THE CHEMICAL AND BIOLOGICAL COMPONENTS OF RAINWATER:

A CASE STUDY FOR THE HABITABILITY OF THE ATMOSPHERE

By

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

MASTER OF SCIENCE IN GEOLOGY

with a
MAJOR IN HYDROGEOLOGY

WASHINGTON STATE UNIVERSITY
School of Earth & Environmental Sciences

DECEMBER 2007

To the faculty of Washington State University:

The members of the committee appointed to examine the thesis of PHILLIP LLOYD RUST find it satisfactory and recommend that it be accepted.

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ACKNOWLEDGEMENTS

I wish to thank first and foremost, my committee members, Dr. Dirk Schulze-Makuch, Dr. Susan Childers, and Dr. Kent Keller, for their guidance and help in my lab experiments and in the discussion of results. I would also like to thank Dr. Lessmann, from the Dept. of Chemistry at WSU and Dr. Scott Wood at the University of Idaho for the charitable use of their labs and equipment. I would like to announce my appreciation to Szuszanna Balough, PhD student at WSU, for her advice in water chemistry analysis and her moral support. And last, but not least, I would like to thank the multitude of friends and family that donated their attention, advice and sympathy for my efforts.

DEDICATION

This thesis is dedicated to Colleen Rust, my patient and supportive wife, a fellow geologist, for walking this rough road with me.

THE CHEMICAL AND BIOLOGICAL COMPONENTS OF RAINWATER:

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Abstract

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December 2007

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Particles from the earth's surface are routinely borne into the atmosphere where they have residence times that generally run from days to weeks. Once in the atmosphere, particulate matter interacts with moisture to play a dominant role in controlling rainwater chemistry. Microbes from the earth's surface, either free floating or attached to inorganic particles, are admitted into the atmosphere for extended periods of time. Atmospheric conditions are generally considered to be too inhospitable for long term microbial survival, but life is relatively abundant in the atmosphere. Despite this abundance, atmospheric ecology is still a little understood component of the atmosphere and the earth's extended biosphere. How microbes endure, adapt and utilize scarce resources in the atmosphere is likewise poorly understood. This thesis attempts to shed some light on the following questions. What species of microbes are more likely to survive in the atmosphere? What species are more prevalent? Are they pathogenic? Is there a relationship between the chemical and biological components of rain water? In the present study, 52 rainwater samples were collected at eight locations around the globe to be studied chemically and biologically. Select samples were chosen for a phospholipid fatty acid (PLFA) analysis for insights into the microbial population size and diversity present in the samples, as well as knowledge of their stress/starvation levels. Population levels were found to be high, with cell counts ranging from 5.43E+03 to 1.49E+06 cells/ml, and were dominated by

Proteobacteria and secondly by eukaryotes. Some samples possessed small amounts of anaerobic metal reducers, SRB (sulfate reducing bacteria), and Actinomycetes. Culturing experiments on LB (Luria-Bertani) or Starkey media led to the identification of 74 organisms by their 16S rRNA gene. The culturing results were dominated by Gram-positive organisms from soil and freshwater sources. 44% of the microbes cultured were pathogenic or opportunistic pathogens of fungi, plants and animals. Elemental sulfur (S_8) was analyzed in all the samples as a possible source of microbial UV protection using fluorescent spectroscopy. S_8 was not ubiquitous, and thus readily available, but it was detected in some samples. The other chemical constituents in rainwater can also vary greatly between locations on the globe, with Ca^{2+}, K^+, Mg^{2+} and Na^+ being major cations. Sulfate levels ranged from 4.3-63 μeq/L and nitrate levels ranged from 81-608 μeq/L. The differing concentrations of these important microbial nutrients did not demonstrate an observed effect on microbial population densities or on the types of organisms found. A comparison of the microbial communities with the chemistry of the rainwater showed no correlation between types of microbes present and chemistry. Although limited amounts of the nutrients and micronutrients needed by organisms can be found in rainwater, these limited nutrients may only assist microbe survivability for short durations, rather than meet the requirements necessary for long-term atmospheric residence times.

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CHAPTER 1: INTRODUCTION

1.1 Significance of the Study

The study of rainwater chemistry has developed to very sophisticated levels. The mobile components of dissolved matter and the mineralogy of solid particulates have been scrutinized in many places of the world. Previous research studying the chemistries of rain water focused primarily on deposition at a single collection site over time or over a few collection sites in a relatively localized region due to the great variation in chemistry that occurs between regions. This variation is in turn due to the fact that rain water chemistry is heavily influenced by local geologic, marine and anthropogenic variables (Zunkel et al, 2002; Ceron et al, 2002; Migliavacca et al, 2005; Shaheen et al, 2005; Rastogi et al, 2007).

The biologic element of the atmosphere has, until recently, been a little understood part of ecology. The atmosphere is far from being 'clean' and is rife with biologic as well as inorganic components (Anthes, 1992). Dust, ash, pollen, microbes and an assortment of other particulates of low mass, are quite prevalent in the atmosphere (Anthes, 1992). Although it has been known for more than a century that microorganisms could be found in clouds, the study of aerobiology is relatively limited and most studies investigate a single collection event or a series of events at a specified location. There is still very little known about the diversity, structure and function of these communities or how they interact with their environment.

This brings up a few interesting questions. How long are some organisms aloft in the atmosphere? How much distance do they cover during aerial transport? Do they scavenge nutrients from cloud water? Do they use wind and rain for dispersal? Do microbes grow and reproduce in the atmosphere? If so, to what extent does this occur?

These important questions are beyond the scope of this study, but to initiate research in their pursuit, this study steps forward and collectively analyzes the chemical and biological components of rain water over a three month span of time from eight locations around the globe. The objective of this study is to determine if there is a relationship between the chemical and biological components of rainwater at each site. To this author's knowledge, no similar studies of this scope have been published.

1.2 Previous Work on Atmospheric Chemistry and Microbial Transport

Precipitation chemistries can differ substantially between locations, across seasons and even between individual precipitation events at the same location (Ceron et al, 2002; Kim et al. 2006; Hao et al., 2007; Rastogi and Sarin, 2007; Tipping et al, 2007). Factors that commonly affect rain water chemistry at a particular location are proximity and intensity of maritime effects, proximity and magnitude of urban areas within the region, mineralogy of dust particulates, contributions from natural sources, volcanic emissions, ground cover, biomass burning, the interlude between rain events, wind direction and anthropogenic activities, such as industrial emissions, agricultural activity, fertilizers application, and road use emissions (Qurol et al., 1998; Zhang and Friedlander, 2000; Zunckel et al., 2003; Migliavacca et al., 2005; Shahee, et al., 2005; Rastogi and Sarin, 2007)

Rainwater chemistry is affected by terrestrial and marine sources of contamination at the local, regional and global scale. Rain water chemistry at a single collection point can change dramatically between rain events, from season to season, even from day to day. This randomness is caused by a number of factors but mostly due to the prevailing weather conditions at the time, which can introduce whole new water chemistries resulting from different source contaminants (Tyson and D'Abreton, 1998; Galpin and Turner, 1999; Zunckel et al, 2000, 2003).

Although the sources for specific chemical elements in rain water and the magnitudes of the contributions differ between locations on the globe, the origin of most contaminants are similar. Winds that bring dust and other pollutants and particulate matter from further inland land masses are more likely to be dominated by anthropogenic and terriginous pollutants. Likewise, sea breezes that arrive from the ocean should bring an influx of marine elements.

Previous studies of the chemistry of atmospheric precipitation concerned with discerning the sources of major ions arrived at the following conclusions. The origin of Na^+ is primarily from sea aerosols (Ceron et al. 2002; Zunckel et al., 2003, Migliavacca et al. 2005). Ca^{2+}, Mg^{2+}, K^+, and SO_4^{2-} can be attributed in part to sea-salt aerosols (Ceron et al. 2002) but are dominantly from local mineral dust and anthropogenic sources, such as agriculture, road dust, biomass burning and coal burning (Migliavacca et al. 2005). Ca^{2+} and Mg^{2+} contributions can originate from terrestrial sources such as wind blown dust from exposed soils and plowing, and from movement over dirt roads. K^+ is dominantly attributed to contributions from wood burning and wood smoke particulates although some contributions from marine areas are possible (Zunckel et al., 2003).

Studies on metals in the atmosphere and in rain water have noted that metal concentrations increased with temperature in an area and increased with duration between rain events, indicating that metals accumulate in the atmosphere between rain events or indirectly accumulate as a result of drier conditions and subsequent dust increases (Shahee, et al., 2005; Qurol et al., 1998). Metals in the atmosphere, in both dissolved and particulate forms, are primarily derived from industrial processes, soil dust, and traffic/road dust (Shahee, et al., 2005). Iron is one of the most abundant trace metals found in rainwater in both dissolved and particulate forms (Kieber et al., 2005). Vanadium, while originating from soil sources can also be derived

from the combustion of heavy residual oil as is often used in large ships (Nriagu and Pacyna, 1988).

