/dark treatments based on substrate availability. As expected, the bacterial community exposed to the fulvic acid fraction isolated from Pony Lake water 2004/05 (WW XAD 04/05) showed a clear response to the substrate and a shift in community structure was evident (Fig. 5.6.). DGGE banding patterns found in the initial inocula and at the end of the 12 day incubation period were different. Nonetheless, a comparison between light and dark treatments revealed that the banding patterns at each time point in both bioassays resembled each other and evolved uniformly. This finding was confirmed by image analysis using GelComparII software (Applied Math; results not shown). Although DNA samples from the initial inoculum for the Pony Lake whole water sample (WW 04/05) were not collected, the DGGE profiles obtained after twelve days also demonstrated that the natural Pony Lake community amended to the light exposed and dark control evolved identically.

It has been demonstrated that variations in the DOM composition and sources influence the bacterial community structure and control biogeochemical processes (Judd et al., 2006). Bacterial communities amended with photochemically altered DOM or dark

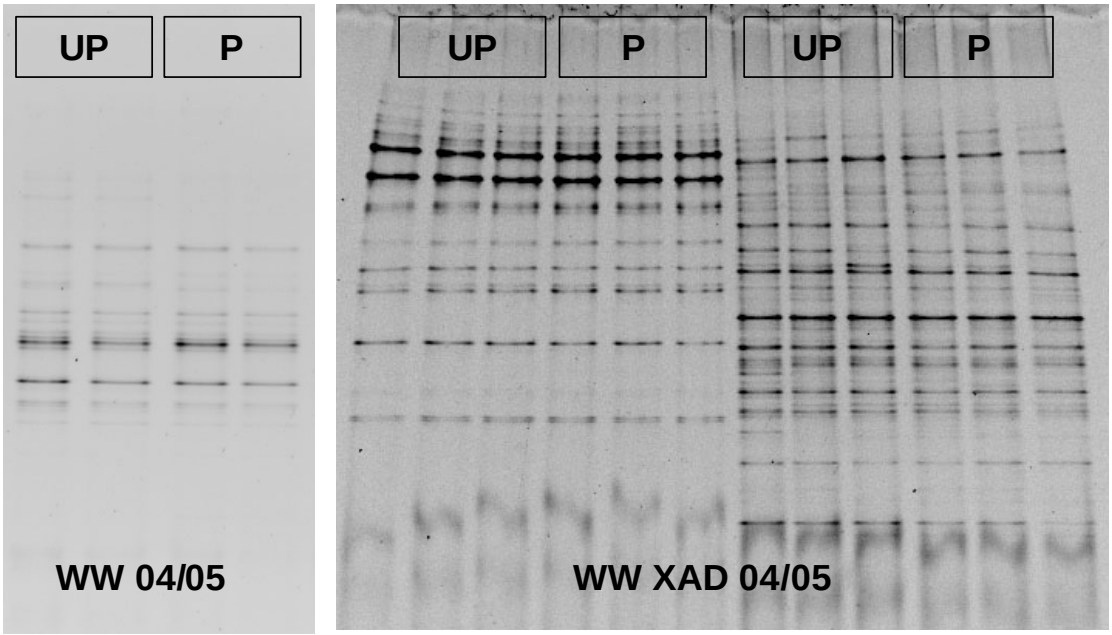

Figure 5.6.: DGGE profiles of natural microbial assemblages and enrichment cultures amended with different organic material from Pony Lake exposed to 24 hrs of natural Antarctic solar irradiation. Profiles indicate a shift in the microbial community structure during the twelve day incubation experiment. Left: Bacterial community profile after 12 days of incubation in Pony Lake whole water samples (WW 04/05). Right: Pony Lake fulvic acid supplemented with an enrichment culture (WW XAD (04/05)). First six columns show the initial amendment, second six columns demonstrate how the bacterial community shifted during the twelve day incubation assay. Photolysed (P), unphotolysed (UP).

controls evolved differently over time related to DOM characteristics and bioavailability (Judd et al., 2007, Pérez & Sommaruga, 2007, Abboudi et al., 2008). This is in clear contrast to our findings and the similarity in banding patterns between light exposed and dark controls in this study supports the idea that the net effect of solar radiation exposure on the bioavailability of organic matter fractions from Pony Lake is rather small.

Summary

The present study demonstrates that solar radiation affects the bacterioplankton community in Pony Lake in several ways; (i) directly via negative impacts of solar radiation on biological processes and cell physiology, (ii) via photobleaching of DOM which may alter the radiation climate of Pony Lake by allowing solar radiation to penetrate deeper in the water column, and (iii) indirectly via photochemical transformation of DOM. However, reduced bioavailability of DOM to bacterial uptake after solar radiation exposure was more apparent in the isolated fulvic acid fraction. In contrast, the effect of solar radiation on the bioavailability of Pony Lake bulk DOM supplemented with a natural bacterial community from Pony Lake was minimal, indicating that the photochemically induced alterations in the aromatic humic acid fraction were compensated for the bulk DOM sample. The structural analysis of phototransformed DOM verified that photochemical processes had little effects on the overall quality of the organic matter in Pony Lake (Guerard et al., in preparation). Thus, we propose that the limited response of the bulk DOM in Pony Lake to photodegradation is the result of past accumulation of aged dissolved organic matter, which is less susceptible to further abiotic transformations than the generation of fresh DOM.

CHAPTER 6

CAROTENOID PIGMENTATION IN ANTARCTIC HETEROTROPHIC BACTERIA AS A STRATEGY TO WITHSTAND ENVIRONMENTAL STRESSES

Adapted from: Markus Dieser, Mark Greenwood and Christine M. Foreman (Draft for submission to Arctic, Antarctic and Alpine Research)

Abstract

Bacterial strains isolated from Antarctic environments were used to assess the role of carotenoid pigments as cryo- and solar radiation protectants. Isolates were subjected to one hundred 12 hrs freeze-thaw cycles and exposed to ambient simulated solar radiation (300 Wm^{-2}) with growth recovery evaluated after pre-set time intervals. Differences in survival were observed between carotenoid pigmented and non-pigmented strains in response to the different stresses based upon the enumeration of colony forming units. On average carotenoid pigmented strains were more resistant to freeze-thaw cycles as compared to the non-pigmented strains. Survival for non-pigmented strains decreased precipitously from 2×10^7 to 1.5×10^4 cells ml^{-1}, on average, within the first 20 cycles. Similar results were found in the solar radiation experiments. After 2 hrs of solar radiation exposure, 61% of the pigmented organism survived versus 0.01% for the non-pigmented isolates. We applied an additive mixed model to estimate differences between the carotenoid pigmented and non-pigmented bacterial groups. Modelled results confirmed a positive effect of pigmentation on survivability and provide evidence that

carotenoid pigmentation in heterotrophic bacteria isolated from Antarctic environments increases resistance to environmental stressors.

Introduction

Organisms that exist in icy environments must possess mechanisms to protect themselves from extreme environmental conditions that would otherwise cause severe damage to non-adapted organisms. Cells revived from ice core samples have often endured desiccation, intense solar irradiation while at the surface, freezing, a long period of frozen dormancy, and eventual thawing. Therefore, it is not surprising that a large number of the isolates recovered from icy environments belong to bacterial groups that form spores (e.g., *Bacillus* and *Actinomyces*) or have thick cell walls and polysaccharide capsules (Priscu et al., 2006). These structures can help overcome the stresses associated with water loss, namely increased intracellular solute concentrations, decreased cell size, a weakened cell membrane, and physical cell rupture caused by freezing and thawing. The high frequency of pigment production in recovered isolates from ice cores (Zhang et al., 2008), glaciers (Foght et al., 2004), or marine surface waters (Agogue et al., 2005) suggests that pigmentation plays a role in adaptation to cold environments. Moreover, carotenoid pigments may play a role in the modulation of membrane fluidity in bacteria growing under low temperature conditions (Jagannadham et al., 2000).

Solar radiation is highly variable over a range of scales and wavelengths, and short wavelength radiation can be more damaging to biological systems than longer wavelengths (Ekelund, 1992; Ross & Vincent, 1998; Cockell & Knowland, 1999). Direct

and indirect inhibitory effects of UV-B (280-320 nm) and UV-A (320-400 nm) radiation are well documented (Ekelund, 1992; Haeder et al., 1998; Jeffrey et al., 2000; Buma et al., 2001; Agogué et al., 2005; Haeder & Sinha, 2005). However, organisms have developed a number of successful strategies to cope with the damage caused by UV radiation. One such approach is known as DNA repair and involves photo-reactivation and dark repair mechanisms (Haeder & Sinha, 2005). The second strategy employs UV absorbing or screening compounds such as carotenoid pigments, scytonemin or mycosporine-like amino acids (MAA's) (Cockell & Knowland, 1999). These screening compounds provide a level of protection to phytoplankton (Cockell & Knowland, 1999); and it has been suggested that carotenoid pigments may provide protection in many non-photosynthetic bacteria (Arrage et al. 1993; Zenoff et al., 2006a); however, this has not been empirically tested.

Although adaptation of microbial life to a variety of cold temperature environments has been studied for the past few decades, and detailed information exists about many of their physiological characteristics (Gounot, 1991) and adaptations (Nadeau & Castenholz, 2000; Mueller et al., 2005), our understanding of the effects of environmental stresses on natural bacterial assemblages is still limited. The purpose of this study was to evaluate the role of carotenoid pigments in heterotrophic bacteria as potential cryo- or solar radiation protectants. Carotenoid pigmented and non-pigmented heterotrophic bacterial isolates from Antarctic ecosystems were exposed to freeze-thaw cycles and simulated ambient solar radiation with the effects of these physical stressors examined.

Materials and Methods

Sample Collection and Bacterial Isolation

Water samples were collected during the austral summer season 2004/2005 from two Antarctic sites: Pony Lake which is a shallow, coastal lake located at Cape Royds (77° 33' S, 166° E) and from a supraglacial fluvial system on Cotton Glacier, which lies in the Transantarctic Mountains (77° 07' S, 161° 50' E). Both systems experience complete freeze-up during winter and are ice free for several weeks during the summer. Samples were immediately (within 4 hrs) transported back to the Crary Laboratory at McMurdo Station, Antarctica. Bacterial strains were isolated on R2A agar media plates. Briefly, 100 µl of water from each site were streaked onto agar plates and incubated at 4°C in the dark. Single colonies from each site were selected according to morphological characteristics including color, size and colony shape. These colonies were subsequently sub-cultured to obtain purified isolates. Isolates were stored in 40% glycerol at -80°C in the dark. All isolates were tested for catalase activity and Gram stain status.

Phylogenetic Analysis

Bacterial isolates were sent to Laragen (www.laragen.com) for 16S rRNA sequence analysis using an Applied Biosystems ABI3730 (www.appliedbiosystems.com) automated sequencer. Nearly full length 16S sequences were obtained with multiple reads. Nucleotide sequences were assembled and aligned in BioEdit (Hall, 1999) using the ClustalX function with data obtained from GenBank using the BLAST search tool (Version 2.0; ncbi.nlm.nih.gov/BLAST/, Altschul et al., 1990).

Pigment Extraction

The steps for pigment extraction preparation were carried out rapidly on ice under minimal light conditions to avoid degradation of pigments. Bacterial isolates were thawed and centrifuged (5000 rpm for 5 min at 4°C). The resulting cell pellets were transferred to 1.5 ml 95% methanol (HPLC grade) in sterile Eppendorf tubes and gently sonicated in an ice water bath (two 30 sec. bursts at 17 W). Samples were allowed to extract for 12hrs at -20°C. After extraction the supernatant was collected by centrifugation (5000 rpm for 5 min at 4°C). If the sample still appeared to be cloudy the centrifugation step was repeated. Absorbance scans (220 to 750 nm) were measured using a spectrophotometer (Nanodrop ND 1000) with a UV-VIS absorbance module. Additionally, bacterial isolates were sent to Horn Point Laboratory (www.hpl.umces.edu) for HLPC pigment analysis.

Freeze-Thaw Experiments

Carotenoid pigmented and non-pigmented isolates (Table 6.1.) from glycerol stocks were enriched in R2A broth media at 6°C. After enrichment a concentrated bacterial stock solution for each organism was prepared. 500 µl bacterial stock solutions were added to Falcon tubes containing 9.5 ml of R2A broth media. Triplicate broth cultures of each bacterial strain point were prepared for each sampling point and subjected to a series of standardized 12 hour freeze-thaw cycles (0, 1, 2, 6, 10, 15, 20, 30, 40, 50, 70, and 100 cycles). Samples were rotated between a -20°C freezer and a 6°C dark incubator every 6 hours. At the end of each thawing period the samples were gently inverted to counteract sedimentation of the bacteria. Triplicate control samples were incubated at 6°C in darkness. At each sampling point serial dilutions were made in

1X PBS buffer for each cell suspension. 100 µl of each sample dilution were spread plated onto R2A agar plates. Colony forming units (CFUs) were enumerated, as a measure of the quantity of bacteria that survived the treatment. Based upon individual grow characteristics, plates were incubated at 6°C in the dark for 3 days for ANT 1, ANT 4; 4 days for ANT 3; 5 days for ANT 11, ANT 12; and 8 days for ANT 16, ANT 20 in order to allow for optimal growth of the organisms.

Solar Radiation Experiments

The same bacterial strains used in the freeze-thaw experiments were also used in the solar radiation experiments. Triplicate 10 ml enrichment cultures (9.5 ml broth media plus 0.5 ml stock solution) were prepared as above. Samples were then transferred to 15 ml quartz tubes, to ensure full scale light penetration, and sealed with parafilm. The samples were exposed to simulated solar radiation in an environmental solar simulator (Russells Technical Products, Inc., Model: WMD-230-5-5), using a metal halide (MHG) lamp (K.H. Steuernagel) to simulate solar radiation between 280-3000nm. This lamp produced a dense multiline spectrum of the rare earth, which is comparable to a continuous spectrum of light (www.khslight.com). Samples were gently agitated using an orbital shaker. A temperature probe was placed inside one quartz tube to record the temperature (~4°C) throughout the experiment. To determine the potential protective role of pigmentation as solar radiation protectants, isolates were exposed to a simulated solar radiation dose of 300 W m^{-2} for 12 hours. The spectrum can be broken into its individual constituents (UV-B (0.9 W m^{-2}), UV-A (19.9 W m^{-2}), PAR (125.2 W m^{-2}), NIR (110.1 W m^{-2}), and IR-B (43.9 W m^{-2})). A dosage of 300 W m^{-2} was chosen to

reflect ambient Antarctic conditions measured in the nearby McMurdo Dry Valleys (Dana et al., 1998). Samples were analysed at the beginning of the experiment, and after 2, 6 and 12 hrs. Similar to the freeze-thaw experiments, dilutions from triplicate samples were made. 100 µl of sample was spread plated on agar media plates, incubated and CFUs were enumerated.

In order to determine potential dark repair mechanisms present in the strains in response to solar radiation damage, irradiated cells were subjected to a longer incubation period of five days after the initial count of CFUs. After the five additional days the number of CFUs was enumerated again.

Statistical Analyses

For both the freeze-thaw and the solar radiation experiments, the experimental design involved repeated measurements on each strain across experiments (cycles or solar radiation exposure time) and triplicate measurements of each observation. To address this design a mixed model structure, as in Pinheiro & Bates (2004), was used incorporating a random intercept for each bacterial strain in each group (pigmented and non-pigmented, treatment and control groups). This framework allowed us to account for the random variation in the initial CFUs of each experiment and for comparing different fixed effects (pigmentation and control group vs. treatment, and cycles or exposure time). The random intercept component of the model also induced a correlation structure similar to compound symmetry for the repeated measurements within a strain. Compound symmetric correlation is often used in repeated measures models. Thus, this model provided a structural design where all the measurements on a bacterial strain were

positively correlated. Additionally, the changes over time were observed to be relatively smooth within each group (pigmented, non-pigmented, controls), which led to the use of a nonparametric trend (Wood, 2006) to capture the change in mean CFUs for each group in the mixed model structure. Penalized cubic regressions splines were used to estimate these smoothing effects.

For the solar radiation exposure experiment, non-constant variance was detected in the initial model diagnostics. In order to accommodate this additional aspect of the data set, a different variance coefficient was estimated for each of the four different exposure levels and incorporated in the mixed model framework.

Other models, including more typical repeated measures models, were considered as well. However, model selection using Akaike's Information Criterion (AIC) (Akaike, 1973) led to final models which incorporated additive effects and differences based on pigmentation. All models were estimated using the statistical software package R (R Development Core Team, 2009) and either the nlme package (Pinheiro et al., 2009) or the mgcv package (Wood, 2006).

During the statistical method development for the freeze-thaw cycle experiment we encountered problems due to the distinct treatment response of ANT 11; subsequent residual diagnostics suggested that ANT 11 is an outlier relative to the other pigmented, treatment observations. Instead of completely removing this unusual observation, we accommodated ANT 11 by placing ANT 11 in its own group (initially we worked with two categories: pigmentation present or absent). Based on the low pigment concentration detected in ANT 11 compared to the other Antarctic bacterial strains (see HPLC analysis)

and the categorization of ANT 11 as an outlier in its response to freeze-thaw cycles, we are able to justify our regrouping of strains. Nonetheless, since the other three bacterial strains in each group (pigmented, non-pigmented) showed a similar response to the freeze-thaw cycle regime, we focus on the interpretation of the results for the pigmented groups that did not include ANT 11.

Results

Phylogeny of Bacterial Isolates

The phylogenetic characterization of selected bacterial isolates from Antarctic environments is summarized in Table 6.1. The closest identified relatives were reported as being psychrotrophic strains. Isolates ANT 11 and ANT 12 were most closely related to *Flavobacterium segetis* AT1048 and *Flavobacterium weaverense* AT1042, respectively (Yi & Chun, 2006). *Flavobacterium segetis* AT1048 produced an orange pigmentation and *Flavobacterium weaverense* AT1042 appeared yellow when growing on R2A agar (Yi & Chun, 2006). ANT 16 was most closely related to *Arthrobacter agilis* MB8-13, a dark rose-red pigmented bacterium (Fong et al., 2001). ANT 20 showed highest similarity with the yellow pigmented bacterial strain of *Sphingomonas echinoides* (Denner et al., 1999). Non-pigmented ANT 1 was related to *β-Proteobacterium* Wuba70, ANT 3 was taxonomically affiliated with *Janthinobacterium* sp. HHS7, and ANT 4 (Foreman et al., in review) was most closely related to *Flavobacterium xinjiangense* AS 1.2749.

Table 6.1.: Pigmentation characteristics of Antarctic bacterial isolates associated with growth on R2A media and their closest relatives based on 16S rRNA sequencing.

Isolate	Closest relative and ascension #	Bacterial group	Pigment
ANT 11	*Flavobacterium segetis* AT1048, AY581115	*Bacteroidetes*	orange
ANT 12	*Flavobacterium weaverense* AT1042, AY581114	*Bacteroidetes*	yellow
ANT 16	*Arthrobacter agilis* MB8-13, U85896	*Actinobacteria*	dark rose-red
ANT 20	*Sphingomonas echinoides*, AJ012461	*α-Proteobacteria*	yellow
ANT 1	*β-Proteobacterium* Wuba70, AF336360	*β-Proteobacteria*	none
ANT 3	*Janthinobacterium* sp. HHS7, AY846272	*β-Proteobacteria*	none
ANT 4	*Flavobacterium xinjiangense* AS 1.2749, AF433173	*Bacteroidetes*	none

The isolates used in this study shared similar characteristics (e.g. colony coloration) with their nearest relatives. Furthermore, all isolates were catalase positive and, with the exception of ANT 16, Gram negative.

Characteristics of Extracted Pigments

The UV-VIS absorption spectra of methanol extracts from the Antarctic isolates were measured to determine the presence of pigments. All pigmented strains showed multiple absorption peaks between 400 and 550 nm (Fig. 6.1.), which are characteristic of carotenoid pigments (Chauhan & Shivaji, 1994; Mueller et al., 2005; Du et al. 2006). Maximum absorption peaks for *Flavobacterium segetis* AT1048 and *Flavobacterium weaverense* AT1042, the closest relatives to ANT 11 and ANT 12, were reported at 452 nm and 472 nm and at 451 nm and 479 nm, respectively (Yi & Chun, 2006); thus, closely resembling the spectra from our isolates. ANT 16 showed similar absorption spectra as reported for *Arthrobacter agilis* MB8-13 with double *cis*-absorption peaks that are typical

of an acyclic or monocyclic chromophore (Britton, 1995; Fong et al., 2001). ANT 16 also exhibited an additional peak at 320 nm. This peak at 320nm and similar absorption spectra were found to be carotenoid pigments in *Micrococcus roseus*, a psychrotrophic Antarctic bacterium (Chattopadhyay et al., 1997). It has been reported that members of the *Arthrobacter* and *Micrococcus* lineages are closely related and phylogenetically intermixed (Koch et al., 1994, Koch et al., 1995). The absorption spectra of ANT 20 resembled the ones reported for *Pseudomonas echinoides* (Czygan & Heumann, 1967). The yellow pigment in *Pseudomonas echinoides* (reclassified as *Sphingomonas echinoides*; Denner et al., 1999) has been identified as the carotenoid nostoxanthin (Jenkins et al., 1979). The three non-pigmented isolates (ANT 1, ANT 3, and ANT 4) did not have any absorption peaks between 220 and 750 nm. None of the isolates yielded spectral evidence for bacteriochlorophyll, which was also confirmed by HPLC analysis. HPLC analysis further revealed that no pigments were present in ANT 1, ANT 3, and ANT 4. ANT 11 and ANT 12 contained zeaxanthin, and other unknown carotenoids and zeaxanthin-like degradation products respectively. ANT 16 showed several small carotenoid peaks, all of similar spectra and ANT 20 had large amounts of an unknown carotenoid similar in spectral signature to zeaxanthin.

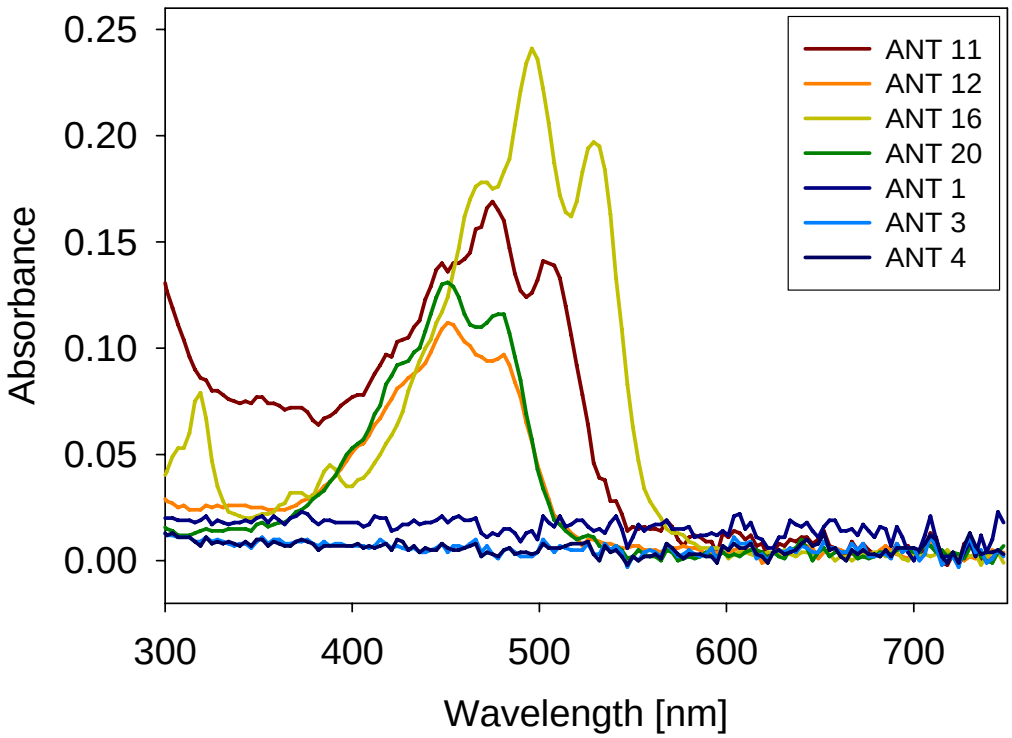

Figure 6.1.: Absorption spectra of selected pigmented and non-pigmented heterotrophic Antarctic bacterial isolates.

Freeze-Thaw Cycles

Carotenoid pigmented and non-pigmented isolates were exposed to 100 12hr freeze-thaw cycles (Fig. 6.2.). On average, the carotenoid pigmented isolates experienced a slight decrease in viability in response to temperature fluctuations (-20 and 6°C). In contrast, the viability of non-pigmented bacteria was significantly decreased by the freeze-thaw regime during the first 20 cycles. All three non-pigmented strains responded similarly with a drop in CFUs from 2 x 10^7 to 1.5 x 10^4 cells, on average. Over the remaining 80 cycles, viability in the non-pigmented isolates levelled off. Within the carotenoid pigmented group each bacterial strain displayed a unique response to the

repeated freeze-thaw cycles. ANT 12 increased in CFUs during the first 50 cycles. CFUs for ANT 16 increased during the entire experiment whereas CFUs for ANT 20 decreased almost linearly by three orders of magnitude over the time course of the experiment. An exception within the carotenoid pigmented group was ANT 11, it is unclear why ANT 11 behaved differently from the other pigmented isolates. During the first 6 cycles CFUs for ANT 11 increased by one order of magnitude, but after 40 cycles this strain was no longer culturable. It has been reported that polar carotenoids confer more rigidity to lipid membranes as compared to non-polar carotenoids (Jagannadham et al., 2000). One can speculate that differences in polarity, or configuration, and differences in the position or orientation of the pigment may exist in the membranes between ANT 11 and the remaining pigmented isolates, which may have substantial effects on the properties of the membrane (Britton, 1995b). A possible explanation could also be provided by the HPLC analysis results. ANT 12 contained six times more zeaxanthin compared to ANT 11 and the unknown peak in ANT 20 was about six times larger than the zeaxanthin peak in ANT 12. The behavor of ANT 11 in the control group was distinctly different from the other pigmented and non-pigmented isolates. Whereas all the other bacterial strains increased in CFUs during the 50 day incubation period at 6°C, ANT 11 remained constant during incubation and dropped approximately two orders of magnitude between 35 and 50 days.

The response of the pigmented isolate ANT 11 to repeated freeze-thaw cycles was not only different within its assigned group, but also in comparison to the non-pigmented strains. Since the closest relative to ANT 11 has been designated as a psychrophilic strain

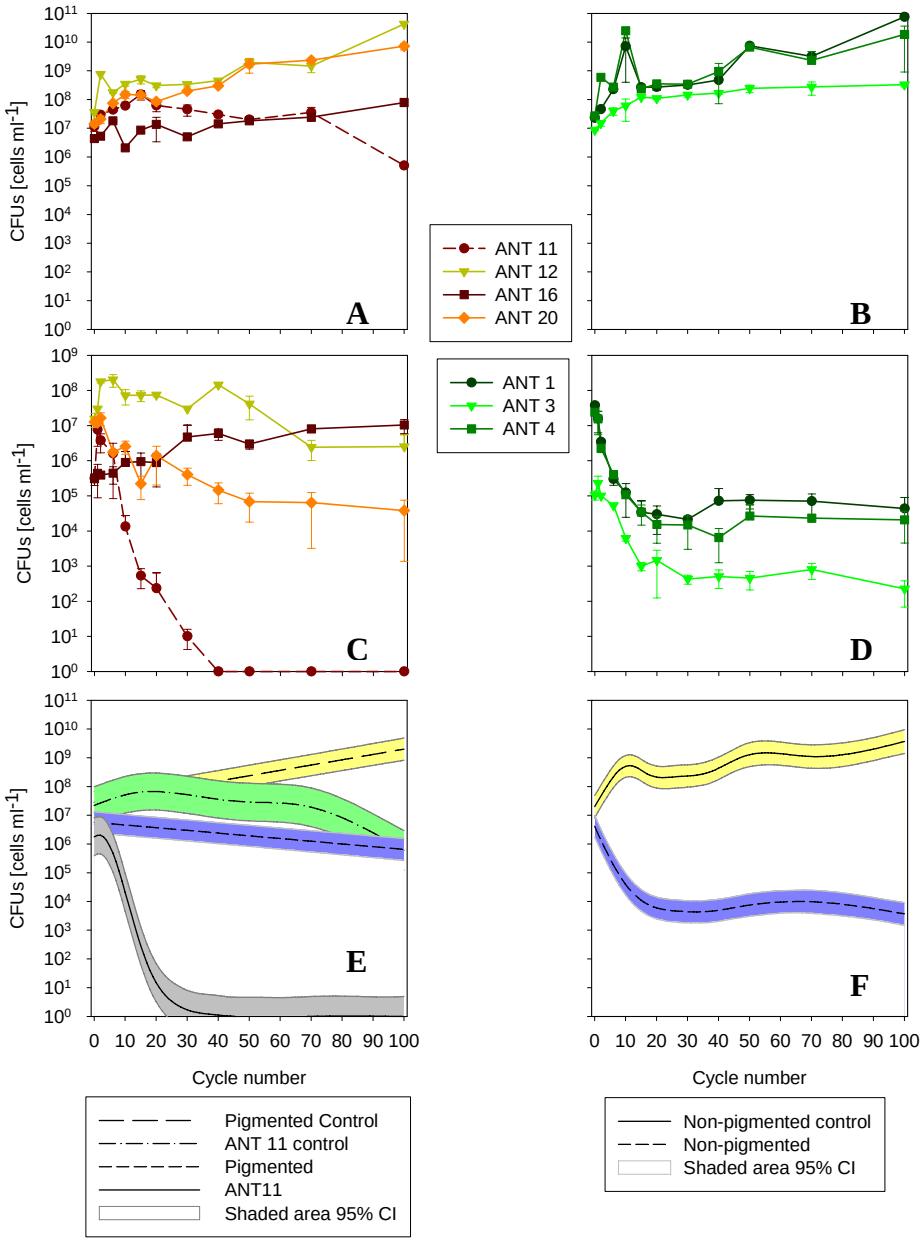

Figure 6.2.: Response of bacterial isolates to freeze-thaw cycles. A and B: Control group. Pigmented (ANT 11, 12, 16, 20) and non-pigmented (ANT 1, 3, 4) isolates incubated for 50 days at 6°C. C and D: Treatment effect on pigmented and non-pigmented bacteria strains. E and F: Additive mixed model results showing the estimated means for the pigmented group and non-pigmented groups. Shaded area shows the approximate 95% confidence intervals (CI) for the estimated mean values obtained from the additive mixed model. Error bars show one standard deviation from the mean.

with a theoretically minimum growth temperature of -29.7°C, its low tolerance to the freeze-thaw regime was unexpected. Instead, a comparative study revealed that ANT 11 resembled the decreasing pattern in CFUs of *Escherichia coli* K12 in response to the freeze-thaw cycle regime (see APPENDIX). Consequently, due to its unusual response to freeze-thaw cycles ANT 11 was defined as an outlier and treated as a separate, pigmented 'group' in our analysis.