Of the anions found in rainwater, nitrate and sulfate are indicative of industrial emissions and agriculture applications of fertilizer (Zunckel et al., 2003). Both nitrate and sulfate tend to be higher in regions adjacent to significant industrial populations (Migliavacca et al., 2005). Sulfur species in the atmosphere are considered to be mainly derived from the combustion of fossil fuels, especially coal (Zhang and Friedlander, 2000). Increased levels of nitrate in the atmosphere are also linked with biomass burning and can be very high in association with regional forest or grass fires.

Phosphate is not usually in rainwater samples at levels above 1ppm, but when higher levels are present in rainwater samples the source is usually the result of fertilizer applications rather than the weathering of phosphate bearing minerals on the land surface (Chen et al., 1985; Callaway et al., 1991; Ahn, 1999; Migon et al., 1999; Pollman et al., 2002; Zunckel et al., 2003).

Rastogi and Sarin (2007) analyzed rain water collected at one location over a three year period. Their results showed that rain waters with low solute levels occurred during periods of excessive precipitation or during periods of extended precipitation. Conversely they noted that rain waters with high concentrations of solutes occurred after an extended dry period and the chemistry was dominated by mineral dust and sea salts. More specifically, the contributions of anthropogenically derived constituents NH_4^+, NO_3^- and SO_4^{2-} were larger in low solute events and the contribution of species from natural sources, such as Na^+, Ca^{2+}, Mg^{2+} and Cl^- were dominant in high solute events.

Since the advent of the study of microbiology it has been known that organisms can be collected from air samples. Louis Pasteur cultured microbes from air samples collected atop mountains (Griffin, 2004). Similar lines of research continued in the early 1900's with the aid

of atmospheric balloons (Rogers and Meier, 1936). In the 1970's, Imshenetsky *et al*. (1978) studied atmospheric samples collected at altitudes of 48-77 km with the aid of rockets. Amazingly, viable bacteria and fungi samples were recovered. Microbial population densities in the atmosphere are highly variable in both space and time, with population estimates for cloud water ranging from 1500 to 355,000 bacteria/ml (Sattler *et al*., 2001; Bauer *et al*., 2002; Amato *et al.*, 2005).

The potential of wind and rain as a vector for pathogen transport is of great concern, because the risks to humans, animals, agriculture, and natural ecosystems are not well understood. More recent work in aerobiology has attempted to characterize the pathogenicity of the biologic component associated with wind blown dust in order to better understand the potential threats to public health and ecosystem effects downwind (Griffin, *et al*, 2001; Griffin, *et al*, 2003; Kellogg *et al*., 2004; Mouli *et al*., 2005). As a possible threat to human or animal health, a few studies have delved into the potential of widespread atmospheric pathogen transport. One such study by Griffin *et al* (2001) noted that a number of human infectious agents have been attributed to migrating intercontinental dust storm events. Another study of African dust transported to the Caribbean by Griffin *et al* (2001), found that 28% of their isolates were plant pathogens and 10% were opportunistic human pathogens. A similar study characterizing the bacteria and fungi of African dust found that 10% were animal pathogens, 5% were plant pathogens and 27% were opportunistic human pathogens (Kellogg *et al*, 2004). Shinn *et al* (2000) found that African dust is implicated in coral reef decline in the Caribbean. The implications of these findings are particularly important in undeveloped countries where many people still store untreated rainwater for personal consumption and agricultural purposes.

The aerial transport of particulates is naturally a function of wind dynamics at both the micro and macro scales. Given sufficient wind velocity dust, pollen, microbes and other

particulates can be sent aloft for extended periods of time depending on their size, shape and density. Most particulates and bioaerosols will have a relatively short atmospheric residence time, however. Within a few days most atmospheric particulates will typically either settle in response to gravity or be drawn down with precipitation.

Figure 1, produced by Rose et al (2001), shows that particulates can spend years in the atmosphere before settling to the surface. But this study was based on particulates settling from an altitude of only 10,000m and some particulates can achieve a staggering altitude and cover enormous distances (Imshenetsky, et al., 1978; Lysenko, 1979; Moulin, et al., 1997; Griffin, et al., 2003; Kellogg, et al., 2004; Schlesinger, et al., 2006). This phenomenon has been observed in the dust events originating in Africa that send dust aloft to be carried across the Atlantic Ocean to the Americas. Some estimates claim the Sahara/Sahel region of Africa contributes as much as one billion metric tons per year to the atmosphere (Moulin et al., 1997). This airborne dust can host an impressive collage of microbes that are inadvertently transported as well. Since precipitation will pull particles from higher elevations, it is logical to look in rain water for atmospheric organisms.

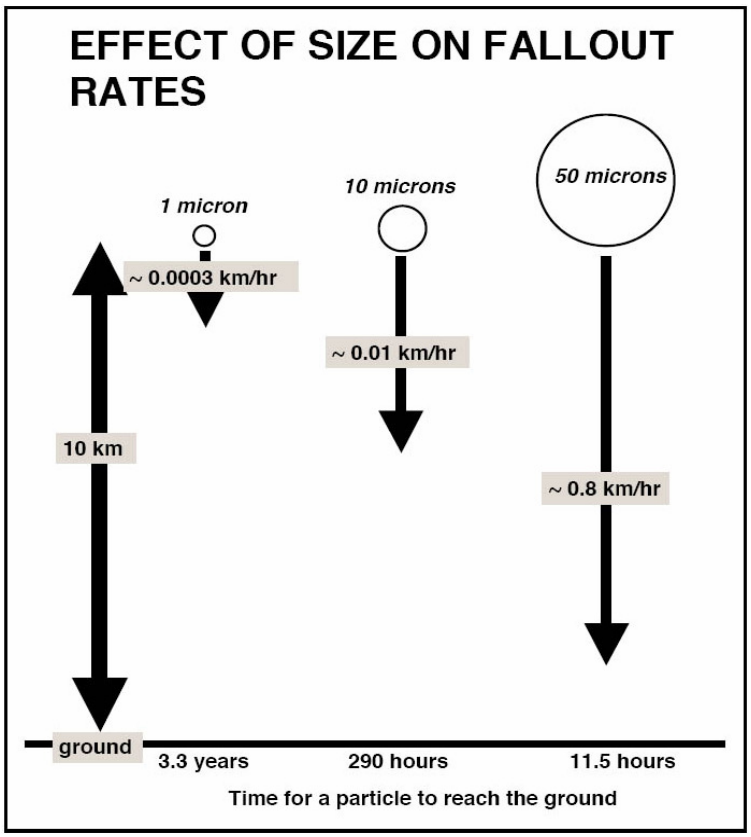

Figure 1: Approximate atmospheric residence times of particles as a function of size from a height of 10km in Earth's atmosphere. (Reproduced from Rose et al, 2001).

Organisms transported through the atmosphere, even for a short duration, face a daunting set of obstacles in regards to their survival. UV radiation, low atmospheric pressure, freezing temperatures, desiccation and a nutrient poor environment can all retard microbial growth and limit survival. Until very recently it was thought that atmospheric conditions were too harsh to allow for significant microbial survival. Yet many microorganisms possess, or have evolved, various and impressive means of coping with these conditions.

Some bacteria sporulate or restrict their metabolism when environmental extremes do not favor growth. Sporulation and/or metabolic modifications are a means of coping with UV radiation, dry conditions, freezing temperatures or limited nutrients (Setlow, 2001; Saffary *et al*, 2002). A recent study of the lower atmosphere found that many of the organisms identified were

spore forming and UV resistant (Griffin *et al*, 2001, 2003); thus allowing for survival for extended atmospheric residence times. But the types of microbes found in the atmosphere are not limited to spore formers, as was once thought.

Microbes possess other mechanisms to counteract the effects of UV radiation as well. Pigments within the membrane of some species help protect internal DNA. Advanced DNA repair mechanisms help monitor and repair damage to genetic material from radiation. In addition, a higher Guanine+Cytosine (G+C) content of the DNA helps protect the DNA better than a low G+C content (Singer and Ames, 1970; Setlow, 2001; Nicholson, et al., 2000).

In an early atmospheric study, Lysenko (1979) collected, cultured and identified six organisms at altitudes ranging from 48-77 km. Five of the six organisms possessed pigments that provided resistance to the damaging affects of UV radiation. A more recent study by Griffin (2004) collected and identified 71 microorganisms from an altitude of 20,000 m. All of the isolates identified were spore-forming, pigmented fungi or bacteria of terrestrial origin. An earlier study by Griffin et al, (2001) found that 91% of the bacteria identified from air samples collected atop Mamey Peak on St John Island during an African dust event, were pigmented. This prevalence of protection suggests that such forays into the upper atmosphere are so common that an element of natural selection favoring atmospheric adaptations is at work. Microbial forays into the atmosphere may be a common mode of propagation for microbes. These findings imply that the organisms most likely to survive such high altitude transport also possess some form of DNA protection from the effects of UV radiation. If UV screening pigments are indeed one adaptation to surviving atmospheric transport, one wonders what other possible adaptations may exist. Schulze-Makuch et al. (2004) have suggested the possibility of microbes utilizing elemental sulfur (S_8) as an UV screen.