Solar Radiation Exposure

Exposure of the isolates to ambient levels of Antarctic solar radiation clearly showed that a protective function was conferred on the organisms that possessed carotenoid pigments (Fig. 6.3.). The viability of carotenoid pigmented isolates was higher, on average, when compared to the non-pigmented bacteria after 12 hrs exposure to 300 W m^{-2} at ~4°C. After a 6 hr exposure non-pigmented isolates decreased in CFUs from 1 x 10^7 to 8 x 10^1. Furthermore, after 12 hrs of exposure the non-pigmented bacteria were no longer culturable. In contrast, the carotenoid pigmented strains showed only a slightly negative response to solar radiation during the first 6 hrs of exposure. ANT 11 and ANT 12 were unaffected by solar radiation exposure after 6 hrs and did not show a significant change in viability. The culturability of ANT 16 and ANT 20 decreased two and four orders of magnitude, respectively, over the same time interval. Although long term exposure (12 hrs) markedly decreased the viability of the pigmented strains, the organisms were still viable, with CFUs of 2 x 10^1-4.5 x 10^3 cells ml^{-1}. Analogous to the freeze-thaw cycle experiments, the non pigmented isolates all showed a similar negative response to solar radiation, whereas representatives within the pigmented group showed a

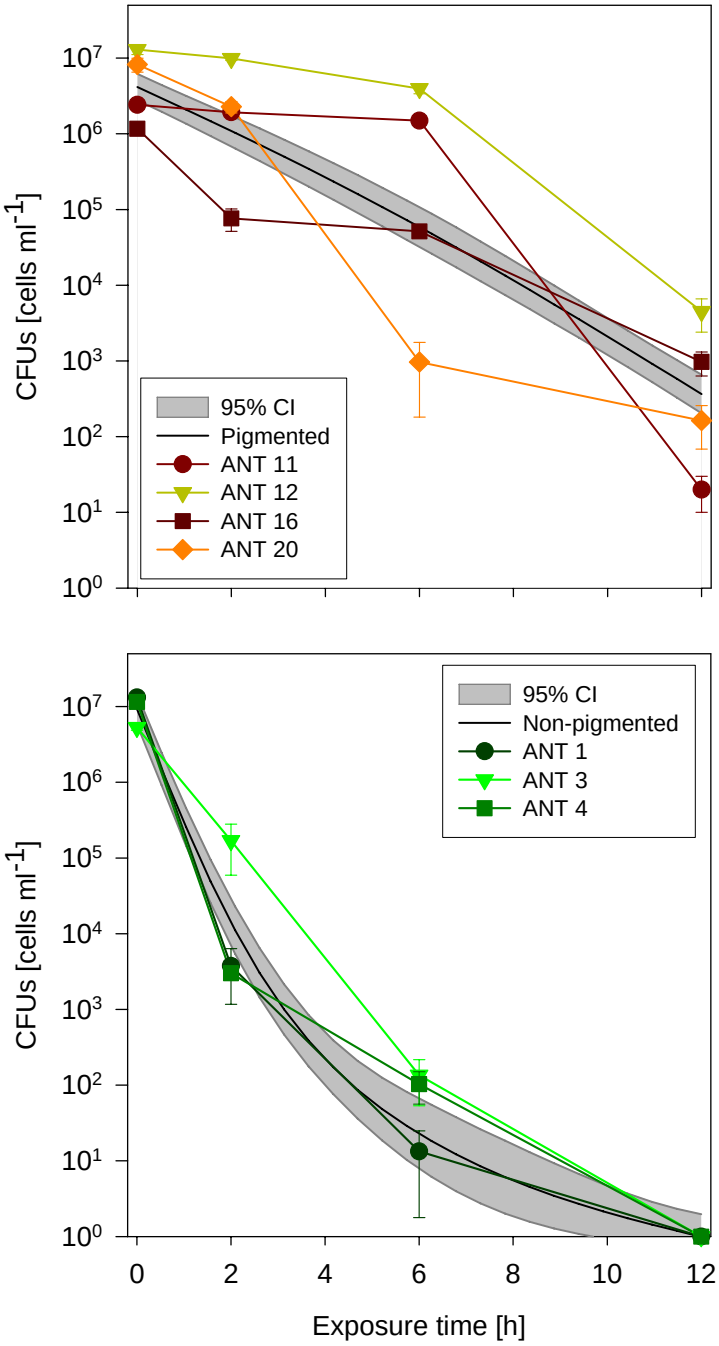

Figure 6.3.: Response of bacterial isolates to solar radiation exposure (300 W m^{-2}). Top: Pigmented isolates. Bottom: Non-pigmented isolates. Error bars show one standard deviation from the mean. Shaded area shows the approximate 95% confidence intervals (CI) for the estimated mean values obtained from the additive mixed model.

Table 6.2.: Response of Antarctic bacterial isolates to exposure to ambient, simulated solar radiation as demonstrated by colony forming units (CFUs), and evidence for dark repair mechanisms as shown by recovery of the isolates after a 5 days incubation period following exposure. CFUs represent counts after 12 hrs of exposure to solar radiation.

Sample ID	**CFUs [ml^{-1}] after exposure**	**CFUs [ml^{-1}] after 5 additional days**
ANT 11	20	325667
ANT 12	4517	49250
ANT 16	975	6100
ANT 20	163	280
ANT 1	0	10
ANT 3	0	0
ANT 4	0	20

range of responses. This range of responses within the pigmented isolates supports the idea that carotenoid pigments may function differently in cell membranes. Although 6 hrs exposure caused damage in two of the carotenoid pigmented strains, the presence of these pigments still conferred an enhanced level of protection from solar radiation when compared to the non-pigmented strains.

After CFUs were enumerated for the final time point, the bacterial isolates were incubated in the dark for an additional period of five days to determine if these organisms could recover from the damage caused by solar radiation. Although damage was observed in both bacterial groups after a 12 hrs period of exposure, the carotenoid pigmented isolates achieved higher recovery, on average, compared to the non-pigmented group. The pigmented bacterial strains increased by approximately one order of magnitude over the additional 5 days of dark incubation. With the exception of ANT 11, none of the isolates recovered to their initial numbers at the beginning of the experiments. ANT 11 increased CFUs by four orders of magnitude and achieved almost full recovery

to pre-exposure numbers. Conversely, the non-pigmented strains showed almost no evidence of dark repair mechanisms (Table 6.2.).

Statistical Analysis

To address differences between the carotenoid pigmented and the non-pigmented group we applied an additive mixed model using nonparametric fixed effects along with random effects to describe the repeated measurement structure of the experimental design. The favored models evaluated by AIC model selection criterion demonstrate a positive carotenoid pigment effect and provide supportive evidence for the increasing resistance of pigmented bacteria to environmental stressors such as temperature fluctuations and solar radiation. After adjusting for different, random starting points for each bacterial strain, for each experiment the modelled, estimated mean and the approximate 95% confidence intervals are displayed in Fig. 6.2.E, F and Fig. 6.3. The different responses of the carotenoid pigmented and non-pigmented groups are illustrated by the estimated effects for each group in each graph. The carotenoid pigmented group displayed a linear trajectory with a slightly negative slope in the freeze-thaw cycle experiment (Fig. 6.2.E) whereas the non-pigmented group dropped off precipitously within the first 20 cycles before levelling off (Fig. 6.2.F). Fig. 6.2.E also clearly highlights the distinct different response of ANT 11 to the freeze-thaw cycle regime. In contrast to the sight linear decrease in the estimated mean for the pigmented group with higher higher numbers of freeze-thaw cycles, ANT 11 decreased in CFUs exponentially and become undetectable after 30 cycles.

A positive carotenoid effect can also be seen in the model-based estimated means for the two groups (pigmented, non-pigmented) in response to solar radiation exposure. The carotenoid pigmented group in the solar radiation experiment followed a convex path whereas the non-pigmented group showed a pronounced concave curve reaching undetectable levels after 12 hrs of exposure (Fig 6.3.).

The estimated contrasts between the pigmented (excluding ANT 11) and non-pigmented group in their response to either freeze-thaw cycles or solar radiation exposure are displayed in Fig. 6.4. These contrasts explicitly point towards a positive pigment effect in heterotrophic bacteria by displaying how much larger the CFUs in the pigmented group were compared the non-pigmented group. With the exception of the starting point, when no treatment was applied, at no point during either treatment did the CFUs of the non-pigmented group exceed the counts of the pigmented group, signifying a remarkably higher level of resistance among the pigmented strains.

Discussion

Freeze- Thaw Cycles

Temperature and the availability of liquid water are critical parameters for life, and set the limits for life processes. Only within a small range, ~5 to 50°C, have most organisms found their niches (Bölter, 2004). These two factors become even more important when environmental conditions exist at the far extremes. Freezing and thawing, along with changes in nutrient availability and drought, are common processes in cold regions on Earth, including the polar and high-altitude alpine regions. Temperature

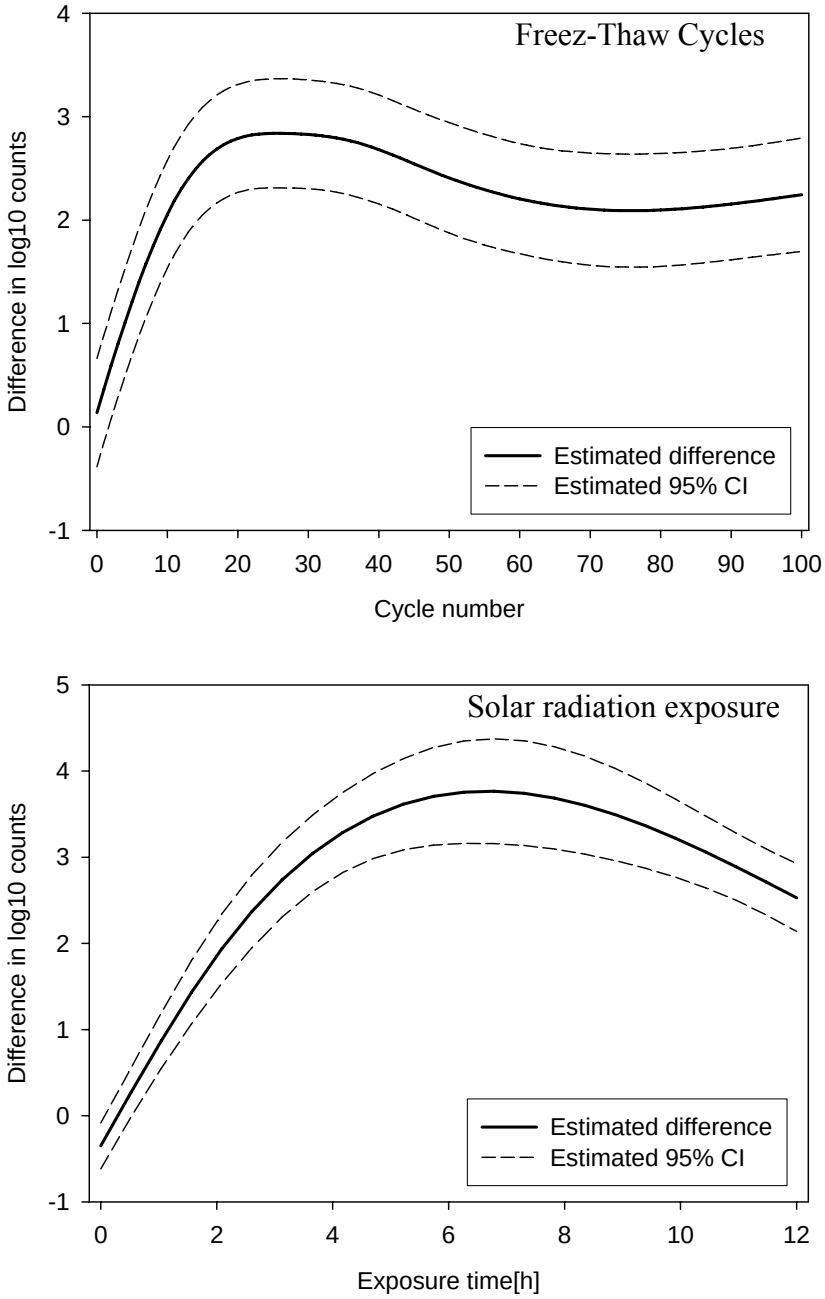

Figure 6.4.: Estimated contrast in CFUs between the pigmented and non-pigmented group in their response to the treatments and the estimated 95% confident intervals (CI). Top: Freeze-thaw cycle regime (100 cycles = 50 days). Bottom: Solar radiation exposure experiment (12 hrs). Both models display a positive effect of carotenoid pigmentation in heterotrophic bacteria to increase the resistance to environmental stresses such as freeze-thaw cycles and solar radiation.

fluctuations of 30°C and more may occur within one day in coastal continental Antarctica (Bölter, 2004). Temperature is a physical factor that immediately affects a cell. Thermal stresses have strong impacts on the membrane lipids of organisms that influence membrane structure and function (Hazel, 1995). The transition from the fluid to the gel phase reduces the activity of membrane bound enzymes, slows the rate of lateral protein diffusion within the plane of the membrane bilayer, and induces cluster formation of integral membrane proteins (Hazel, 1995). Living organisms typically encounter temperature changes throughout their life cycle and most microorganisms can tolerate a variety of changing conditions and stresses in their surrounding environment. Several defense mechanisms exist against temperature stresses, such as the production of cold and heat shock proteins and the alteration of membrane fatty acids (Gounot, 1991). These strategies allow nutrient exchange and enzyme activity to occur, and most importantly prevent intracellular ice nucleation. Recent studies have suggested that pigments such as carotenoids may play a role in the modulation of membrane fluidity in bacteria (Jagannadham, et al. 2000). Jagannadham et al. (2000) speculated that when cells grow at low temperatures (5°C) the unsaturated and branched chain fatty acids increase the fluidity of the membrane, whereas polar carotenoids counterbalance this effect by stabilizing the membrane. For the closest relatives to the pigmented (ANT 11, ANT 12, and ANT 20) and non-pigmented (ANT 4) isolates, abundant monounsaturated and branched fatty acids in the membranes were reported (Denner et al., 1999; Zhu et al., 2003; Yi & Chun, 2006). Since these membrane modulations were reported for close relatives in both groups, one can speculate that additional features contributed to the

different response to freeze-thaw cycles. It has been demonstrated that lower cultivation temperatures promoted an increased carotenoid production in psychrotolerant bacteria (Jagannadham et al., 2000). Similarly, Yokoyama et al. (1996) reported that bacteria growing under extreme conditions (e.g. low temperatures) have adopted carotenoids suitable for membrane stabilization of the cell wall. Since the bacterial cytoplasmatic membrane must always be maintained at a critical level of fluidity (Chauhan & Shivaji, 1994), the pigments appear to contribute to membrane stabilization by modulating membrane fluidity (Chattopadhyay et al., 1997; Jagannadham et al., 2000; Fong et al., 2001). Furthermore, it has been shown that carotenoid production was greater in cells growing at 5°C than at 25°C (Chattopadhyay et al., 1997; Fong et al., 2001). Although the exact function of carotenoids in membranes is still unknown, it has been demonstrated that bacterioruberin binds to membrane vesicles and increases the rigidity of the membrane (Strand et al., 1997). Bacterioruberin is a long chain (C-50) carotenoid and its presence has been identified in *Arthrobacter agilis* MB8-13 (Fong et al., 2001), the closest relative to ANT 16. To date, bacterioruberin has only been isolated from extremophilic bacteria and several archaea (Fong et al., 2001). Our data, and the additive mixed model, contribute to the functional understanding of carotenoids; whereby, carotenoid pigmented heterotrophic bacteria tolerate low temperatures and episodic freezing more successfully than non-pigmented bacteria. These findings strongly suggest that carotenoids may act in the modulation of membrane fluidity and contribute to membrane stability, a necessary adaptation to withstand the physiological stress incurred under low or freezing temperatures.

Solar Radiation Exposure

The impact of solar radiation on microorganisms depends upon the spectral composition of the light, the time pattern of exposure, and the presence of protective mechanisms (e.g. UV absorbing compounds, active or passive migration) (Ekelund, 1992; Haeder et al., 1998; Ross & Vincent, 1998; Cockell & Knowland, 1999; Buma et al., 2001; Haeder et al., 2003; Haeder & Sinha, 2005; Agogué et al., 2005). Considering that bacteria my account for up to 90% of the cellular DNA in aquatic environments (Joux et al., 1999) and that these organisms constitute a fundamental link in carbon flow, especially in microbially dominated systems, understanding the influence of solar radiation on bacteria is essential. Results from field studies on marine bacteria indicated that exposure to natural solar UV radiation resulted in a decrease in total cell abundance, a reduction in amino acid uptake, a depression of the activity of enzymes, and a significant inhibition of protein and DNA synthesis (Jeffrey et al., 2000).

Zenoff et al. (2006a) exposed bacterial isolates from high-altitude wetland waters from the Andean region, Argentina, to 4.49 W m^{-2} UV-B irradiance over 36 hrs. A general resistance to UV-B radiation was found among the majority of the culturable community. Most of these bacterial isolates were Gram-positive, spore-forming and/or showed pigmentation; characteristics that are expected to provide increased adaptation and resistance to UV radiation. With the exception of ANT 16, all of the Antarctic isolates in this study were Gram negative. Further, closest relatives assigned to our Antarctic isolates were non-spore forming organisms (Koch et al., 1995; Denner et al., 1999; Yi & Chun, 2006), highlighting the important role of pigmentation as solar

radiation screening compounds. The large proportion of pigmented bacteria, primarily reds and yellows, found in oceanic surface layers (Tsyban, 1971; Hermansson et al., 1987; Du et al., 2006), may provide indirect evidence of resistance to intense solar radiation, but this protective effect of pigments has never been experimentally demonstrated. In contrast, Agogué et al. (2005) isolated 90 bacterial strains from sea surface layers and underlying waters of two sites in the northwestern Mediterranean Sea, but their results show no correlation between pigmentation, solar radiation levels, and the occurrence of bacteria in the surface microlayer. They argued that pigmentation may only have an indirect effect on the resistance of bacterial cells to solar radiation.

Zenoff et al. (2006b) found a correlation between UV-B resistance and the environment from which the bacteria were isolated. Organisms isolated from environments exposed to high levels of solar radiation showed high UV-B resistance and/or repair abilities compared to those isolated from less irradiated environments. Nevertheless, after a 20 min exposure to UV-B light (3.3 W m^{-2}) only 12-48% survival was observed. Similar results were found by Joux et al. (1999) after exposure of marine bacterial isolates to UV-B light (2.3 W m^{-2}).

Although previous experiments have mainly focused on the inhibitory effect of different fractions of the radiation spectrum, we exposed bacterial strains to simulated solar radiation between 280-3000 nm. We assume that complex interactions in the spectral composition may induce cell damage. Hernandez et al. (2004) reported that mortality values resulting from PAR + UV-A + UV-B treatments were higher than those observed under strictly PAR + UV-A treatments. Helbling et al. (1995) demonstrated that

the impact of UV-A on the viability of bacterioplankton from Antarctic waters was considerably greater than the impact of UV-B radiation. However, because UV-B wavelengths overlap the absorption spectra of DNA it is expected that UV-B is more responsible for DNA damage. It is therefore assumed that the loss of viability in the UV-A range is due to photodynamic reactions involving reactive oxygen species (Helbling et al., 1995). Our results suggest that carotenoid pigmentation provides a natural sunscreen for bacteria allowing them to tolerate increased solar radiation exposure. Furthermore, carotenoid pigmentation may protect the organism against reactive oxygen species like superoxide and free radicals. Reactive oxygen species (ROS) generated from photochemical reactions have been shown to severely damage cellular structures and the physiology of aquatic microorganisms (Fridovich, 1986). Against these toxic oxygen species, microorganisms possess various defense mechanisms such as antioxidants, quenchers, and scavengers including carotenoids and enzymes (Chow, 1988). All pigmented and non-pigmented isolates in this study were found to be catalase positive. Catalase is essential for the decomposition of hydrogen peroxide. Kuznetsov et al. (1979) reported an increase in cell lysis of catalase negative isolates, which they attributed to oxygen toxicity. Although both groups, pigmented and non-pigmented isolates, showed catalase activity, they responded differently during the solar radiation exposure experiments. Therefore, we assume that other cell mechanisms, independent from enzyme production, may contribute to additional resistance in the pigmented group. Mikell et al. (1986) found accelerated cell lysis in a carotenoid-negative mutant compared to its pigmented counterpart. They argued that cell lysis resulted from

membrane disruption by autocatalytic lipid peroxidation independent of superoxide dismutase activity and catalase production, and that carotenoids in the pigmented isolate quenched intermediates of this process. Although ROS production was not measured during the solar radiation experiments, carotenoids may have positively counteracted cellular damage by toxic oxygen species as previously suggested (Mikell et al., 1986; Clocksin et al., 2007). Hernandez et al. (2002) detected no effect of hydrogen peroxide on the yellow pigmented strain *Arthrobacter* UVvi. Similarly, Arrage et al. (1993) found a higher resistance of pigmented bacterial isolates to reactive oxygen species when compared to non-pigmented strains. Our study shows that after an initial short term exposure (2 hrs) to a full scale solar radiation flux of ~300 W m^{-2}, 61% of the carotenoid pigmented isolates survived, as compared to only 0.01% of the non-pigmented isolates. Between 6 and 12 hrs of exposure non-pigmented bacteria experienced a complete loss of culturability, whereas pigmented isolates were able to tolerate these levels of solar radiation. The additive mixed model confirmed these findings and the positive effect of carotenoid pigmentation. The modelled means of each group showed an inverse shape of the graph, indicating a protective role of carotenoids against solar radiation exposure.

It is well known that microorganisms can enter a viable but non-culturable state (Reszak & Colwell, 1987). Therefore, the loss of culturability of non-pigmented isolates would not necessarily imply mortality. The loss of culturability only implies that an organism is damaged, whereas respiratory chain reactions and morphological integrity may remain almost unchanged (Muela et al., 2000). As a result, bacteria have developed several repair mechanisms to overcome damage. In response to UV radiation, repair

mechanisms are usually classified into dark repair and photoreactivation, with recovery strategies differing widely between different bacterial strains (Haeder & Sinha, 2005; Zenoff et al., 2006a). Kashimada et al. (1996) proposed an asymptotic model assuming that the reactivation phenomenon (dark repair and/or photoreactivation) follows a saturation first order reaction and that the inactivation and reactivation curve is a function of time. Agogué et al. (2005) reported that bacterial strains that were highly resistant to solar radiation exposure had a different response with respect to the lag time of the growth curve. After 5 hrs of exposure the lag time could last 24 hrs longer as compared to normal growth.

If organisms have developed mechanisms to compensate for DNA damaging effects of solar radiation it is more likely that the DNA damage in the three non-pigmented strains was accumulated over the time interval of the experiment and that the repair mechanisms were insufficient to undo the damage. Furthermore, enzyme based repair mechanisms, like DNA repair, are generally temperature dependent and colder temperatures decrease the efficiency of these repair mechanisms (Ross & Vincent, 1998; Buma et al., 2001). Nevertheless, under cold incubation conditions (~6°C) carotenoid pigmented isolates in our experiment recovered more effectively than the non-pigmented isolates. Consequently, our data suggest that pigmentation attenuated the intensity of DNA damage, although, with the exception of ANT 11, none of the isolates were able to reach their initial abundance.

Summary

In this study we demonstrate that carotenoid pigmented, heterotrophic bacteria, isolated from Antarctic lake and river systems are more resilient to environmental stressors. We applied common physical stressor such as freeze-thaw cycles and solar radiation. In each treatment the culturability of non-pigmented bacterial strains was more effected than of the pigmented group. These distinctly different behavioral patterns and the positive pigmentation effect were clearly highlighted by the applied additive mixed model. Although the bacterial isolates, especially within the non-pigmented group, exhibited a similar response to the treatments, generalizations should be handled with care, since the sample size in each group (pigmented and non-pigmented) was small. The unique behavior of the bacterial isolate ANT 11 during the freeze-thaw regime emphasizes the need for caution. Although pigmented, the response of this bacterial strain neither resembled the general response pattern detected in the pigmented group nor the one in the non-pigmented group and eventually became unculturable after 30 cycles. Nonetheless, when exposed to ambient simulated solar radiation, ANT 11 showed a similar resistance to radiation as detected for the other pigmented strains.

Our results indicate that carotenoid pigmentation increases the resistance of heterotrophic bacteria to environmental stressors. Differences in the response to the physical stressors and the varying extent of the treatment effects on pigmented strains suggest that numerous factors such as pigment structure, their location within the membrane, and pigment concentration may play a role in protecting cells against these stresses.

CHAPTER 7

VIABLE/DEAD DISTINCTION OF MICROBIAL COMMUNITY STRUCTURE WITHIN THE PERMANENT ICE COVERS OF ANTARCTIC LAKES USING PROPIDIUM MONOAZIDE

Adapted from: Markus Dieser, Andreas Nocker, John C. Priscu and Christine M. Foreman (Submitted to Applied Environmental Microbiology)

Abstract

The permanent ice covers of the McMurdo Dry Valley lakes, Antarctica, are colonized by a diverse microbial assemblage. We collected ice cores from Lakes Fryxell, Hoare and Bonney. Propidium monoazide (PMA) in combination with quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA genes was used to examine membrane integrity of prokaryotes in these extreme environments. PMA selectively penetrates cells with compromised membranes and results in the suppression of DNA detection. A difference in the fraction of cells with intact and compromised cell membranes was found for Lakes Fryxell and East Lobe Bonney, both expressed in qPCR signal reductions and differences in DGGE banding patterns. Limitations in the ability to distinguish between intact or compromised cells occurred in samples from Lakes Hoare and West Lobe Bonney due to low initial DNA template concentrations. Despite the presence of certain levels of membrane-damaged cells with site-specific differences, our results suggest that the permanent ice covers of the McMurdo Dry Valley lakes support a high proportion of intact 'viable' microbes, and that diversity and survival increases in association with sediment aggregates within the ice covers. Apart from viability

information, this study provides a comparison and discussion of similarities and heterogeneities in DGGE profiles from these unique lake systems.

Introduction

Permanently ice covered lakes are a prominent feature of the McMurdo Dry Valleys, Antarctica, which represents the coldest and driest desert on our planet. Diverse microbial consortia and microplankton dynamics have not only been described in the liquid water columns beneath the ice cover (Takacs & Priscu, 1998; Roberts et al., 2000; Van Trappen et al., 2002; Roberts et al., 2004; Glatz et al., 2006), but also embedded within the ice cover of these lakes (Fritsen & Priscu, 1998b; Olson et al., 1998; Paerl & Priscu, 1998; Priscu et al., 1998; Gordon et al., 2000; Mosier et al., 2007). The primary mode of introducing bacteria and nutrients into the ice cover of these lakes is aeolian transport and deposition from surrounding soils (Psenner & Sattler, 1998; Gordon et al., 2000). It has been demonstrated that soil particles and organic matter serve as water forming microzones supporting diverse microbial life within the ice covers (Paerl & Priscu, 1998; Priscu et al., 1998; Gordon et al., 2000). Fritsen & Priscu (1998b) reported that cyanobacterial biomass was absent in sediment free ice covers from Lakes Vanda and Morning, stressing the association of bacterial activity with wind-seeded soil particles. Gordon et al. (2000) have shown that the lake ice microbial communities are not exclusively restricted to their icy ecosystem, but are also associated with epilithic and endolithic particles originating from the surrounding terrestrial regions. This seeding mechanism of microorganisms by windblown particles is characteristic of icy habitats

and has also been well documented for cryoconites on glaciers in the McMurdo Dry Valleys (e.g. Christner et al., 2003; Porazinska et al., 2004; Foreman et al., 2007).

Once microorganisms are incorporated into the ice, they experience a unique environment characterized by a multitude of seasonally occurring freeze/thaw cycles. These harsh environmental conditions naturally raise questions about the ability of these cells to survive and to maintain membrane integrity as a prerequisite of metabolic activity. While it is known that bacteria play a key role in nutrient cycles and transformation of organic matter in icy systems (Paerl & Priscu, 1998), little is currently known about the viability status of these ice-entrapped populations. Previous studies investigated activities associated with aggregates in the permanent ice cover (Olson et al., 1998; Paerl & Priscu, 1998; Priscu et al., 1998). These observed metabolic activities can be assumed to be linked to cells with intact membranes. Although the presence of an intact cell envelope is only an indirect parameter of viability, it implies that the cells have the physiological ability to withstand the harsh environment and the potential for biogeochemical activities, even if they spend considerable time in a dormant or senescent state.

The objective of this study was to gain insight into the extent of membrane damage among microorganisms that colonize the ice layers of these perennially ice covered lakes. For this purpose a relatively new sample pretreatment technology was utilized based on exposure of cells to the chemical propidium monoazide (PMA) prior to subsequent molecular analysis of extracted DNA (Nocker et al., 2006; Nocker et al., 2007b). PMA has been reported to only penetrate cells with comprised membranes

(Nocker et al., 2006) and once inside the cell, PMA intercalates into the DNA to which it can be covalently cross-linked upon exposure to light. This irreversible DNA modification results in suppression of PCR amplification. In other words, PMA sample treatment allows for selective analysis of the microorganisms with intact membranes in a mixture of live and dead cells. PMA was used in this study both in combination with quantitative PCR (qPCR) and end point PCR-denaturing gradient gel electrophoresis (DGGE) as two distinct downstream DNA analysis methods (Nocker et al., 2006; Nocker et al., 2007b). The application of this method to samples from McMurdo Dry Valley lakes appeared very appealing given the high likelihood that the exposure of cells to these challenging environmental conditions in the ice over extended time periods could induce membrane damage.

Materials and Methods

Sample Collection and Processing

Ice samples were collected in January 2008 from the upper 1.2 m of Lakes Fryxell, (FRX) Hoare, (HOR), East Lobe of Lake Bonney (ELB), and West Lobe of Lake Bonney, (WLB) using a 10 cm diameter SIPRE ice corer. All collections were made near the centers of the lake basins, well away from seasonal moat ice. With the exception of ELB, none of the ice cores collected from the lakes showed sediment pockets. Because the bottom cores from ELB contained larger sediment enclosures (1-2 cm across), cores from ELB were further divided into top and bottom sections (each ~60 cm). Before processing, the ice cores were mechanically cleaned by removing approximately 1 cm of

the outer surface using a sterile blade. Cleaned core sections were melted at 4°C in acid rinsed and autoclaved glass containers. One liter of ice core melt water was concentrated via centrifugation (5,000 rpm for 20 min) to a final volume of 50 ml. 6ml of sub-sample were removed from each concentrated ice core melt for use in the PMA experiments described below. The remainder of the sample was dried for 48 hrs at 100°C to obtain a dry weight. The particulate matter was then combusted at 450°C for 5 hr in a muffle furnace to determine the organic matter (OM) fraction.

PMA Treatment

For each lake ice sample triplicate assays were prepared in sterile micro-centrifuge tubes. Each replicate consisted of duplicate 1 ml aliquots where one tube was subjected to PMA treatment following the protocol of Nocker et al. (2007b); the other tube was left untreated. The treated sample (volume of 1 ml) received 2.5 µl of a 20 mM PMA stock solution (phenanthridium, 3-amino-8-azido-5-[(3-diethylmethylammonio)propyl]-6-phenyl dichloride, dissolved in 20% DMSO; PMA from Biotium, Inc., Hayward, California; final concentration, 50 µM). Samples were incubated in the dark for 5 min with occasional gentle mixing. After PMA treatment the tubes were placed horizontally on ice and exposed to bright light (650-W halogen; sealed beam lamp, FCW 120 V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH) for 2 min. The light source was placed approx. 20 cm from the sample tubes and the samples were gently shaken during light exposure by tilting the ice box to ensure optimal light penetration. Cells were pelleted at 5,000 rpm for 5 min followed by DNA extraction.

A mixture of Antarctic isolates from the Cotton Glacier (Foreman et al., in review) was used as a control sample to demonstrate the efficiency of the PMA treatment. Frozen stock samples of the isolates were transferred to 50 ml tubes and grown to log phase in 10 ml of R2A broth at 4°C. A 250 ml aliquot was collected from each enrichment, combined, and killed by heating for 15 min at 72°C followed by the addition of isopropanol (final concentration 70%) for 10 min. Cells were harvested by centrifugation (5,000 rcf for 5 min), and the isopropanol supernatant discarded. Pellets were resuspended in 1 ml 1X phosphate buffered saline (PBS). The PMA treatment described above was then performed on the control sample.

DNA Extraction and DGGE

DNA was extracted using an Ultra Clean Soil DNA Kit (MoBio). Primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 534R (5'-AATACCGCGGCTGCTGG-3') were used to amplify a portion of the 16S rRNA (1996). A 40 base pair GC clamp was added to the 5' end of the 341F primer (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG). PCR reactions were performed in a total volume of 50 µl containing 2 µl of extracted DNA, $MgCl_2$ buffer (final conc. 1X), Taq Master (final conc. 1X), PCR nucleotide mix (final conc. 800 µM), and Taq DNA polymerase (final conc. 0.025 u μl^{-1}) (all components from 5 Prime, Eppendorf), upstream and downstream primer (final conc. 0.5 µM), and nuclease free water (Promega). The cycling parameters for endpoint PCR included a hot start (94°C for 4 min) and a touchdown program. During the touchdown phase, the initial annealing temperature of 65°C was decreased by 1°C in each of the first eight cycles. For the

following 22 cycles the annealing temperature was kept at 58°C and a final elongation step was performed at 72°C for 10 min. PCR amplifications were carried out in an automated thermal cycler (Mastercycler ep, Eppendorf). In order to achieve sufficient PCR products for DGGE analysis, three PCR reactions for Lakes Hoare and West Bonney were conducted and pooled via ethanol precipitation. DGGE was performed with a BioRad D Code™ system as described by Murray et al. (1996). PCR products were loaded onto 8-12 % polyacrylamide gels. The gel contained a 40-70 % denaturing gradient. The gels ran in 1X TAE at 60 V for 17 hrs. Gels were stained with SYBR Gold (Invitrogen) for 15 min and viewed with a Alpha Innotech FluorChem™ 8800 system.