Other microbial adaptations exist to cope with atmospheric conditions other than UV radiation. Some bacteria produce biosurfactants on their external membranes that forces vapor water to condense before atmospheric supersaturation can occur (Ahern et al, 2007). This has the advantage of helping to protect the cell from desiccation, but also leads to cloud droplet formation and subsequent precipitation fall out. Other microbes, such as *Pseudomonas syringae*, produce proteins on their outer membrane that nucleate ice at higher temperatures and also encourage precipitation and deposition (Warren and Wolber, 1991; Graether and Jia, 2001; Sattler *et al*, 2001).

Though limited studies of atmospheric ecology have been performed, Sattler et al, (2001) demonstrated that bacteria at high altitudes are not just surviving, but actively growing and reproducing within super-cooled cloud droplets at temperatures below 0°C, with generation times ranging from 3.6-19.5 days. This limited metabolic activity allows organisms to consume and/or alter various chemical constituents in the air and in rainwater. (Sattler et al, 2001; Priscu and Christner, 2002). Cao et al. (2004) observed between 100 and 200 $\mu g/m^3$ (air) of atmospheric organic carbon and that carbonaceous aerosol accounted for approximately a third of the aerosols collected, so there are clearly some nutrients available for atmospheric microorganisms. Liquid water, once thought rare in high altitudes, is known to be able to maintain a metastable liquid state at temperatures well below 0°C (down to -40°C in some cases) until an ice nucleation reaction triggers ice formation (Diehl, *et al* 2000). The perception of this accumulated research is that the only limiting factor for perpetual biological niches in the atmosphere is one of residence time, since most atmospheric particulates on earth generally settle out over a span of days or weeks.

For decades, microbiologists and atmospheric scientists have suspected that air-borne microorganisms that are relatively abundant at high altitudes play critical roles in atmospheric

processes that affect rain water chemistry (Sattler *et al*, 2001; Ariya *et al*, 2004; Amato *et al*, 2005). Very little is understood, though, about the biological impacts of atmospheric microbes on rain water chemistry or how they interact with their environment and the extended biologic community.

1.3 Purpose and Objectives

The purpose of this study is to explore the potential of earth's atmosphere to act as a suitable habitat for microbial life. Not all aspects of habitability can be covered in any single experiment. This study attempts to identify microbial species present in rainwater and to quantify some of the chemical components in rain water that are biologically relevant. Of primary importance are sulfate, nitrate, phosphate, Ca^{2+} , K^{+} , Mg^{2+} , Na^{+} and of secondary importance are the cations Fe, Mn, Cr, V, Mo, Cu, and Sr.

The objective of this study was to analyze fifty two rain water samples collected from eight locations around the globe for their biological and chemical constituents. Further, this data is used to determine if there is a relationship between the organisms present and the unique rain water chemistries.

CHAPTER 2: LABORATORY EXPERIMENTS

2.1 Materials and Methods

2.1.1 Collection and Storage

Fifty two rain water samples were collected during rain events between May 31, 2004 and August 31, 2004 by volunteers at nine locations globally, representing six continents (Figure 2). One volunteer in Madrid, Spain observed no significant rainfall during this time period and no samples were collected at that location. Also, the one and only sample from South Africa was collected outside of this time frame on November 11, 2004.

Collection and storage of the samples were performed using methods similar to Ceron et al. (2002) and Ahern et al. (2007), with the exception that the sample containers were not rinsed with DI water prior to collection. Rain water samples were stored in new polyethylene or glass containers (South Africa sample only). Volunteers were asked to only handle the bottles with gloves to reduce contamination. Sterile plastic bottles and gloves were offered to volunteers without access to these supplies. Volunteers were asked to wait for a heavy rain shower lasting at least 10 minutes to restrict ground based contamination. After 10 minutes of consistent rain, collection could ensue, with efforts to minimize undesirable particulate contamination from the ground. A 'sterile' funnel was used to shunt a greater volume of water into the sample containers and collection continued until the rain stopped. No "Field Blanks" were collected.

Samples were refrigerated and then shipped on ice to Washington State University. Once the samples were received at Washington State University they were stored in a refrigerator at a temperature ranging from 1˚ to 4°C.

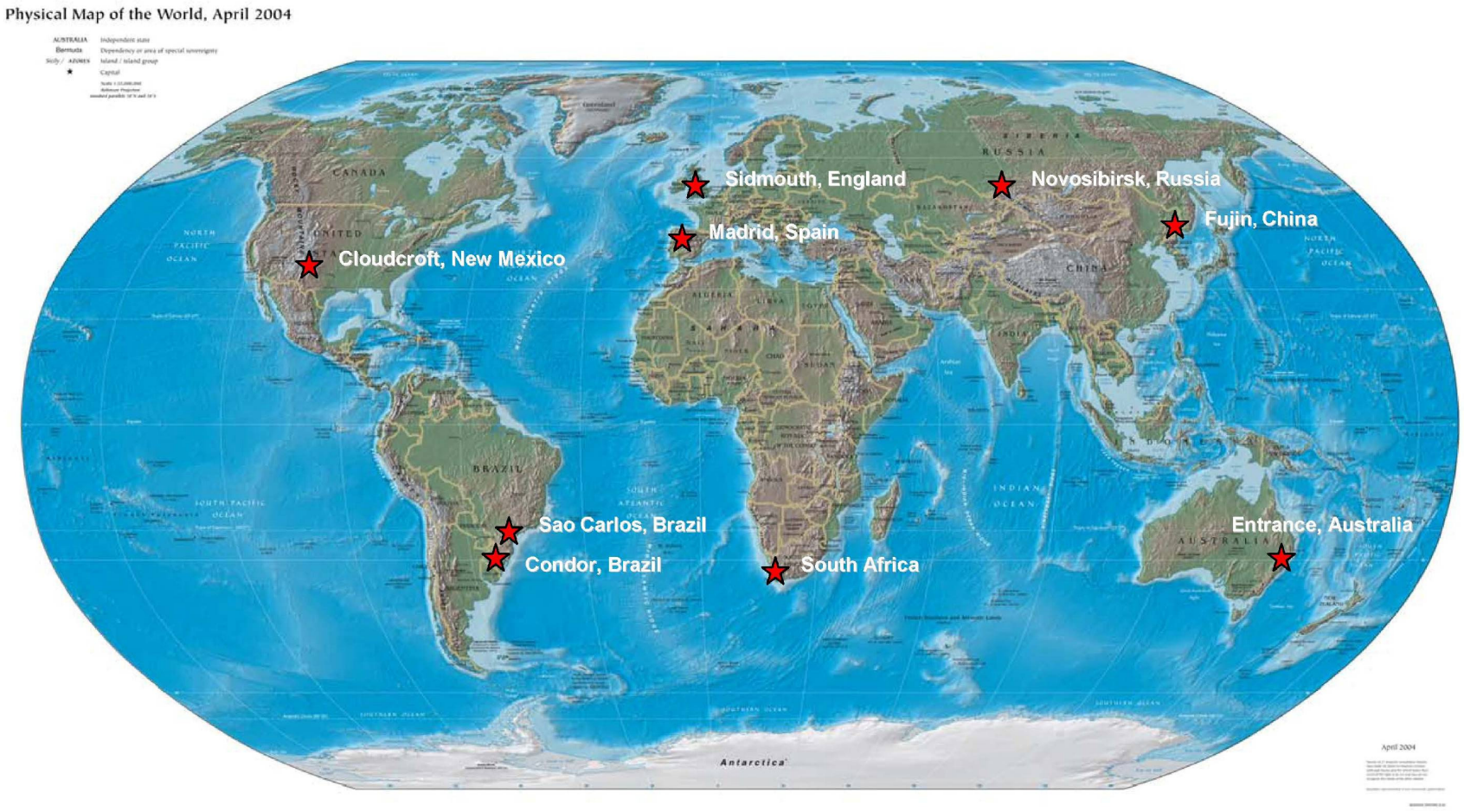

Figure 2: Map of the world displaying the eight sample collection points.

2.1.2 ICAP Methods

All 52 rainwater samples were tested with a Thermo Jarrell Ash – model ICAP-61 Inductively Coupled Plasma Spectrometer to determine the concentration of 14 cations within the samples. The fourteen cations were analyzed for Ca, K, Mg, Na, Si, Fe, Mn, Cr, V, Mo, Cu, Al, Sr and P. All samples were filtered prior to testing with 0.2µm membrane filters, via vacuum suction. The vacuum container was cleaned and rinsed thoroughly between each use with Nanopure DI water with the sample water transferred to a clean and sterile tube. During the analysis, argon was used as a carrier with a flow rate of 2.2ml/minute. Separate standards were used to assess concentrations of different cations as shown in Table 2.