Quantitative PCR

To amplify the prokaryotic 16S rRNA we used primers 1070F (5'-ATGGCTGTCGTCAGCT-3') and 1392R (5'-ACGGGCGGTGTGTAC-3') following the protocol described by Nocker et al. (2007b). Each 25 µl qPCR reaction mixture contained 1 µl of extracted genomic DNA, 12.5 µl of 1X Power SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA), 1 µl of each primer (10 pmol/µl), and 9.5 µl molecular grade water (Ambion). The cycling protocol included an initial enzyme activation step of 9 min at 95°C, followed by 45 cycles of 30 sec at 95°C, 20 sec at 56°C, and 25 sec at 72°C. The analysis was performed using a SmartCycler II (Cepheid, Sunnyvale, CA). The cycle threshold (C_T) values were calculated by the SmartCycler II software using a 30-fluorescence unit threshold.

Statistical Analysis of DGGE Banding Patterns

The structural diversity of the microbial community in the non-PMA treated samples was examined using the Shannon index. Bands were visually detected and scored based upon presence or absence (Gafan et al., 2005). The presence of bands in each sample was denoted by a value of 1, while a value of zero was assigned when bands were not shared in common. The index was calculated using the following equation (1):

$$\text{Shannon index (H')} = -\sum_{i=1}^{s}(p_i)*(\log p_i) \qquad (1)$$

where s is the number of species in the sample and p_i is the proportion of species i in the sample.

Results

Ice cover characteristics from each lake are summarized in Table 7.1. Cores from FRX and HOR consisted of solid, clear ice with a few scattered grains of particles. In contrast, cores from ELB and WLB contained large amounts of interstitial water and were composed of ~60 % slush. Fine grained sediments were dispersed throughout the cores; however clearly visible soil aggregates (1-2 cm across) were only found in the bottom 60 cm core section from ELB. Sediment load and organic matter content within the ice cover of the lakes ranged between 0.12 to 5.26 g L^{-1} and between 0.7 to 25.9 µg L^{-1} respectively (Table 7.1.). Highest concentrations of both fractions were found in the bottom core section of ELB.

DGGE profiles (Fig. 7.1.) revealed distinct banding patterns between the individual lakes, with the exception of the HOR and WLB profiles, which were similar.

Eleven similar bands were found in each lake profile based upon migration length in the gel. The greatest number of unique bands occurred in the ice core samples from ELB (bottom 60 cm), which contained the largest amount of sediment. A pronounced difference in the total number of bands as well as in the banding pattern was apparent between the top and bottom 60 cm sections from ELB. The elimination of cells with compromised cell membranes due to the PMA treatment resulted in a loss of, or weakened intensity of bands (Fig. 7.1.). Most noticeably, PMA-induced shifts in banding patterns were seen in samples from FRX and ELB Top. Smaller differences between intact and compromised cells were observed for ELB bottom and WLB, while no visible effects were apparent in the community profiles from HOR. The addition of PMA to the killed control sample validated the successful removal of cells with compromised cell membranes.

Differences between DGGE profiles with and without PMA treatment agree well with the data obtained from PMA-qPCR. Calculated threshold cycle (C_T) values from PMA treated samples were subtracted from the corresponding C_T values from the non-PMA treated samples. The greatest signal reduction was seen for ELB (top 60 cm) with a difference of 3.23 ± 0.11 cycles between PMA-treated and untreated aliquots (Fig. 7.2.B). The signal reduction for FRX, which showed the second largest PMA-caused differences in the DGGE profiles, was 1.12 ± 0.09 cycles. There was less than one cycle difference between the PMA-treated and non-treated samples for HOR and ELB (bottom 60 cm). PMA treatment in the WLB sample resulted in a signal decrease of 1.5 ± 0.15cycles. For the WLB and HOR samples it should be noted that the obtained C_T values were above 30

Table 7.1.: Ice core characteristics from McMurdo Dry Valley lakes collected January 2008. Ice cores represent the top 120 cm of the ice cover. Cores collected from East Lobe Bonney were divided into top and bottom 60 cm sections. Lake Fryxell (FRX), Lake Hoare (HOR), East Lobe Bonney (ELB), West Lobe Bonney (WLB), and organic matter (OM).

Lake	**Sampling date 2008**	**Ice thickness [m]**	**Matrix**	**Sediment [g L^{-1}]**	**OM [µg L^{-1}]**
FRX	20.Jan.	4.66	clear ice	0.12	3.0
HOR	22.Jan.	3.34	clear ice	0.15	0.7
ELB (top)	27.Jan.	3.40	ice and slush	1.21	6.0
ELB (bottom)	27.Jan.	3.40	ice and slush	5.26	25.9
WLB	30.Jan.	3.54	ice and slush	0.46	2.2

cycles, and close to or even larger than the threshold values in the qPCR blank sample (sample with no DNA template). For this reason qPCR signals for HOR and WLB were deemed to be unreliable for further interpretation.

Discussion

Knowledge of the ratio of live:dead cells provides important information about microbial assemblages in icy environments where many organisms are present because of physical processes rather than *in situ* growth. These subzero environments can preserve cells for millions of years making it difficult to tell if cells are growing slowly in ice grain boundaries (Price, 2000) or are “freeloaders”. PMA treatment, prior to the DNA extraction and amplification steps, has been suggested to be beneficial in the field of microbial ecology for samples that show substantial differences in the viability of microorganisms (Nocker et al., 2007b). Therefore, most applications employ the PMA treatment to assess disinfection efficacy, where an active killing treatment is applied (Nocker et al., 2007a; Rieder et al., 2008; Lee & Levin, 2009; Wahman et al., 2009) and

the difference between living and dead cells is expected to be significant. Studies utilizing PMA treatment on untreated environmental samples are rare. Nocker et al. (2007b) profiled the microbial community of three environmental samples (municipal wastewater, estuarine benthic mud, and marine sediment) using PMA-DGGE. Only the estuarine samples showed a PMA-caused difference in intensity for one prominent band, while the patterns were identical for municipal wastewater and marine sediment. These finding are similar to the DGGE profiles obtained from HOR and WLB in this study. The effect of PMA treatment on these samples was either not visible or in the case of WLB very small (difference in intensity for two bands). Nocker et al. (2007b) argue that this finding is likely explained by the nature of endpoint PCR, as the probability that no significant numbers of membrane-compromised cells were contained in those samples is low. Whereas C_T values in qPCR correlate well with initial DNA template concentrations, the quantitative aspect is lost with increasing cycle numbers in end-point PCR (Suzuki & Giovannoni, 1996). Higher cycle numbers increasingly favor the amplification of low-abundance templates whose amplification products tend to catch up and finally reach the same (or even higher) levels as amplification products from templates with higher initial abundance. This phenomenon can be seen for many qPCR amplification curves. Thus, different from qPCR, endpoint PCR may not represent the initial amount of starting templates due to its dependence on the final number of cycles. In order to minimize PCR artifacts and biases, endpoint PCR was carried out for only 30 cycles in this study. However, the use of fewer cycles proved difficult in endpoint PCR

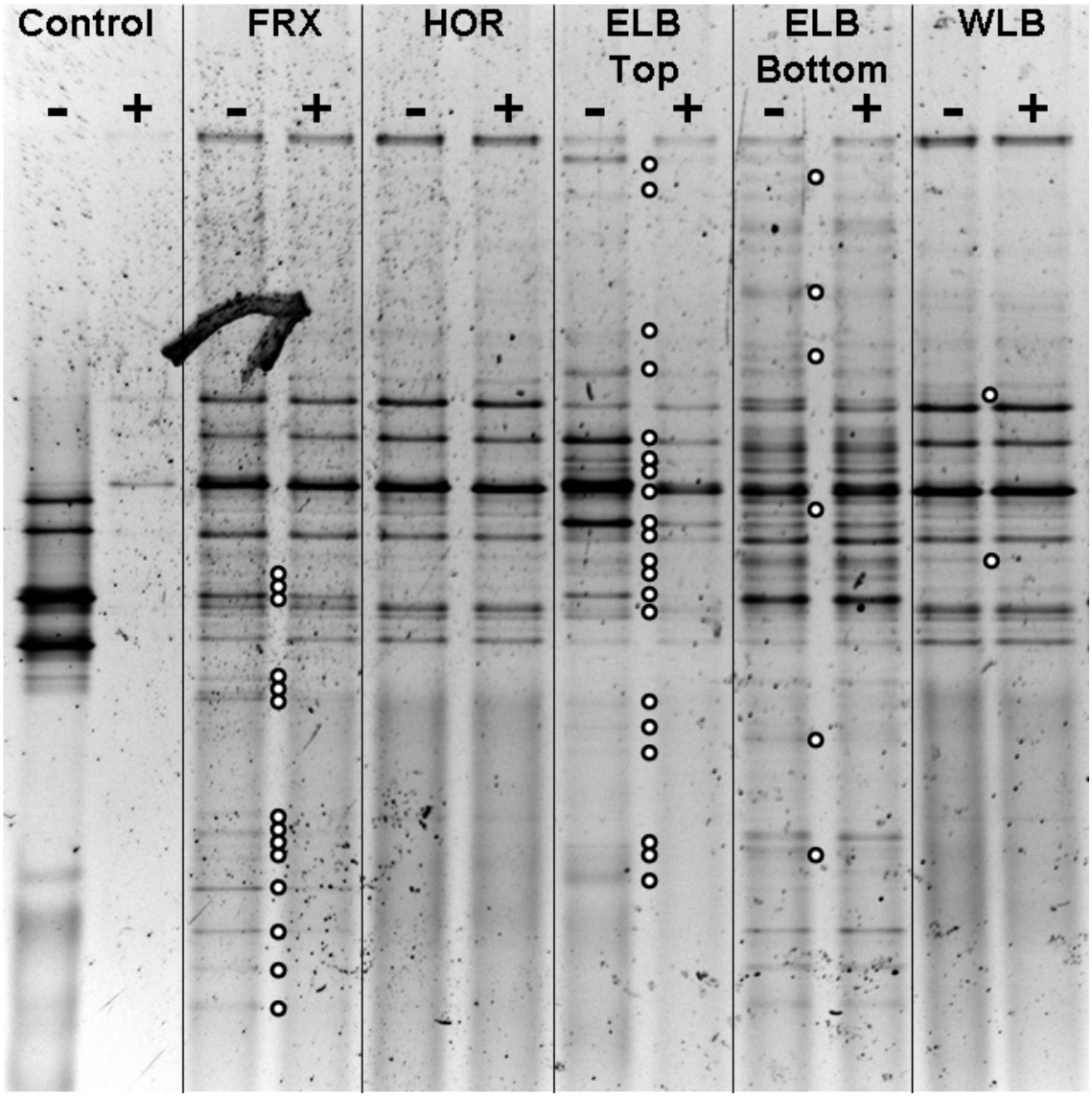

Figure 7.1.: Differences in DGGE profiles between the microbial communities from McMurdo Dry Valley lake ice core samples (top 120 cm) based on 16S rRNA gene amplification. Image colors were inverted, but no other modifications were made to the gels. The control sample consisted of a cultured Antarctic isolate and served as an indicator for PMA treatment efficiency. For each profile, minus signs (-) indicate samples without PMA treatment; and plus signs (+) indicate PMA treatment. Circles highlight bands that show a difference in intensity caused by the elimination of cells with compromised cell membranes due to the PMA treatment. The comparison of profiles from PMA-treated vs. non-PMA treated samples shows that the ice covers of Lakes Fryxell and East Lobe Bonney (top 60 cm) are comprised of a large fraction of cells with compromised cell walls. Lake Fryxell (FRX), Lake Hoare (HOR), East Lobe Bonney (ELB top and bottom), and West Lobe Bonney (WLB).

due to the very low template concentrations typically encountered in these samples. C_T values for HOR and WLB were above 30 cycles; hence endpoint PCR amplification products (3 sets of 30 cycles for these lakes) had to be pooled in order to obtain sufficient product for DGGE analysis.

Aside from these technical considerations, the effect of DNA damage on PCR amplification might minimize differences in PMA-treated and untreated samples. This is particularly important since PMA specifically addresses membrane integrity. Sikorskya et al. (2004) tested the hypothesis that single lesions in the DNA would be sufficient to block polymerase progression on synthetic 90 base oligonucleotides containing normal or modified DNA bases. They concluded that the reduction in polymerase progression depends on the type of DNA damage and the relative position of lesions within the template. Microorganisms that exist in icy environments have endured desiccation, high solar irradiation (especially in surface layers), freezing and thawing, potentially long periods of dormancy, and/or reduced nutrient availability, with all of these being environmental factors that increase the chance of DNA damage. Considering that single lesions in the DNA could already reduce polymerase progression, this could clearly have an impact on DNA amplification. Conversely, significant DNA damage is more likely in dead cells that have lost the ability to repair damaged nucleic acids. In the case of DNA damage in the amplified region, PMA modification might not lead to a further inhibition if the DNA damage per se already impacted amplification potential. In case of HOR and WLB samples the initial DNA extracts were most likely insufficient to accurately differentiate between ‘viable’ and dead cells via endpoint PCR, as emphasized by the

need to pool PCR samples for DGGE analysis and the fact that high cycle numbers were required for obtaining sufficient PCR product as indicated by C_T values. Both parameters lower the probability of seeing PMA-induced differences in profiles for the reasons mentioned before. In contrast to HOR and WLB, a pronounced PMA-caused difference was seen in the DGGE profiles from FRX and ELB (top 60 cm) (Fig.7.1.) suggesting that there existed a significant proportion of membrane-compromised cells in these samples. Due to the environmental stresses on bacteria inhabiting the ice covers of the dry valley lakes, this decrease in membrane integrity should not be surprising. From a wide variety of aquatic habitats it is recognized that only a small portion (<30%) of the bacteria are viable (Choi et al., 1996; Luna et al., 2002; Davidson et al., 2004).

Despite separation of several kilometers between these lakes, eleven identical DGGE bands were present in all lake ice covers. This accounted for 50% of the bands identified in DGGE profiles from Lakes HOR and WLB. Previous studies have described phylogenetically and metabolically diverse microbial assemblages within regions of the ice covers associated with sediment particles where liquid water inclusions were shown to exist (Fritsen & Priscu, 1998b; Paerl & Priscu, 1998; Priscu et al., 1998; Olson et al., 1998; Mosier et al., 2007). It is believed that these particles are of terrestrial origin (Paerl & Priscu, 1998; Priscu et al., 1998; Gordon et al., 2000) and that the strong katabatic winds in the valleys may act as the major dispersal mechanism (Lancaster, 2002). This mechanism could lead to phylogenetic commonality among the lake ice covers. However, phylogenetic surveys of different Antarctic soil samples uncovered highly diverse prokaryotic communities (e.g. Smith et al., 2006; Niederberger et al., 2008). Studies on

soil samples from the dry valleys showed a high level of species heterogeneity between distinct soil biotopes and altitudes (Smith et al., 2006). Due to the spatial distance between the three studied lakes it seems unlikely that the wind seeded microbial community was solely of the same origin. Although the similarity in banding patterns between WLB and HOR may suggest a homogeneous origin of organisms, banding patterns from FRX and ELB corroborate the assumption of heterogeneous sources. Thus, the occurrence of identical bands more likely indicates the selective mechanism of these environments. Once soil particles melt into the lake ice they are isolated from atmospheric inputs. Since the ice cover could provide more favorable growth conditions (e.g. seasonal liquid water associated with soil particles) compared to the highly saline, arid, and exposed soils, the development of a unique, specialized community could be induced. For instance, little biologically available nitrogen enters these lake ice systems and nitrogen has been found to be the limiting nutrient (Fritsen et al., 1998a; Psenner & Sattler, 1998); hence microorganisms capable of nitrogen fixation may have a selective advantage (Olson et al., 1998; Paerl & Priscu, 1998). It appears that a less diverse microbial community exists within the ice when larger soil aggregates are absent while diversity flourishes in the presence of larger sediment enclosures as in the case of ELB (bottom 60 cm). Calculated diversity indices support this assumption (Fig. 7.2.A). With the exception of the bottom section of ELB (H' = 1.35), diversity indices in the ice cores were <1. A moderate positive correlation was found between the organic matter content and the Shannon index (Spearman's rho = 0.4). Microorganisms in the ice covers can experience high radiation, desiccation, freeze/thaw cycles and low temperatures.

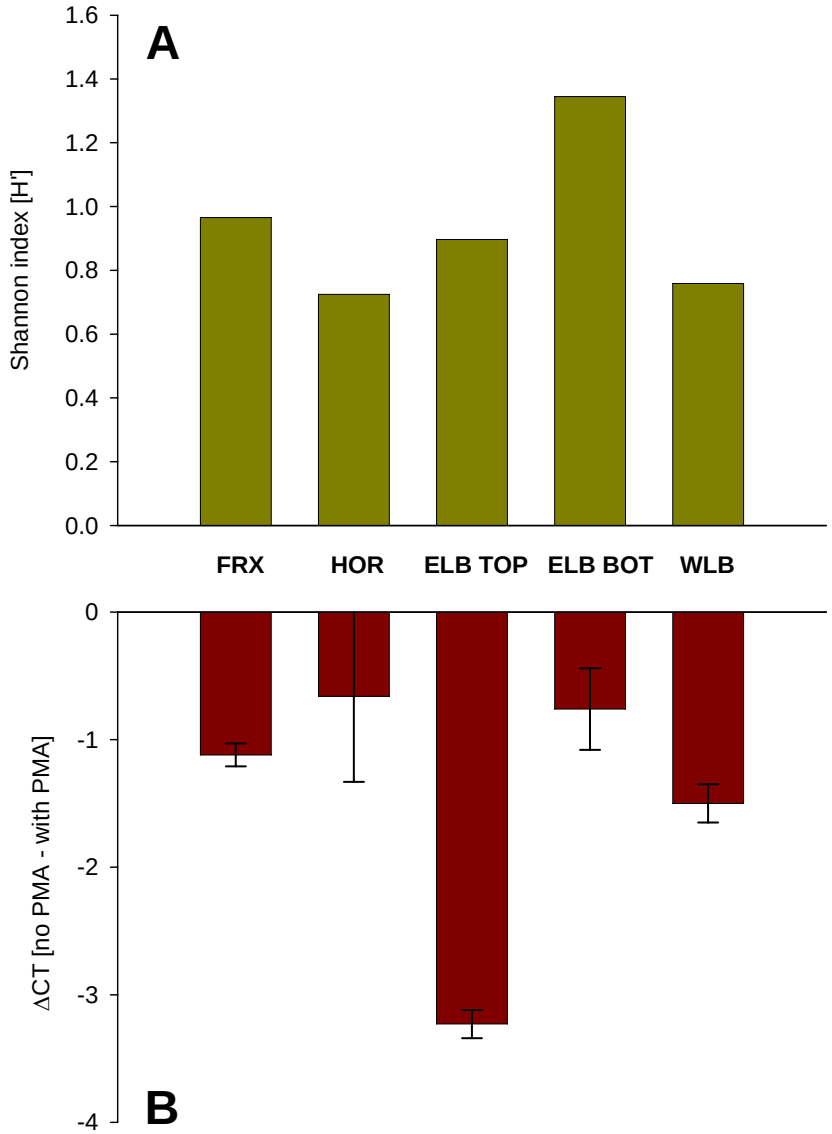

Figure 7.2.: A: Structural diversity of the microbial community within the ice covers of McMurdo Dry Valley lakes using the Shannon index. B: Calculated qPCR signal reduction for McMurdo Dry Valley lakes. The illustrated signal reduction is the average of three qPCR runs. Signal reduction is a result of the inhibition of DNA amplification from cells with compromised membranes. Higher values indicate a more pronounced difference between intact cells and cells with compromised membranes. Lake Fryxell (FRX), Lake Hoare (HOR), East Lobe Bonney (ELB top and bottom), and West Lobe Bonney (WLB).

Therefore, soil aggregrates embedded in the ice create a source of liquid water and nutrients (Paerl & Priscu, 1998). Fritsen et al. (1998a) calculated that liquid water associated with these soil aggregates exists for ~150 days per year, all within the summer months when continuous sunlight persists. Since nutrient availability and the presence of liquid water are key factors for microbial survival, these aggregates provide an "oasis" for life within the ice cover (Priscu et al., 1998). Although the ice cover on ELB and WLB contained interstitial water and consisted of a slushy matrix, only ELB (bottom 60 cm) displayed increased microbial diversity; thus, providing evidence for the stimulating effect of soil aggregates on microbial communities. Presumably the extended presence of liquid water associated with sediments would favor a longer growth season that would allow more cells to propagate. This may result in higher genome copies, which would translate into increased diversity. Fritsen and Priscu (1998b) discovered in ice cores from nine dry valley lakes that more than 98 % of the microalgal biomass was associated with ice core sections that contained sediments. Similarly, results obtained by Paerl & Priscu (1998) demonstrated that the vast majority (>95%) of prokaryotic cells in the ice cover of ELB were confined to sediment aggregates and metabolite exchange between microbial consortia has been implicated (Paerl & Pinckney 1996; Paerl & Priscu, 1998; Olson et al., 1998). During the summer months when liquid water inclusions exist within the ice this mutually beneficial coexistence would provide a hospitable refuge for growth in what would otherwise appear to be an inhospitable environment. In this coexistence, phototrophs, like cyanobacteria, may fix N_2 and provide reduced carbon to the environment. Conversely, heterotrophic bacteria may mineralize potentially limiting

nutrients (e.g. P and Fe) and contribute to localized O_2 consumption, which is essential for O_2 sensitive processes such as N_2 fixation (Paerl & Priscu, 1998; Priscu et al. 1998; Olson et al., 1998). Although we detected a strong correlation between sediment load and the organic matter content (Spearman's roh = 0.7), organic matter concentrations were generally low (~0.5% relative to dry weight). However, this is a typical feature of dry valley soils where the organic matter content is generally less than 0.1% (Campbell et al., 1998). Only the percentage of organic matter found within the ice core melt water from Lake Fryxell was higher (2.5%), but still within the range of those reported within cryoconites from Canada Glacier, Antarctica (Foreman et al., 2007). The low atmospheric input of organic matter emphasizes the importance of photosynthetically derived carbon as a source to the heterotrophic microfauna. Thus, the consortial interactions within the ice cover between producers and consumers associated with soil particles appear to be the fundamental life sustaining mechanism which promotes biogeochemical processes and nutrient cycling.

Summary

Assuming that the presence of cells with intact membranes is correlated with viability, our results provide further confidence that the ice cover of the McMurdo Dry Valley lakes supports viable microbial populations. Life in the ultra-oligotrophic ice cover of these lakes is controlled and constrained by annual variations in local climate conditions (e.g. light, temperature) and suitable habitats for microorganisms are driven by internal melt processes in close vicinity to sediments (Priscu et al., 1998). Our results

substantiate that there is increased diversity and survivability of organisms found in association with sediment aggregates within the ice covers. However, determining phenotypic characteristics of microorganisms or their role in ecosystem processes are challenging tasks especially in the case of uncultured microbial species. Alternatively, gene clone libraries are used to interpret biogeochemical dynamics thereby applying the phenotypical description of close relatives. Consequently, limiting the molecular fingerprint to the living microbial fraction will greatly help to address important ecological questions (e.g. linking phylogenetic characterizations to biogeochemical processes) more precisely and will provide new insights into ecosystem function in these extreme environments.

CHAPTER 8

SUMMARY

Antarctic environments have often been used as an analog to extraterrestrial environments (e.g. Pearl & Priscu, 1998; Priscu et al., 1998), as exobiology studies have mostly been focused on polar-like environments. It is believed that Antarctic environments, especially the polar desserts, hold the clues to where and how extraterrestrial life could be explored. Besides modeling and linking microbial dynamics in Antarctic environments to extraterrestrial life, Antarctica itself serves as a laboratory for Martian research. Physical and chemical conditions on Mars are reconstructed from Martian meteorites found on the Antarctic continent and actually support our understanding of Antarctica itself as a Mars analog (Cockell, 2007). Besides the speculations regarding extraterrestrial life, I believe that studies on physiological adaptations of microorganisms and ecological processes in microbially dominated Antarctic environments are of great significance. In a region where the macroclimate provides hostile environmental conditions, lakes, such as the permanently ice covered dry valley lakes can provide an "oasis" for life (Priscu et al., 1998). As in the present work, research opportunities exist for studying the microbial aspects in important biogeochemical processes in polar lakes. For instance, lacking higher trophic levels, the microbial contribution to carbon, nitrogen or sulfur cycles in lake systems can be investigated more precisely. It is also generally accepted among scientists that ecosystem studies in Antarctica are useful models for a better understanding of global changes since

it is believed that the Antarctic will be among the first places to feel the effects of these changes (National Research Council, 1993).

Pony Lake is a small lake located at Cape Royds, Ross Island, Antarctica. Although Pony Lake has not been intensely investigated it has been of interest to researchers since 1908 (West & West, 1911). More than 80 years later McKnight et al. (1994) affirmed the early reports from West & West (1911) and documented *Chlamydomonas intermedia* Chodat as the dominant algal species in Pony Lake. Brown et al. (2004) demonstrate a shift in algal community during the transition from an ice covered to open lake system with cryptophytes being more abundant in late summer.

Isolated from inflows, the closed basin of Pony Lake transforms periodically to a frozen solid block of lake ice during winter, while being an open water lake system during summer. These two distinct phases of Pony Lake during the course of the year impose permanently changing environmental conditions on the microbial community. Pony Lake was investigated during two consecutive seasons 2004/05 and 2005/06. In each season ice core samples were collected in early summer when Pony Lake was frozen to its base. Lake water was sampled periodically over the course of the austral summer to characterize the transition from a frozen to an open lake system.

Pony Lake ice cores showed a chemical stratification of the ice column. During ice formation and refreezing, cryo-concentration partially forces solutes and dissolved salts towards the base of the lake thereby creating a more pure ice matrix. The result is the formation of a highly concentrated brine solution in the residual water column on the bottom of the lake prior to complete freeze up as indicated by the observed dark yellow

coloration of the bottom ice cores and the many-fold increase in the major ion concentrations. It can be assumed that this concentrated basal brine would ultimately cause an extreme osmotic stress for microorganism and would drastically reduce the freezing point, exposing organisms to temperatures well below 0°C. Besides a chemical stratification, the strong odor of H_2S indicated anoxic conditions in the bottom ice cores, dividing the ice column into an oxygenated upper section and micro-oxic or anoxic bottom section. The microbial community identified within the ice column followed this oxygen gradient. Based on 16S rRNA sequence analysis, the closest relatives to Pony Lake clones isolated from the basal section of the ice cores were described as obligate anaerobic or anaerobic bacteria. Production assays on the microbial community revealed that once released from their icy entrapment they were highly metabolically active, demonstrating that they successfully withstood these more extreme physical and chemical stressors within the ice column. Chemical analysis of the dissolved organic matter showed evidence of freshly produced dissolved organic matter, suggesting that the phytoplanktonic assemblage remained active beneath the ice cover prior to complete freeze up.

The time frame of the open water period of Pony Lake is controlled by air temperature, wind, and snow accumulation during the short Antarctic summer. The dependence on weather conditions strongly affects the physicochemical appearance of this lacustrine system and year to year differences can be observed. For instance, during 2004/05 most of the lake became ice free by the end of December and strong winds caused mixing of the water column. On the other hand, 2005/06 was a snow rich summer

and peripheral melt occurred by the end of December. More importantly, calm weather conditions caused a more stable water column. Along with these annual variabilities, Pony Lake evolved differently over the course of the summer in each year. Lacking inflows and outflows, evaporation gradually increased the concentration of the chemical constituents in Pony Lake during 2004/05. pH increased by phytoplanktonic activity. The planktonic community consisted of bacteria, viruses, phytoflagellates and a small population of protozooplankton. Following the physicochemical alterations in the lake, a shift in the bacterioplanktonic community structure was detected. Bacterial production rates were strongly affected by the quality of the microbially derived dissolved organic matter; thus, it seems more likely that the shift in the bacterial composition was related to their carbon requirements. Only twelve months later when Pony Lake was investigated during 2005/06, environmental conditions were distinctly different. Not only was the ice free area comparatively small, conductivity and pH remained constant during the observation period. The stable pH implies a less active phytoplankton community. Also bacterial production rates were one order of magnitude less compared to the previous season. Moreover bacterioplankton appeared more consistent in abundance and structural composition during 2005/06. These drastic, annual, environmental changes highlight the sensitivity of Antarctic environments and the close coupling between environment and biological processes. However, a comparison to previous reports (McKnight et al., 1994; Brown et al., 2004) showed that the overall character of Pony Lake remained constant. The lake is Cl dominated and shows abundant concentrations of Na and SO_4 ions. Phytoplankton is dominated by *chlorophytes* and *cryptophytes*. Nonetheless, between

1992 (McKnight et al., 1994) and 2006 the habitat characteristics of Pony Lake changed markedly. Major ion concentrations for Cl, Na, and SO_4 decreased by 68, 79, and 91% respectively, dissolved organic carbon levels were >60% reduced, and the dominant algal species were several orders of magnitude less abundant. Mechanisms and processes that triggered these changes are yet unresolved and would require long term investigation.

Besides the physicochemical and biological characterization of Pony Lake an important aspect of this work involved the study on the bioavailability of the dissolved organic matter pool in Pony Lake. The source of organic matter in Pony Lake is algal derived, thus being considered of young age, which can be rapidly hydrolysed or remineralized by bacterioplankton. However, when these biomolecules undergo polymerization and condensation reactions induced by solar radiation exposure they will become less susceptible to enzymatic cleavage (Harvey et al., 1983). In particular, this study focused on the bioavailability of photochemically transferred Pony Lake bulk dissolved organic matter and fulvic acid isolates. The different organic matter fractions were exposed to natural Antarctic sunlight for 24 hours and subsequently amended with bacterial cultures. These bioassays were then incubated for 12 days to examine the difference in substrate availability to bacteria in light exposed samples and dark controls. Photobleaching was observed in all light exposed Pony Lake bulk dissolved organic matter samples, indicating changes in absorptivity and changes in the humic substance composition. Consequently, it was also the fulvic acid fraction that exhibited the strongest inhibitory effect on the bioavailability to bacterioplankton. However, the decrease in bacterial activity in the fulvic acid fraction might have partially been related

to H_2O_2 production. Conversely, the effect of solar radiation on the bioavailability of Pony Lake bulk DOC supplemented with a natural bacterial community from Pony Lake was minimal, indicating that the photochemically induced alterations in the aromatic humic acid fraction were compensated for in the bulk DOC sample. The structural analysis of phototransformed dissolved organic matter demonstrated that the impact of photochemical processes on the overall quality of the organic matter in Pony Lake was minimal (Guerard et al., in preparation). Thus it is believed that the limited response of the bulk DOC in Pony Lake to photodegradation is the result of past accumulation of aged dissolved organic matter, which is less susceptible to further abiotic transformations compared to freshly produced algal dissolved organic matter.

Adaptations of microorganisms to the imposed physical stresses typically encountered in Antarctic environments are broad and involve modulations of cell membranes, modification of intracellular biomolecules or the formation of spores and cysts to survive extended periods of time in unfavorable conditions. In this study the role of carotenoid pigmentation in heterotrophic bacteria was investigated as potential biomolecules to protect cells against common Antarctic environmental stresses such as freeze-thaw cycles and solar radiation. Extensive exposure to 100 freeze-thaw cycles over 50 days and simulated solar radiation (~300 W m^{-2}) for 12 hours clearly demonstrated an increased resistance of the selected carotenoid pigmented Antarctic bacterial isolates to these treatments compared to non-pigmented isolates. Nonetheless, since the sample size in each group (pigmented and non-pigmented) was very small, generalizations should be handled with care. Whereas bacterial strains in the non-pigmented group exhibited a

more uniform response to the applied stresses, pigmented bacteria demonstrated a wider range of treatment effects. Thus, these results suggest that a combination of numerous factors such as pigment structure, their location within the membrane, and pigment concentration may play a synergistic role in protecting cells against environmental stresses.

Physical stressors are ever-present in Antarctica regulating microbial life. Despite the fact that microorganisms seem to have successfully adapted to these environmental constraints, the presence of liquid water and nutrients are crucial determinants for their existence. In contrast to a eutrophic system such as Pony Lake, where nutrients are abundant to support life, habitat suitability becomes limited in polar deserts. The ice cover of the perennially ice covered McMurdo Dry Valles lakes exhibits these life limiting features. Ultra-oligotrophic and frozen solid for most of the year, this environment minimizes the odds of survival. Nonetheless, soil particles of aeolian origin found within the ice can provide nutrient enriched microzones and since these particles absorb more heat, they can create seasonally liquid water pockets within the ice. Investigations presented in this study on the 'viability' of microorganisms using propidium monoazide, to distinguish between cells with intact vs. compromised cell membranes, revealed that increased diversity and 'viability' of microorganisms found within the ice covers were association with sediment aggregates. Whereas microorganisms over-winter in the ice column of Pony Lake, outlasting unfavorable conditions until they are released from their entrapment during melt season,

microorganisms found within the ice cover of the McMurdo Dry Valley lakes have successfully colonized this icy environment.