The standard, STD1, was retested periodically during the analysis and later was used as a baseline for correcting for equipment "drift" with usage. The equipment was restandardized approximately two-thirds of the way through the analysis. Because STD1 was only used twice more after restandardization, a reliable trend line for monitoring "drift" could not be calculated or applied to the latter third of the samples. However drift appeared negligible during this time. Additionally, because only STD1 was used repeatedly, only Ca, K, Mg, and Na could be corrected for drift in all the samples (Table 2).

Table 1: The detection limits for the ICAP-61 of the following elements, in ppm.

Ca	K	Mg	Na	Si	Fe	Mn	Cr	Cu	Al	P
0.05	0.70	0.05	0.20	0.08	0.05	0.01	0.01	0.01	0.05	0.20

Table 2: List of standards used in conjunction with the cation analysis of the rainwater samples.

STANDARDS			
Standard	**Cation**	**Conc. (ppm)**	**medium**
STD1	Ca	10	in 5% HNO_3
	K	10	
	Mg	10	
	Na	10	
STD2	Sr	1	in 5% HNO_3
	Si	1	
	P	1	
Fe-Std	Fe	1	in 5% HNO_3
STD-18	Mn	2	in 5% HNO_3
	Cr	3	
QC-19	V	1	in 5% HNO_3
	Mo	1	
	Cu	1	
QC-7	Al	1	in 5% HNO_3

2.1.3 Spectrophotometer Methods

All 52 rainwater samples were tested using fluorescence spectrophotometry to detect the presence of elemental sulfur (S_8). All samples were vacuum filtered prior to testing through 0.2µm pore size membrane filters. The vacuum container was cleaned and rinsed thoroughly between each use with Nanopure DI water with the sample water transferred to a clean and sterile tube.

A Hitachi F-3010 Fluorescence Spectrophotometer, with a xenon lamp, was used to scan the samples for suspended elemental sulfur (S_8). Since S_8 has an Excitation (EX) of 275nm (Rao, 1975) the spectrophotometer parameters were set to an EX of 275nm and the Emission bandwidth was scanned from 290nm to 500nm.

A sulfur standard was created for comparison at 50ppm. Since S_8 is insoluble, very fine sulfur powder was suspended in Nanopure water. There was a tendency of the sulfur to flocculate but this did not appear to have adverse effects on the test. The sulfur used was reagent grade and >99.5% pure α-S_8. However, commercial samples of sulfur are never pure S_8, and contain between 0.1% and 0.5% S_7 as a result of the manufacturing process (Eckert and Steudel, 2003). The standard was tested three times with the results averaged. A blank consisting of NanoPure water was scanned six times during the analysis. The average of the readings was calculated and subtracted from every scan.

2.1.4 ICS Methods

ICS lab experiments, to determine nitrate, sulfate and phosphate concentrations were performed at the University of Idaho under the direction of Dr. Scott Wood. A Dionex ICS-3000 (DP) Model DP-1 was used to detect nitrate, sulfate and phosphate. A 1000ppm standard was made using K_2HPO_4, Na_2SO_4, and KNO_3 in purified deionized water. The 1000ppm standard was further diluted to 50ppm, 20ppm, 10ppm, 5ppm and 1ppm solutions with purified deionized water and dilutions were analyzed as an internal quality control. Standard containers were rinsed thoroughly with purified deionized water prior to making the standards. The eluent for the ICS was 40 milliMolar NaOH in DI water. The eluent was created by Dr. Scott wood in his lab and aerated with argon gas prior to use.

Eighteen select samples, representing all eight sample collection locations, were filtered through 0.2 micron filters to remove possible particulates before analysis. Of the eighteen samples, two randomly chosen duplicates were chosen and analyzed. Two duplicate samples were performed to check for consistency for the Africa 11-Nov and England 31-May samples.

2.1.5 Microbiology Methods

With the goal of investigating the biological component in the water samples, the company, Microbial Insights, Inc. was hired to perform two experiments on select rain water samples. The two methods used were a phospholipid-fatty-acid (PLFA) analysis and denaturing gradient gel electrophoresis (DGGE) analysis.

PLFA:

PLFA involved the extraction of the total lipids in the sample using a modified Bligh and Dyer method (White *et al*., 1979) and then separating the polar lipids via column chromatography. Lipids were recovered, dissolved in chloroform, and fractionated on disposable silicic acid columns into neutral-, glycol-, and polar-lipid fractions. The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane (Ringelberg *et al*., 1994). Fatty acids were confirmed via chromatography/mass spectrometry and equivalent chain length analysis. The detection limit was 7 picomoles of total PLFA.

In an attempt to determine the quantity of viable biomass in eleven select samples, the microbial population communities within the samples and an indication of the stress and starvation levels within the samples, a PLFA analysis was performed on the Australian, English, and both Brazilian sites from selected time periods. Since phospholipids break down relatively quickly upon the death of a cell, PLFA is a very reliable means of determining available living biomass (White et al, 1979).

When many microorganisms experience adverse environmental conditions or starvation, they begin converting their *cis* fatty acids into *trans* fatty acids (Guckert et al., 1985). Therefore, a relative indication of the stress/starvation levels of a community of microbes is to measure the ratio of *trans* fatty acids to *cis* fatty acids. This analysis was performed on each of the eleven samples.

Additionally, generalized phylogenetic groups of microbes possess notably different fatty acids (White et al., 1996; White et al., 1997). Thus, PLFA analysis can determine the relative microbial community components within the samples (Tighe et al., 2000). The community information was separated into six categories.

These categories are:

1. Monoenoic (Monos) – Classified as, Gram-negative bacteria (Proteobacteria). These organisms typically grow and reproduce quickly, utilize many various carbon sources of energy and are more adaptable to a range of environments.
2. Terminally Branched Saturated (TerBrSats) – Characteristic of Gram-positive Firmicutes that have low G+C mol%, but also found in some Gram-negative bacteria (especially anaerobes.
3. Branched Monoenoic (BrMonos) – Indicative of sulfur and iron reducing bacteria and other anaerobes and micro-aerophiles.
4. Mid-Chain Branched Saturated. (MidBrSats) – The PLFAs are found in Gram-positive, high G+C mol% organisms like Actinobacteria and some metal reducing bacteria.
5. Normal Saturated (Nsats) – These PLFAs are found in all organisms.
6. Polyenoic – These PLFAs are found specifically in eukaryotes such as fungi, protozoa, algae, plants and animals.

DGGE:

The procedure for the identification of microbes within the water samples was carried out by denatured-gradient-gel-electrophoresis (DGGE). Two separate gels were run; one for Archaea and one for bacteria. Nucleic acid extraction was performed using a bead-beating method (Stephen et al., 1999). Sodium phosphate buffer, chaotropic reagent, glass beads, and the sample were agitated in a microcentrifuge tube using a high-speed beater. Chloroform was

added, mixed thoroughly, and the tube was recentrifuged. The aqueous supernatant was collected and PCI (24:24:1) extracted. Glycogen was added and the DNA was precipitated from the aqueous phase with an equal volume of isopropanol. DNA was pelletted by centrifugation, washed with 80% ethanol, air-dried, and re-dissolved in Tris buffer, pH 8.0. The DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer.

PCR (Polymerase Chain Reaction) amplification of 16S rRNA gene fragments was performed as described in Muyzer *et al* (1993) with modifications as follows. Thermocycling consisted of 35 cycles of 92ºC for 45 sec., 55ºC for 30 sec., and 68ºC for 45 sec. Using 0.44 units of Clontech Advantage™ 2 polymerase and 12.5 pmole each primer (forward primer contained 40bp GC-clamp) in a total volume of 25 µL, thermocycling was performed using a Robocycler™ PCR block. Two primer sets were used in a nested PCR approach. The first primer set used primers corresponding to *E. coli* positions 341-534. A portion (20%) of each PCR product was analyzed by agarose gel electrophoresis (1.5% agarose, 1X TAE buffer) and ethidium bromide fluorescence. The amount of DNA used for DGGE was standardized to 150 ng by comparison to molecular weight standards using Alpha Imager™ software. DGGE was performed using a D-Code 16/16 cm gel system maintained at a constant temperature of 60˚C in 6L of 0.5X TAE buffer (20mM Tris acetate, 0.5 mM EDTA, PH 8.0). Denaturing gradients were formed at 30-65% denaturant (with 100% denaturant defined as 7 M urea, 40% v/v formamide). A size gradient was imposed on the denaturing gradient by forming 8-10% acrylamide gradient (i.e. double gradient – DGGE) as described by Cremonesi *et al.* (1997). Gels were electrophoresed at 35V for 16hr. Gels were stained with ethidium bromide (0.5mg/L) and destained twice in 0.5X TAE for 15min. each. Gel images were captures using an Alpha Imager™ system. The central 1mm portion of intensely fluorescing DGGE bands were excised

using a razor blade and soaked in 50 µL of purified water overnight. A portion (15 µL) was used as the template in a PCR reaction as described above. The products were purified by electrophoresis through a 1.2% agarose/TAE gel followed by glass-milk extraction (Gene-Clean™ kit). Purified DNA was sequenced with an ABI-Prism automatic sequencer model 377 with a dye terminators. Sequence identifications were performed using the NCBI website BLAST search engine.

2.1.6 Microbial Culturing and Identification

For the purpose of identifying cultivable bacteria, the biosciences company ACGT Inc. was employed to carry out the culturing experiments. Three separate culturing experiments were performed (outlined below) on select samples (Table E1 in Appendix E).

Experimental Set 1 contained samples that represented every collection site, except Fujin, China due to a delay in the receipt of these samples. This experiment consisted of two parts whereby culturing for the samples was attempted at a neutral pH and at a pH of 2.5 to look specifically for acidophiles. The pH levels were adjusted to 7.5 with NaOH or to 2.5 with H_2SO_4. 3-6ml of rain water were plated onto 14cm LB-agar plates and incubated overnight at 37˚C (Exceptions: the sample from Novosibirsk, Russia was taken from a colony cultured at room temperature and the rain water sample from Cloudcroft, NM was incubated at both 37˚C and at room temperature. Three colonies were collected from this room temperature Cloudcroft plate for identification and one colony from the plate incubated at 37˚C). Four bacterial colonies were selected from each of the plates depending on the size and morphology of the colony. No colonies grew at a pH of 2.5.

Genomic DNA was extracted from the colonies with a QIAamp™ DNA mini kit. PCR amplification of the full-length sequences 16S rRNA gene was performed using proprietary

primers. The DNA was sequenced using the dye terminator method and with sequences BLAST searched on the NCBI website for the top ten matches.

Experimental Set 2 contained samples that represented every collection site, except China. In this experiment, 3-6 ml of rain water were plated onto 14cm agar plates with selective mineral-salt thiosulfate Starkey medium at neutral pH and at a pH of 2.5. (See Table D1 in Appendix D for exact nutritional requirements). The pH levels were adjusted to 7.5 with NaOH or to 2.5 with H_2SO_4. Samples were incubated at 37˚C for 10 days. Four bacterial colonies were selected from each of the pH 7.5 plates depending on the size and morphology of the colony. No colonies grew at a pH of 2.5.

Genomic DNA was extracted from the colonies with a QIAamp DNA mini kit. PCR amplification of the full-length sequences 16S rRNA gene was performed using proprietary primers. The DNA was sequenced using the dye terminator method and with sequences BLAST searched on GENBANK for the top ten matches.

Experiment 3 was similar to Experiment 1 but was performed on five additional samples that had not previously been tested. 3-6ml of rain water were plated onto 14cm LB-agar plates and incubated overnight at 37˚C. Four bacterial colonies were selected from each of the plates depending on the size and morphology of the colony.

Genomic DNA was extracted from the colonies with a QIAamp DNA mini kit. PCR amplification of the full-length sequences 16S rRNA gene was performed using proprietary primers. The DNA was sequenced using the dye terminator method and with sequences BLAST searched on GENBANK for the top ten matches.

CHAPTER 3: RESULTS

3.1 ICAP

Table 3 shows that Ca, K, Mg, and Na cations ubiquitously dominated most of the samples chemically, with Na concentrations often being higher at collection sites with proximity to the coast. Ca concentrations ranged from 14.5 μeq/L in Sao Carlos, Brazil, to 3100 μeq/L in Cloudcroft, New Mexico. The seven highest concentrations of Ca were found in the samples from New Mexico. Si was detected in most samples in low amounts, with the highest recording just over 148 μeq /L in Sao Carlos, Brazil. Sr was found at concentrations barely above detection limits only at the New Mexico and Australia collection site. Cr concentrations were below detection limits (0.01ppm) for the ICAP and V and Mo were not detected in any of the samples. Fe, Cu, and P were only barely above detection limits (0.05ppm, 0.01ppm and 0.2ppm respectively) in one sample each. Mn was observed in three samples and at concentrations only barely above detection limits (0.01ppm).

An expanded table of the ICAP cation results is provided in Table 3.

Table 3: Results for the cation analysis with the ICAP. All results are in µeq/L. BDL represents results that were below the detection limits for the machine, but were more than 0.00. This chart does not display Cr since no samples were above detection limits (0.01ppm). V and Mo were also excluded because they were not detected in any of the samples.

	Sample	Ca ±20	K ±20	Mg ±12	Na ±23	Si ±6	Fe ±0.3	Mn ±0.06	Cu ±0.2	Al ±3	Sr ±0.1	P ±21
Fijin,China	23-Jun	256	BDL	36	BDL	18	BDL	BDL	BDL	BDL	0.10	BDL
	1-Jun	46	BDL	31	0	13	BDL	0.07	BDL	BDL	0.07	BDL
	6-Jul	100	BDL	34	BDL	19	BDL	0.09	0.07	BDL	0.03	0.0
	8-Jul	64	BDL	32	BDL	BDL	BDL	0.00	0.03	BDL	0.00	BDL
	1-Aug	236	20	38	0	27	BDL	0.00	0.14	BDL	BDL	BDL
	25-Aug	114	BDL	39	BDL	19	BDL	0.01	0.13	BDL	0.09	BDL
	28-Aug	81	20	40	17	11	BDL	0.04	0.15	5.8	0.07	BDL
Novosibirsk,	21-Jun	163	BDL	39	BDL	BDL	BDL	0.00	0.11	BDL	0.02	0.0
Russia	7-Jul	123	BDL	40	11	BDL	BDL	0.00	0.43	BDL	0.03	BDL
	10-Jul	75	BDL	39	0	BDL	BDL	0.13	0.08	BDL	0.00	0.0
	14-Jul	127	36	78	17	BDL	BDL	0.02	0.05	BDL	BDL	62.7
	4-Aug	116	35	49	56	BDL	BDL	BDL	0.15	BDL	0.10	BDL
Condor, Brazil	13-Jul	57	23	49	2850	BDL	BDL	0.01	0.15	6.1	0.09	BDL
	15-Jul	59	BDL	48	2870	BDL	BDL	0.00	0.07	BDL	BDL	BDL
	8-Aug	56	BDL	49	266	BDL	BDL	0.06	BDL	BDL	0.05	BDL
	24-Aug	68	18	53	3070	BDL	BDL	0.02	0.12	BDL	BDL	0.0
	10-Jun	61	BDL	51	181	0	BDL	0.00	0.05	BDL	0.11	BDL
	23-Jun	61	22	55	94	BDL	BDL	0.00	BDL	BDL	0.02	0.0
	24-Jun	71	19	53	4820	BDL	BDL	0.00	0.04	BDL	BDL	BDL
	1-Jul	65	18	55	52	0	BDL	0.04	0.14	BDL	0.00	BDL
	3-Jul	64	20	59	1570	0	BDL	0.00	BDL	6.2	0.10	BDL
	6-Jul	66	BDL	58	1060	BDL	BDL	0.00	0.03	BDL	0.07	BDL
South, Africa	11-Nov	112	34	74	29	BDL	BDL	0.68	0.13	BDL	0.03	BDL
Cloudcroft,NM	25-Jun	609	20	81	18	BDL	BDL	0.00	0.12	5.8	0.30	BDL
	4-Jun	3100	56	130	58	76	BDL	BDL	BDL	19.5	1.45	BDL
	23-Jun	1000	29	113	29	12	BDL	0.03	0.09	BDL	0.65	BDL
	15-Jul	1260	26	107	19	BDL	BDL	0.01	0.11	6.0	0.53	0.0
	21-Jul	790	27	108	31	35	BDL	BDL	0.11	6.8	0.58	0.0
	29-Jul	120	33	71	17	0	BDL	0.02	0.10	BDL	0.05	BDL
	14-Aug	772	52	96	14	BDL	BDL	0.00	0.12	BDL	0.32	BDL
	14-Aug	443	30	92	13	BDL	BDL	0.00	0.06	BDL	0.25	0.0
Entrance, Aus	3-Jun	105	32	77	15	0	BDL	0.00	0.14	BDL	0.05	BDL
	10-Jun	151	39	80	34	BDL	BDL	0.00	0.02	BDL	0.00	BDL
	11-Jun	229	45	177	479	BDL	BDL	0.00	0.09	BDL	BDL	BDL
	10-Jul	192	50	84	45	BDL	BDL	0.00	0.06	BDL	0.03	BDL
	18-Jul	150	45	209	631	25	BDL	0.00	0.06	BDL	0.23	BDL
	24-Jul	285	52	173	406	13	BDL	0.17	0.11	BDL	0.35	BDL
	16-Aug	117	36	99	101	16	BDL	0.00	0.07	BDL	0.09	BDL
Sidmouth, Eng	31-May	192	48	95	61	BDL	BDL	0.00	0.04	BDL	0.07	BDL
	22-Jun	60	25	40	176	BDL	BDL	0.17	0.06	BDL	0.00	BDL
	2-Jul	130	BDL	25	55	30	BDL	0.00	0.00	BDL	0.00	BDL
	7-Jul	60	35	20	66	0	BDL	0.11	0.01	0.0	0.03	0.0
	8-Aug	83	0	19	31	20	3.3	0.00	0.00	BDL	0.00	BDL
	11-Aug	57	BDL	16	29	26	BDL	0.04	0.05	0.0	0.02	BDL
	16-Aug	57	0	22	41	22	BDL	0.11	0.10	BDL	0.00	0.0
	17-Aug	67	0	25	67	BDL	BDL	0.03	BDL	0.0	0.00	BDL
	22-Aug	44	BDL	25	76	BDL	BDL	0.09	BDL	BDL	0.02	BDL
Sao Carlos, Bra	31-May	25	0	3	BDL	BDL	BDL	0.00	0.01	0.2	0.00	0.0
	3-Jun	19	BDL	4	0	12	BDL	0.00	0.11	BDL	0.00	0.0
	10-Jul	208	46	76	11	148	BDL	0.02	0.03	BDL	BDL	BDL
	18-Jul	35	BDL	11	BDL	12	BDL	0.38	0.04	BDL	0.03	0.0
	19-Jul	15	BDL	5	10	23	BDL	BDL	0.07	BDL	0.00	0.0

3.2 ICS anion analysis

The results of the anion analysis are provided in Table 4. Only data for nitrate and sulfate was obtained because phosphate could not be detected due to an unknown error. To rule out errors in the making of the standard, two additional accepted standards were analyzed and these also failed to detect the phosphate. Reasons for this are unknown.