In conclusion, the results presented in this study mainly described the physical, chemical and biological nature of the shallow, eutrophic, coastal Pony Lake, Antarctica. The data indicate that microorganisms that persist throughout the year in this lacustrine Antarctic environment were capable of surviving dynamic and periodically much more extreme environmental conditions. Changes in the local climate (e.g. retention time of the ice cover) dictated inter-annual variations in the abundances of the planktonic community, biological processes and lake chemistry, as well as the intra-annual progression of these lake parameters. The long term maintenance of a viable microbial community in Pony Lake relies on adequate adaptation mechanisms. This study suggests that the production of carotenoid pigments in heterotrophic bacteria maybe an adaptation mechanism to environmental stresses since it was demonstrated that pigmented Antarctic bacterial isolates were more resistant to common Antarctic environmental stresses such as freeze-thaw cycles and solar radiation exposure. Lastly, the carbon cycle in Pony Lake is closely linked to microbial production and degradation processes. Abiotic processes such as phototransformation of the dissolved organic matter can interfere with this cycle by altering the bioavailability of organic substrates. Nonetheless, the data imply that photolytic reactions of Pony Lake bulk dissolved organic matter had an overall negligible effect on the microbial utilization of these substrates.

APPENDIX A

RESISTANCE OF MICROORGANISMS TO FREEZ-THAW CYCLES AND SOLAR RADIATION EXPOSUE

Introduction

In addition to the pigmented and non-pigmented Antarctic isolates described in Chapter 6, several other bacterial strains were tested for their response to freeze-thaw cycles and solar radiation exposure. We used two laboratory strains, *Escherichia coli* K12 and *Serratia marcescens* MSU 69. *E. coli* and *S. marcescens* are ubiquitous bacteria. Both organisms are mesophiles and have optimal growth temperatures at 37°C and 25°C, respectively. Thus, testing their tolerance to repeated freeze-thaw cycles was of particular interest. Furthermore, *S. marcescens* synthesizes the red pigment prodigiosin (Hejazi and Falkiner, 1997) which may provide a protective function in *S. marcescens* to these treatments.

Further, we subjected the Antarctic isolate ANT 13 to the same environmental stressors. ANT 13 was phylogenetically similar to *Psychrobacter* sp. IC008, a psychrophilic bacterium isolated from Antarctic sea ice, which was subsequently identified as *Psychrobacter glacincola* (Bowman et al., 1997a). ANT 13 displays a pale pink pigmentation, which has been shown to occur in *Psychrobacter* isolates due to accumulated cytochrome proteins (Bowman, 2006). However, no fluorescent pigments were reported for *Psychrobacter glacincola* sp. nov. (Bowman et al., 1997b).

Also included in this section are additional experiments conducted for the seven pigmented and non-pigmented Antarctic bacterial isolates used in Chapter 6. The experimental procedures followed the protocols outlined in Chapter 6. In general, *E. coli* K12 and *S. marcescens* MSU 69 were incubated in Tryptic Soy Broth (TSB) at 37°C and ANT 13 was incubated in R2A-broth at ~6°C. In addition to the two treatments described

in Chapter 6, the bacterial strains were exposed to a solar radiation dose of ~700 W m^{-2} at ~4°C for 12 hrs and ~700 W m^{-2} at ~35°C for 24 hrs. The following section focuses on the major findings.

Results

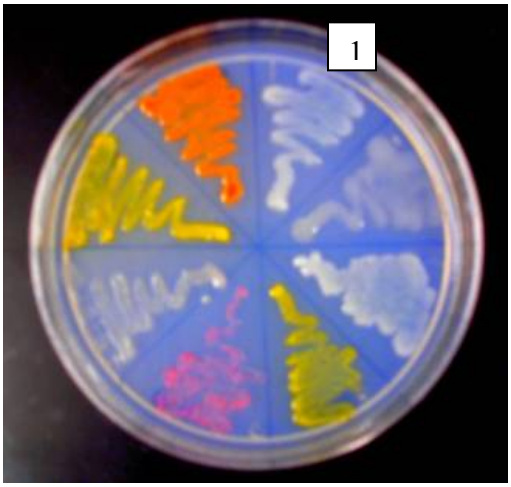

Figure A.1.: This image shows the pigmented and non-pigmented isolates from Antarctic ecosystems. Starting by 1 going clockwise: ANT 4, ANT 3, ANT 1, ANT 20, ANT 16, ANT 13, ANT 12, and ANT 11.

Exposure of *Escherichia coli* K12 and *Serratia marcescens* MSU 69 to Freeze-Thaw Cycles

The two bacterial strains, *Escherichia coli* K12 and *Serratia marcescens* MSU 69, were exposed to repeated freeze-thaw cycles. Both strains displayed a similar response and decreased sharply in CFUs (Fig. A.2.). Although, *E.coli* and *Serratia* were not longer culturable after 40 and 50 cycles respectively, these mesophilic bacteria withstood the freeze-thaw regime much longer than expected, in particular, since growth and survival of a bacterial cell occur within narrow windows of physicochemical conditions. In the

case of temperature, the survival of an organism will be defined by the temperature range within which key cellular functions operate. Temperature-dependent impairment of these key functions will result in a failure at the windows limits. For instance, *E. coli* K12 is a mesophilic bacterium able to grow well in the temperature range of 21–49°C, with an optimum at about 37°C. Its growth is impaired at temperatures below 20°C, and the minimum for measurable growth is around 7.5°C (Ingraham & Marr, 1996). Nonetheless, *E. coli* K12, and *S. marcescens* MSU 69 exhibited 100% mortality or at least a loss of culturability not until after 40 and 50 freeze-thaw cycles respectively. We observed that both organisms produced an enormous amount of biopolymer substances. It has been found that mucoid strains of *E. coli* K12 can produce as much as 30% of the cell dry weight as colanic acid capsule (viscous polysaccharide) and slime (Ophir & Gutnick, 1994). Further, it has been indicated that these exopolysaccharides bind many times their weight in water and may therefore play an important role in maintaining an appropriate humid environment near the outer cell surface (Ophir & Gutnick, 1994). The production of extracellular substances (EPS) observed in *E. coli* K12 and *S. marcescens* MSU 69 in this study as a result of repeated freeze-thaw cycles correlated with these findings and suggests that the EPS prevents cells from desiccation and intracellular ice nucleation. Exopolysaccharides have also been reported to be a natural defence mechanism used to attenuate UV light exposure (Elasri & Miller, 1999) although, the production of these substances was not observed during our solar radiation exposure experiments.

Exposure of *Escherichia coli* K12 and *Serratia marcescens* MSU 69 to Solar Radiation Exposure

E. coli K12 and *S. marcescens* MSU 69 were exposed to different solar radiation doses. In general, the two strains expressed a different solar radiation resistance pattern when exposed to 300 W m^{-2} (measurements in W m^{-2}: UV-B=0.9, UV-A=19.9, PAR=125.2, NIR=110.1, IR-B=43.9) at 4°C and 700 W m^{-2} (measurements in W m^{-2}: UV-B=2.0, UV-A=46.3, PAR=292.2, NIR=257.0, IR-B=102.5) at ~35°C. The response of *E. coli* K12 and *S. marcescens* MSU 69 to solar radiation exposure is displayed in Fig. A.2.B and Fig A.2.C.

S. marcescens MSU 69 decreased about three orders of magnitude in CFUs independently from the solar radiation dosage or temperature over the first six hours of exposure. However, when exposed to 700 W m^{-2} solar radiation dosage at ~35°C, *S. marcescens* MSU 69 recovered from the solar radiation induced damage and reached the initial number of CFUs after 24 hrs. Conversely, exposure to 300 W m^{-2} at 4°C resulted in progressive damage and *S. marcescens* MSU 69 was no longer culturable after 12 hrs.

E. coli K12 was more resistant to solar radiation exposure. Short term exposure for 2 hrs to 700 W m^{-2} at 35°C caused a slight decrease in CFUs. However, *E. coli* recovered from the initial damage and after 6 hrs and 24 hrs CFUs exceeded the initial number by one and three orders of magnitude, respectively. *E. coli* K12 showed no response to the treatment at 300 W m^{-2} at 4°C during the first 6hrs. Nonetheless, the loss of culturability was severe after 6 hrs and *E. coli* K12 was nearly undetectable after 12 hrs of exposure.

Although, both strains exhibited a negative response to solar radiation exposure, a clear counteracting temperature effect could be seen when exposed to solar radiation and a temperature near the optimum. Berney et al. (2007) demonstrated that *E. coli* K12 can express an adaptive response to UV-A radiation when growing in continuous culture. Similar to the UV-A dosage used in this study they exposed *E. coli* to a UV-A irradiation intensity at 50 W m^{-2}. Correlating with our findings, they reported that *E. coli* was able to maintain growth after a transient reduction of specific growth rate and recovery. Since slow growing cells were unable to induce enough protection capacity to maintain growth under UV-A irradiation, they argued that the adaptive response seems to be dependent on the specific growth rate, with the faster growing cells having a higher adaptive flexibility to UV-A light induced stress.

In the case of *S. marcescens*, its response to the solar radiation treatment was more negatively pronounced when compared to *E. coli*. Therefore, it can be assumed that prodigiosin, the red pigment in *S. marcescens*, had no demonstrable function as a solar radiation screening compound. Prodigiosin is a secondary metabolite and its biosynthesis occurs over a relatively narrow range of temperatures (Williams, 1973). Cultures incubated at 24 to 28°C produced the maximum amount of prodigiosin (Williams, 1973). Similarly, light affects prodigiosin production. Ryazantseva et al. (1994) reported that visible light influenced pigmentation without changing the growth characteristics of the culture. Furthermore, Ryazantseva et al. (1995) suggested a correlation between the storage of visible light energy and the pigment prodigiosin, since visible light induced the phototransformation of prodigiosin. Although, Zenoff et al. (2006b) demonstrated that

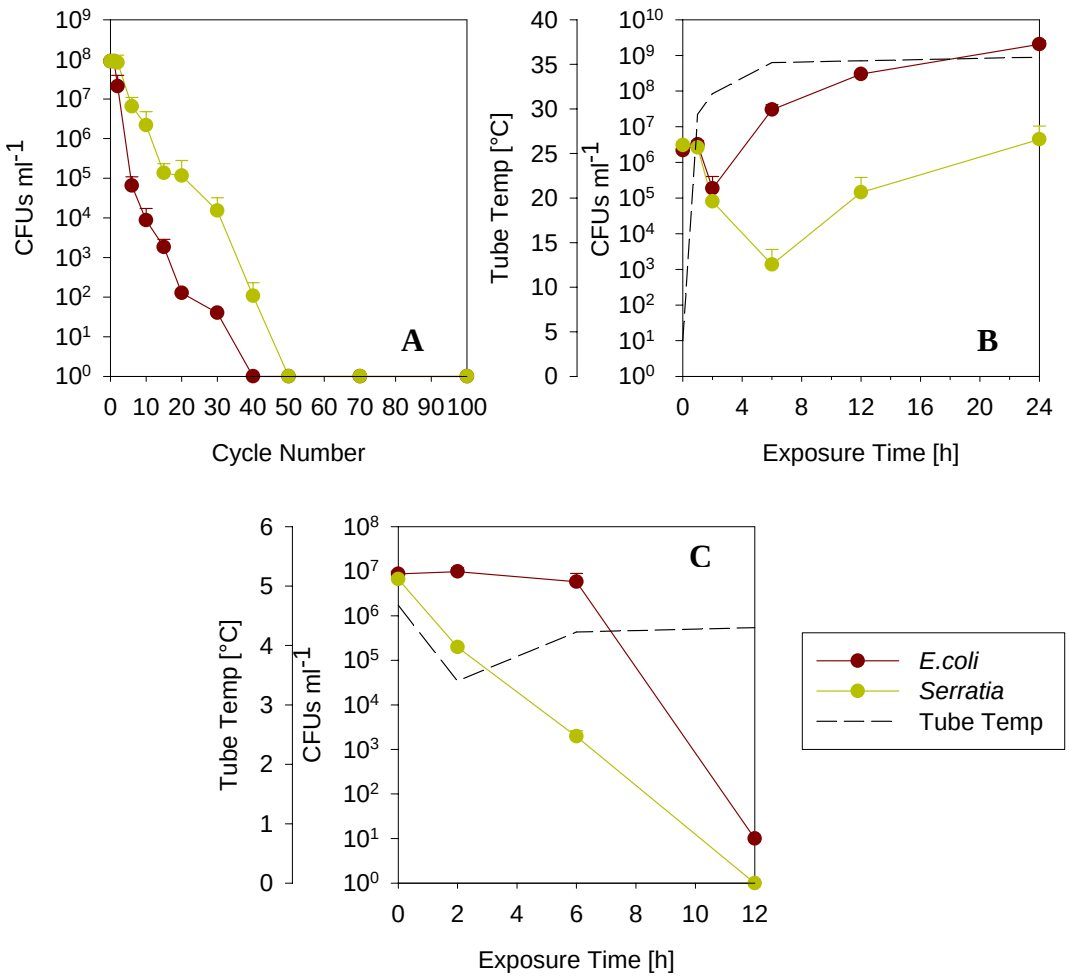

Figure A.2.: Response of *Escherichia coli* K12 and *Serratia marcescens* MSU 69 to simulated stressors. A: freeze-thaw cycles; B: solar radiation (700 W m^{-2}, ~35°C); C: solar radiation (300 W m^{-2}, ~4°C). Notice difference in the scaling of axes.

mutants of *S. marcescens* showed little resistance to UVB exposure alone, the process of phototransformation could have contributed to the recovery of *S. marcescens* during exposure to full light spectrum in this study.

Exposure of Isolate ANT 13 to Freeze-Thaw Cycles
and Simulated Solar Radiation

The Pony Lake isolate ANT 13 showed a close phylogenetic relationship to *Psychrobacter glacincola* (Bowman et al., 1997a). Although little specific information exists about the ecology of *Psychrobacter*, the genus is distinct as *Psychrobacter* species have evolved to tolerate and grow well at low temperatures (Bowman et al., 2006). For instance, *Psychrobacter glacincola* has an optimal growth temperature between 13-15°C, maximum growth occurs between 19-22°C, and the theoretical minimum growth temperature is -18°C (Bowman et al., 1997b). The relative rapid growth at low temperatures gives this group a strong competitive advantage in cold environments. Consequently, the high tolerance of ANT 13 to repeated freeze-thaw cycles was unsurprising. With a small degree for variations in CFUs, ANT 13 grew continuously during the treatment of 100 freeze-thaw cycles and reached CFUs one order of magnitude above the initial counts (Fig. A.3.A). Similarly, when exposed to a solar radiation regime of 300 W m^{-2}at ~4°C, ANT 13 remained unaffected over the first 6 hr of exposure followed by a drop over four orders of magnitude after 12 hrs (Fig. A.3.C). During the high solar radiation regime (700 W m^{-2}) ANT 13 was able to thrive for 30 min when temperature gradually increased from about ~4°C to ~35°C (Fig. A.3.B). The subsequent

rapid decline in culturability of ANT 13 was most likely related to the high temperature in the test tubes.

The results indicate that the ANT 13 was highly successful in tolerating the stressful simulated Antarctic environmental conditions which combined low temperature, periods of freezing and thawing, desiccation, highly variable availability of nutrients, and high solar radiation doses. Moreover, without showing evidence of the protecting mechanism of pigmentation, as being assayed in this study, the physiology of ANT 13 may provide important insights into environmental adaptation and survival strategies.