The nitrate and sulfate data are reliable based on the results obtained using the standards. Detection limits for nitrate and sulfate are approximately 8 μeq/L and 10 μeq/L respectively. In the samples analyzed nitrate levels varied from a low of 71 μeq/L in Sao Carlos, Brazil to a high of 608 μeq/L in Novosibirsk, Russia. Sulfate levels ranged from below detection limits to a high of 84 μeq/L in Entrance, Australia. Sulfate levels were within the limits of previous studies. Nitrate however tended to be noticeable higher than in most previous studies.

Table 4: ISC anion results for selected samples. Detection limits for nitrate and sulfate are approximately 8 μeq/L (1.0ppm) and 10 μeq/L (0.5ppm) respectively. For expanded results, see Table A1 in Appendix A.

Name	Nitrate μeq/L (±5μeq)	Sulfate μeq/L (±3μeq)
Cloudcroft, NM 04-Jun	279	32
Cloudcroft, NM 29-Jul	366	17
Russia-14-Jul	379	16
Russia-4-Aug	608	27
Australia-11-Jun	193	63
Australia-10-11-Jul	195	30
Australia-18-20-Jul	175	84
Sao-Carlos-Brazil-10-Jul	71	4
Sao-Carlos-Brazil-19-Jul	199	6
Condor-Brazil-10-Jun	81	15
Condor-Brazil-15-Jul	81	23
England-31-May	279	25
England-22-Jun	133	33
England-7-8-Jul	196	29
England-11-12-Aug	275	16
China-8-Jul	280	30
China-1-Aug	150	45
Africa-11-Nov	125	28

3.3 Fluorimetry

Fluorescence spectrophotometry was used to determine the presence of elemental sulfur (S_8) in the rain water samples. With an excitation of 275nm, S_8 has two distinctive emission peaks at ~328nm and ~340nm (Figure 3). The fluorescence spectra of S_8 differs markedly from the Emission spectra of other sulfur species like S_1 (EM=366nm), S_2 (EM=362nm, 510nm), S_3 (EM=485nm), S_4 (EM=370nm, 520nm), S_6 (EM=354nm, 373nm) and S_7 (EM=355nm, 370nm) over the observed wavelengths (Wu et al, 1998; Eckert and Steudel, 2003).

Five samples, of the fifty two tested, clearly demonstrated emission spectra similar to that of the S_8 standard. This was confirmed using a *one-tailed t-test* on the emission peaks at ~328nm and ~340nm and comparing them against the standard. Four of these positive samples were from Australia (Figure 4) and had a matching confidence interval of ~98%. The fifth positive sample was from England (Figure 5) and possessed a matching confidence interval of 99.8%. Two samples from China (Figure 6) had indistinct peaks at approximately the same wavelengths as the standard but a quantitative match could not be assigned with reasonable confidence. A complete compilation of the spectra for all locations and samples can be found in Appendix B.

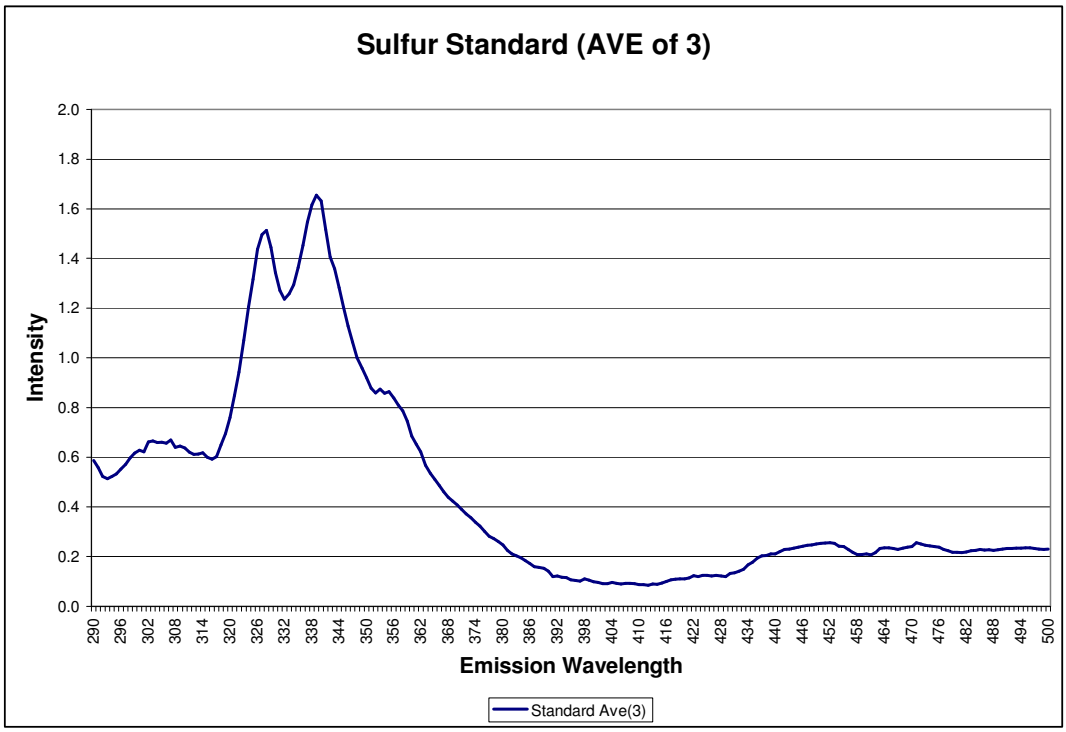

Figure 3: Emission Spectra for the S_8 standard (Average of three readings).

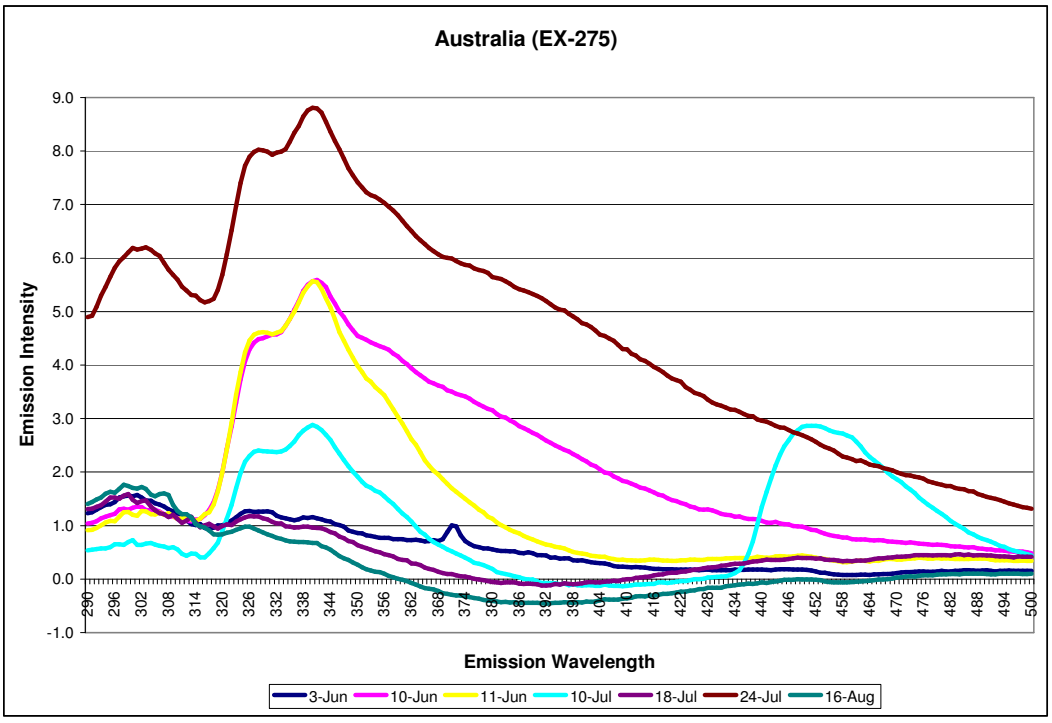

Figure 4: Emission Spectra for the seven samples from Entrance, Australia. Four samples tested positive for S_8.

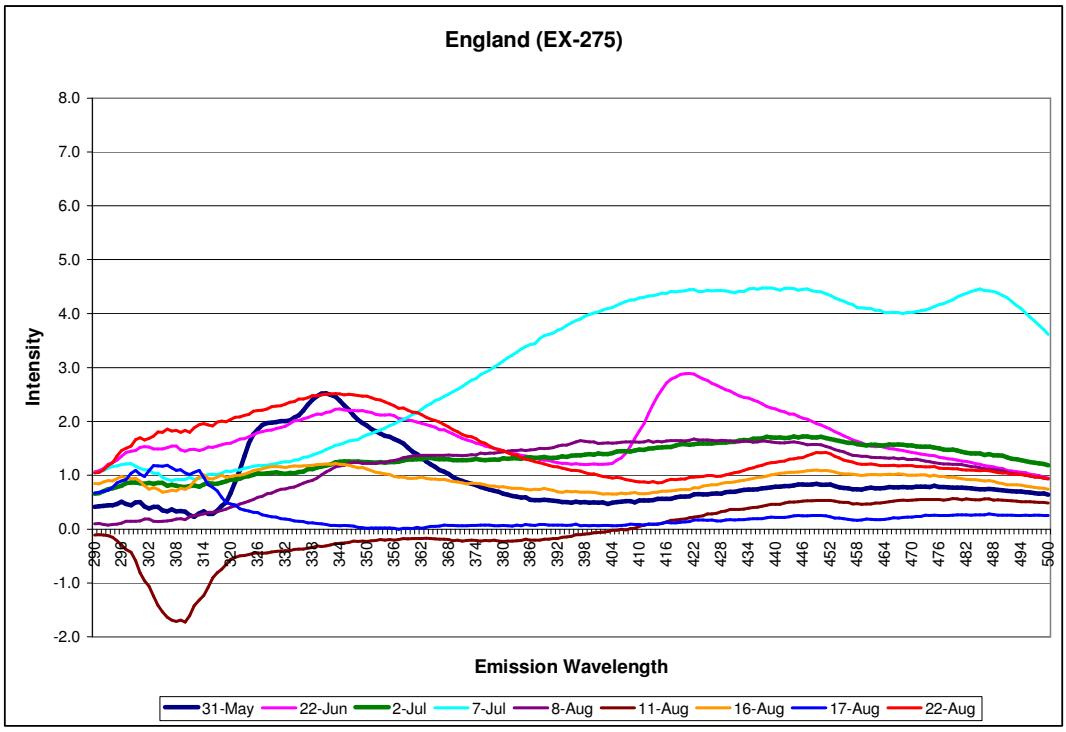

Figure 5: Emission Spectra for the nine samples from England. Only the sample of 31-May tested positive for S_8.

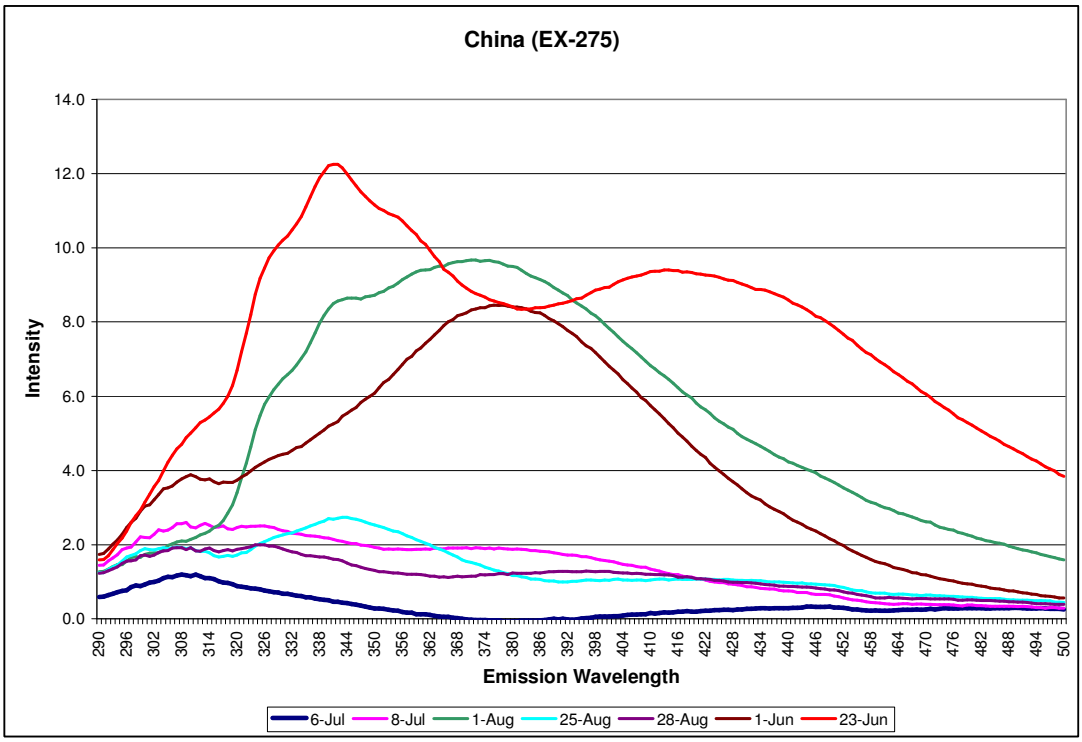

Figure 6: Emission Spectra for China. The samples of 1-Aug and 23-Jun contain conspicuous peaks at ~328nm and ~340nm that indicate the present of S_8.

3.4 Microbial Insight

The results of the PLFA community analysis are summarized in Figure 7. Notation clarifications for the samples are as follows: Eng (England), Aus (Australia) and Bra (Brazil). The PLFA analysis demonstrated that the microbial communities were predominantly Gram-negative Proteobacteria in all but one sample tested and eukaryotes were the second most abundant. Percentages of Proteobacteria ranged from a low of 30.8% in 22-Jun-Eng to a high of 79.1% in 10-11-Jul-Aus. Seven samples contained appreciable amounts of eukaryotes ranging from 11.3-39.3%. No eukaryotes were detected in any of the three samples from Australia. The Firmicutes, representative of Gram-positive bacteria with low G+C%, were detected at levels from 0% to 5.6%. PLFA counts from the general Nsats ranged from 16.1%-45.6%.

Anaerobic metal reducers and SRB/actinomycetes were present in low abundance (0.7%-2.9%) in half of the samples. The same samples tested positive in both categories, with the exception of 19-Jul-Bra, which was only positive for anaerobic metal reducers.