Exposure of Pigmented and Non-pigmented Bacterial Isolates to
High Simulated Solar Radiation Doses at High Temperatures

The four pigmented isolates (ANT 11, ANT 12, ANT 16, and ANT 20) and the three non-pigmented isolates (ANT 1, ANT 3, and ANT 4) (see Chapter 6) were exposed to ~700 W m^{-2} at high temperatures. The temperature in the test tubes increased rapidly from 4°C to 30°C within the first hour and inclined more slowly up to 35°C during the following hours of the experiment. 700 W m^{-2} is more than twice the dosage measured in the McMurdo Dry Valleys (Dana et al., 1998). It is debateable whether the high solar radiation dosage, the high temperature or a combination of both caused the severe and abrupt loss of culturability for most of these Antarctic isolates. Almost certainly we can argue that synergistic effects were accountable for the cell damage and that the high temperature became either a stimulatory or an inhibitory key regulator with the prolonged length of the experiment. In general, a clear distinction could be made between pigmented and non-pigmented isolates during short and long term exposure. Pigmented

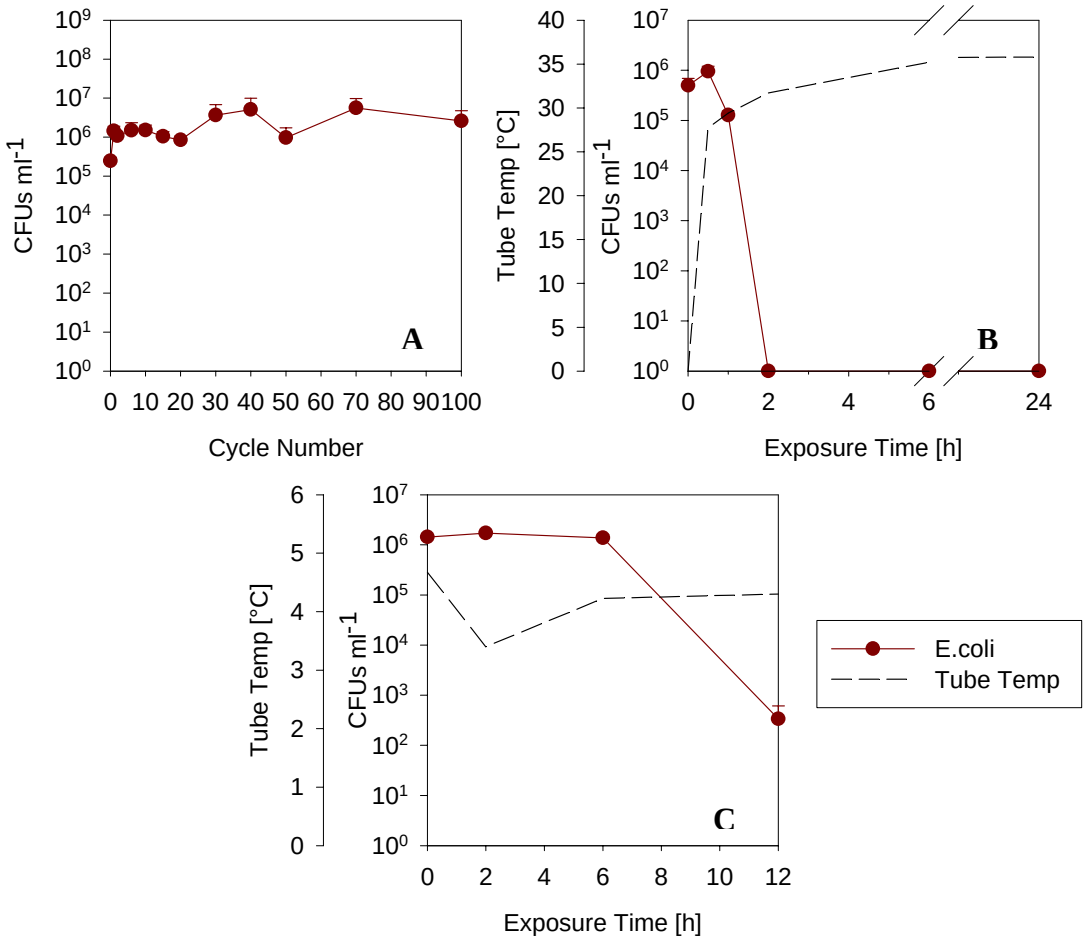

Figure A.3.: Response of the Antarctic bacterial isolate, ANT 13, to simulated stressors. A: freeze-thaw cycles; B: solar radiation (700 W m^{-2}, ~35°C); C: solar radiation (300 W m^{-2}, ~4°C). Notice difference in the scaling of axes.

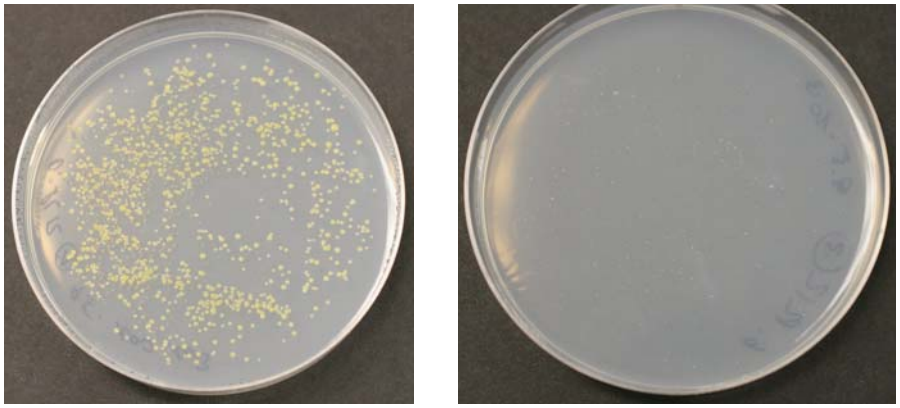

Figure A.4.: These images demonstrate the negative effect of stimulated solar radiation (300 W m^{-2}) on the bacterial isolate ANT 12. The left image shows growth on R2A agar without solar radiation treatment after an incubation time of 5 days. The right image highlight the impact of a six hour exposure to simultated sunlight on colony growth. Colonies appear much smaller after 5 days of incubation.

isolates withstood the solar radiation regime during the first hour whereas a decrease over several orders of magnitude in CFUs was recorded for non-pigmented isolates (Fig. A.5.B, A.5.D), indicating that pigmentation protected the cells from the damaging solar radiation.

Since many of the reported close relatives to the Antarctic isolates have been reported as psychrophilic bacteria and their maximum growth temperatures are below the temperature regime used in the experiment, it seems most plausible that the elevated temperature in the test tubes had a more pronounced effect on the survival of these isolates than the solar radiation dosage, thereby suppressing potential pigment effects. The theoretical maximum growth temperature for the close relatives to the pigmented isolates ANT 11 and ANT 12 is 19.9°C and 18.9°C respectively (Yi & Chun, 2006). These strains became unculturable when the temperature in the test tubes reached 30°C.

On the other hand, the taxonomic relatives to the pigmented isolates ANT 16 and ANT 20 can grow over a wider range of temperatures which in turn could explain their resilience to the treatment. *Arthrobacter agilis* (relative to ANT 16) grows from 5°C to 40°C (Fong at al., 2001) and *Sphingomonas echinoids* (relative to ANT 20) has optimal grow temperatures between 28°C and 30°C (Denner et al., 1999). Maximum growth rate for *Arthrobacter agilis* occurrs at 30°C, although pigment production is low at this temperature and no pigment production is observed at 40°C (Fong et al., 2001). This reciprocal relationship between pigment production and temperature demonstrates the thermal stress on pigment production. Relating the phenotypical characteristics of *Arthrobacter agilis* to ANT 16, ANT 16 could have thrived under the high temperature regime, but concomitantly would have lost its protection against solar radiation which was attributed to carotenoid pigmentation. This becomes obvious since ANT 16 was unaffected by the treatment after 2 hrs of exposure. However, with growth temperatures in the test tubes above 30°C and ongoing exposure to simulated solar radiation, ANT 16 lost culturability after 6 hrs (Fig. A.5.A). ANT 20 lost culturability after 12 hrs (Fig. A.5.A) and will be discussed further below.

In contrast, non-pigmented ANT 1 and ANT 4 initially exhibited a strong decrease in CFUs when exposed to 700 W m^{-2} at ~35°C but recovered from the treatment after 2 hrs. Whereas ANT 1 continuously increased in colony numbers, ANT 4 repeatedly decreased in CFUs after 12 hrs (Fig. A.5.C). This is in strong contrast to the previously described assumptions especially since the closest relative to ANT 4, *Flavobacterium xinjiangense*, has been reported as a psychrophilic bacterium with an optimal growth

temperature at 11°C. Even more surprisingly, no grow occurred for *Flavobacterium xinjiangense* at 20°C (Zhu et al., 2003). However, we hypothesis, that the adaptation to the environmental stressors was related to the temperature in the test tubes. A similar experiment revealed that when ANT 1 was exposed to ~700 W m^{-2} at 4°C the isolate showed a constant loss in viability and was almost unculturable after 11 hrs (Fig. A.6.B). Since the only difference between these two experiments was the exposure temperature in the test tubes, temperatures above 30°C in the first experiment must have stimulated growth or increased growth rate to overcome the initial cell damage.

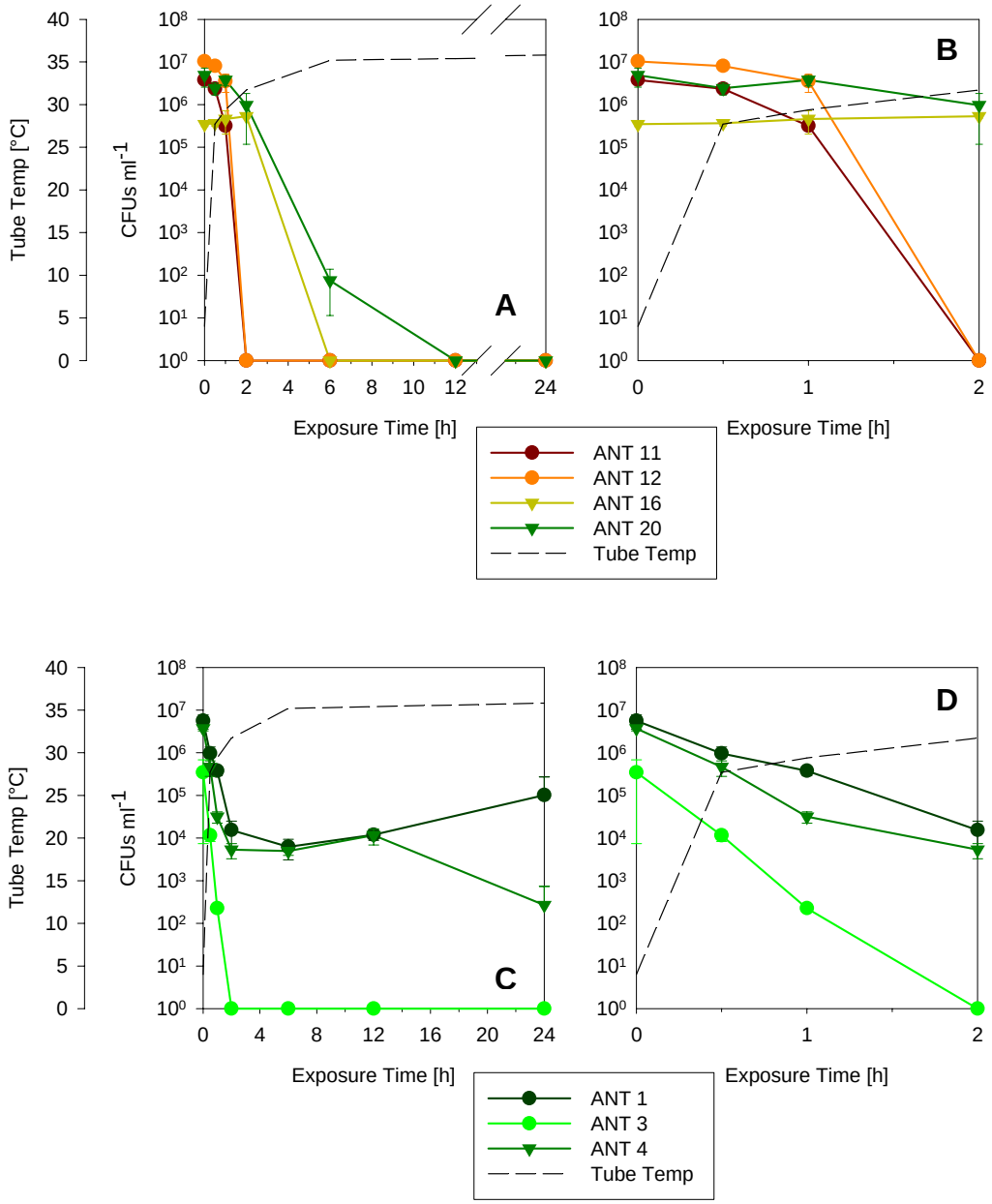

Figure A.5.: Response of pigmented and non-pigmented Antarctic bacterial isolates to simulated solar radiation (700 W m^{-2}) at high temperatures (~35°C). A: pigmented isolates (24 hrs exposure). B: pigmented isolates (first 2 hrs). C: non-pigmented isolates (24 hrs exposure). D: non-pigmented isolates (first 2 hrs). Notice difference in the scaling of axes.

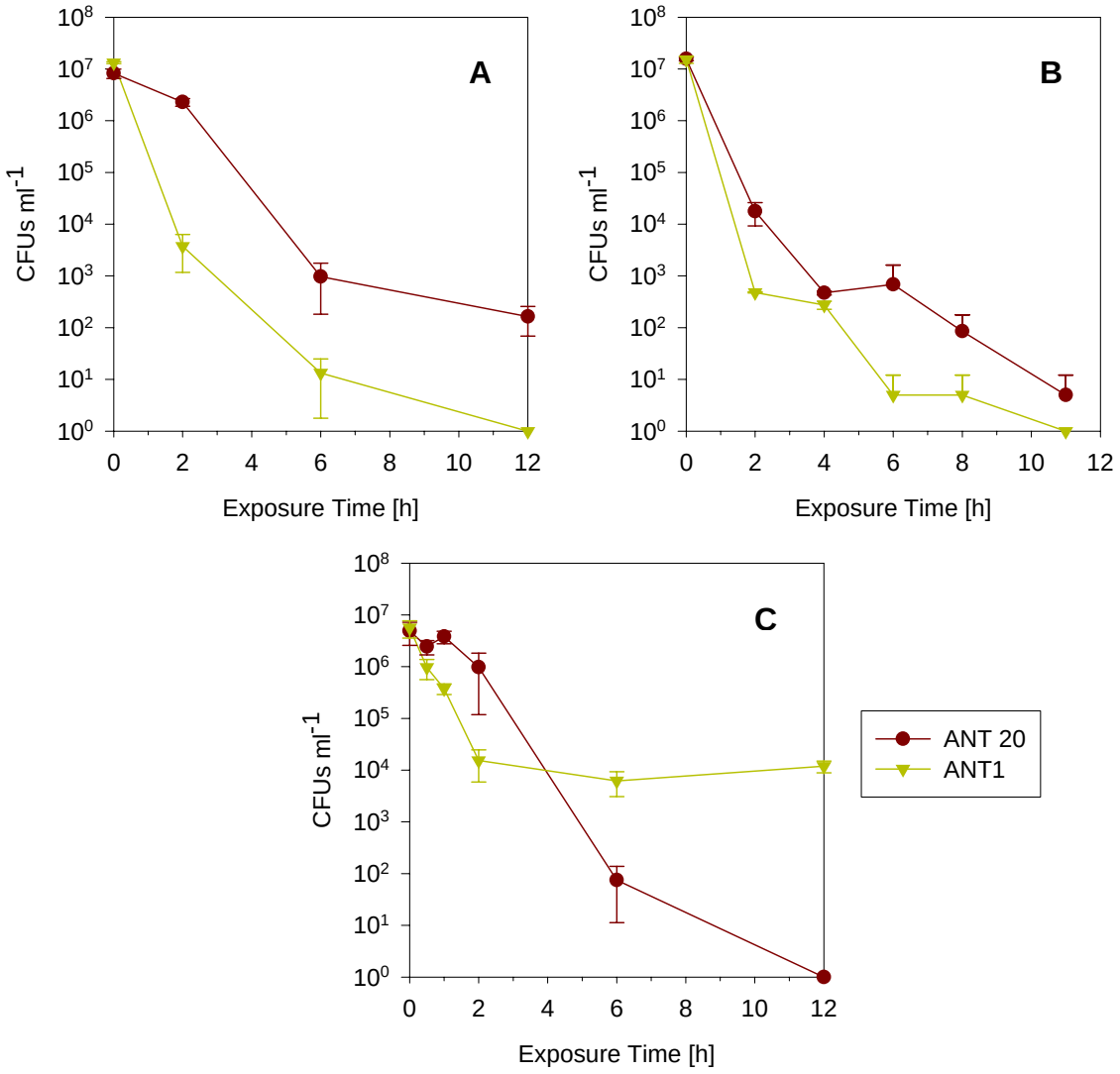

Figure A.6.: Response of Antarctic bacterial isolates, pigmented ANT 20 and non-pigmented ANT 1, to simulated solar radiation. A: ~300 W m^{-2} at ~4°C. B: ~700 W m^{-2} at ~4°C. C: ~700 W m^{-2} at ~35°C

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