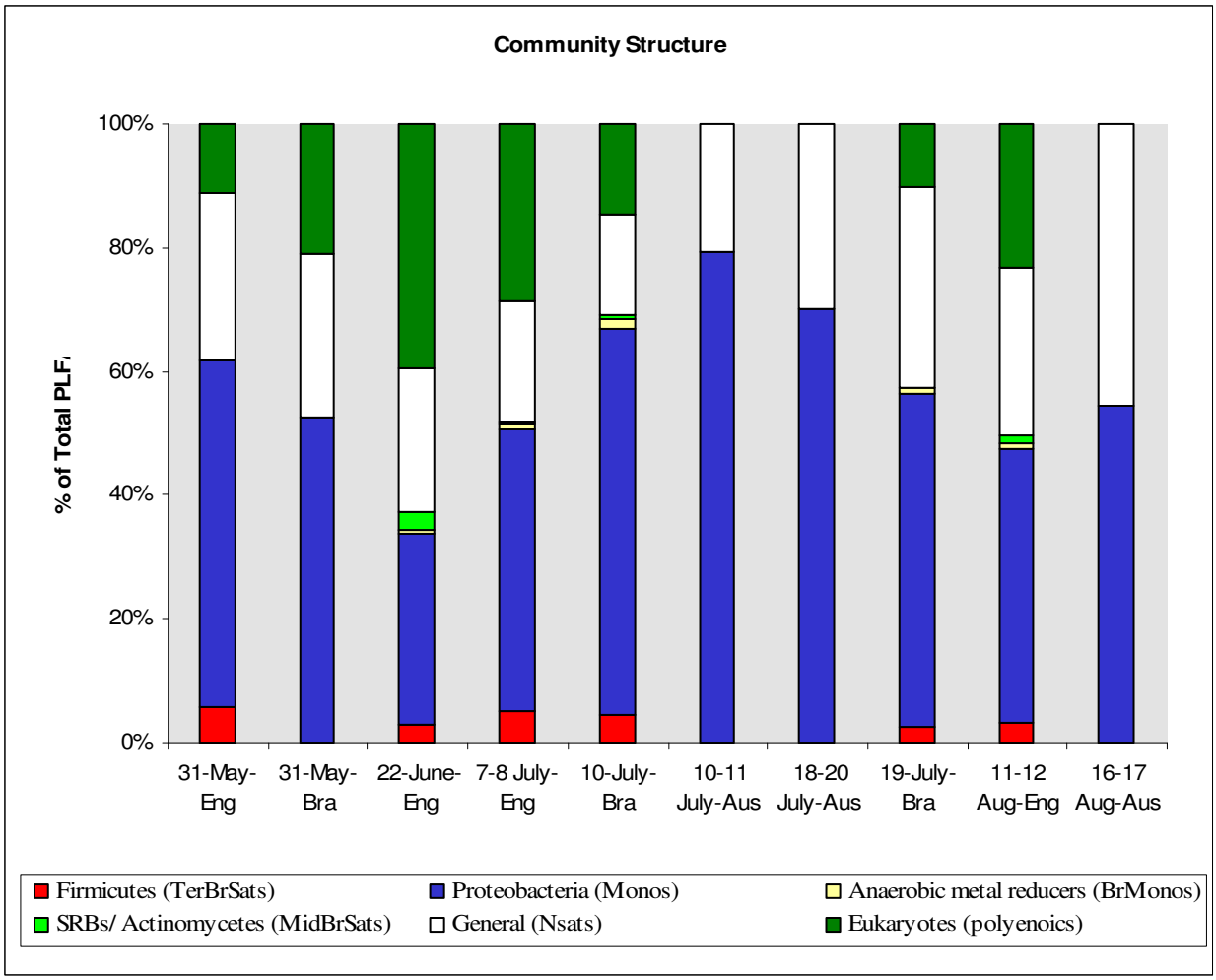

Figure 7: Community Structure listed as a percentage of the total PLFA found within the sample. SRB=Sulfate reducing bacteria. For exact figures see Table C1 in Appendix C. (Reproduced with minor changes from the Microbial Insights report).

PLFA biomass results for the eleven samples ranged from $1.1x10^4$ cells/ml to$1.5x10^6$ cells/ml (Figure 8). The three lowest biomass samples were from Australia and four of the five highest biomass samples were from England. The single sample from Condor, Brazil (13-July) however, did not contain sufficient amounts of PLFA for analysis.

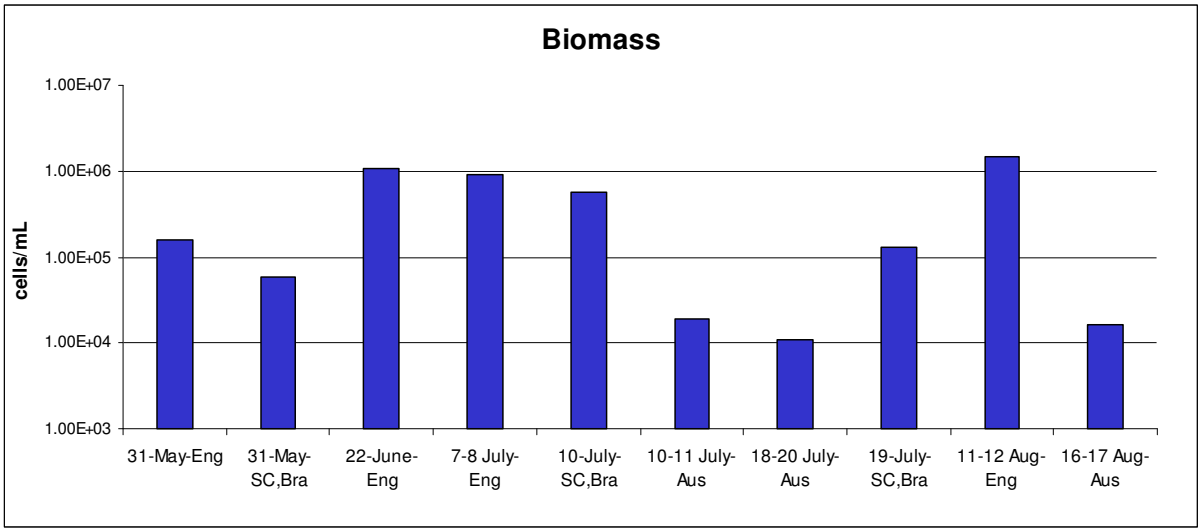

Figure 8: PLFA analysis of available biomass. For exact figures see table C2 in Appendix C. (Reproduced with minor changes from the Microbial Insights report)

During periods of environmental stress or starvation, some bacteria produce specific fatty acids that act as biomarkers indicating their physiological status. The PLFA analysis showed that four samples showed signs of "starvation" with one sample (10-July-Bra) showing extreme starvation. This sample possessed a relatively high biomass of (~$5.7x10^5$ cells/ml) but the sample with the next highest starvation levels (10-11-July-Aus), possessed a mere $2.0x10^4$ cells/ml.

Environmental stress was very minimal in all samples except one; 11-12-Aug-Eng, which also showed significant starvation. This sample was also the sample with the highest biomass (~$1.5x10^6$ cells/ml). However, the sample with the second highest cell counts had very low stress and starvation levels and other samples with high cell counts were similar. Environmental stress was discerned by dividing the amount of trans-fatty acids detected by the amount of cis-fatty acids. The results are summarized in Figure 9.

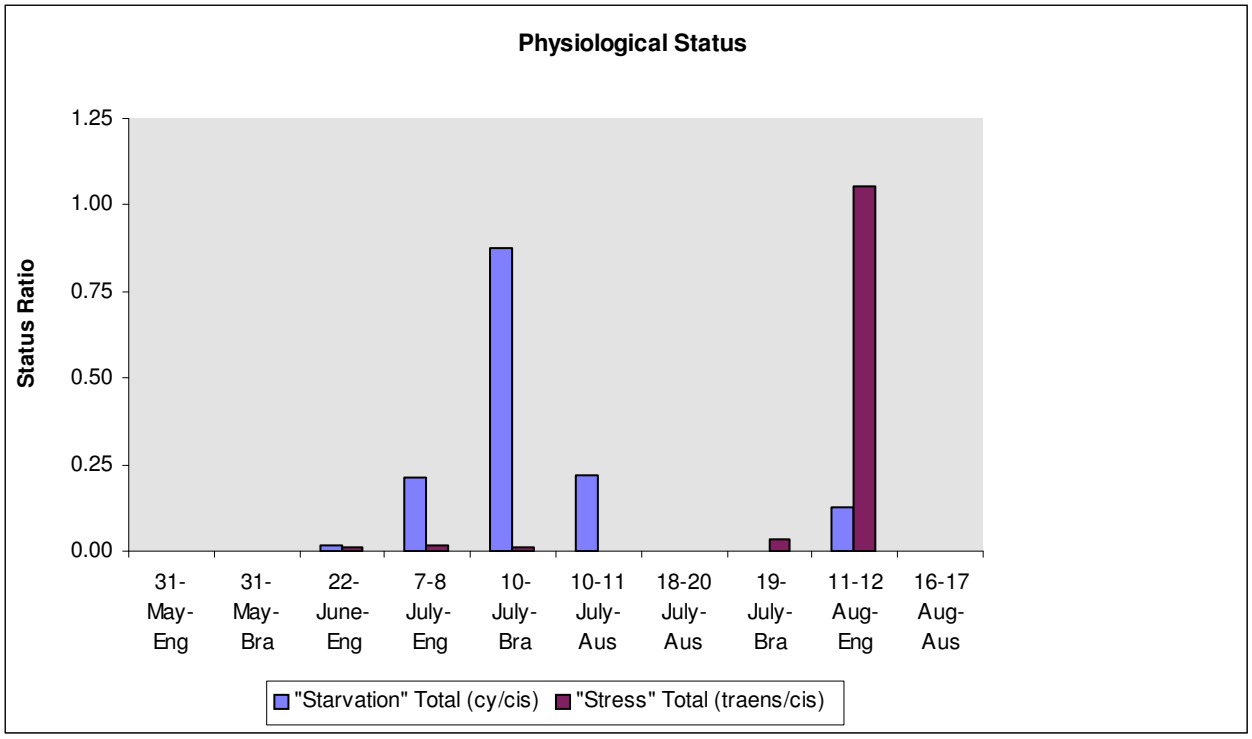

Figure 9: Physiological status can be determined by finding the ratio of *trans*/*cis* fatty acids. Ratios greater than 0.1 are indicative of starvation. For exact figures see Table C3 in Appendix C. (Reproduced with minor changes from the Microbial Insights report)

DGGE:

Figure 10 shows the DGGE gel used to isolate the 16S rDNA fragments. The bacteria represented by each band must have constituted at least 1-2% of the total bacterial community to be visualized. In general, the darker the band, the greater the proportion of that organisms within the community. The distinctive bands that were excised and sequenced produced sequences ranging in size from 276bp in length and smaller. Results from sequencing the various bands are shown in Table 10.

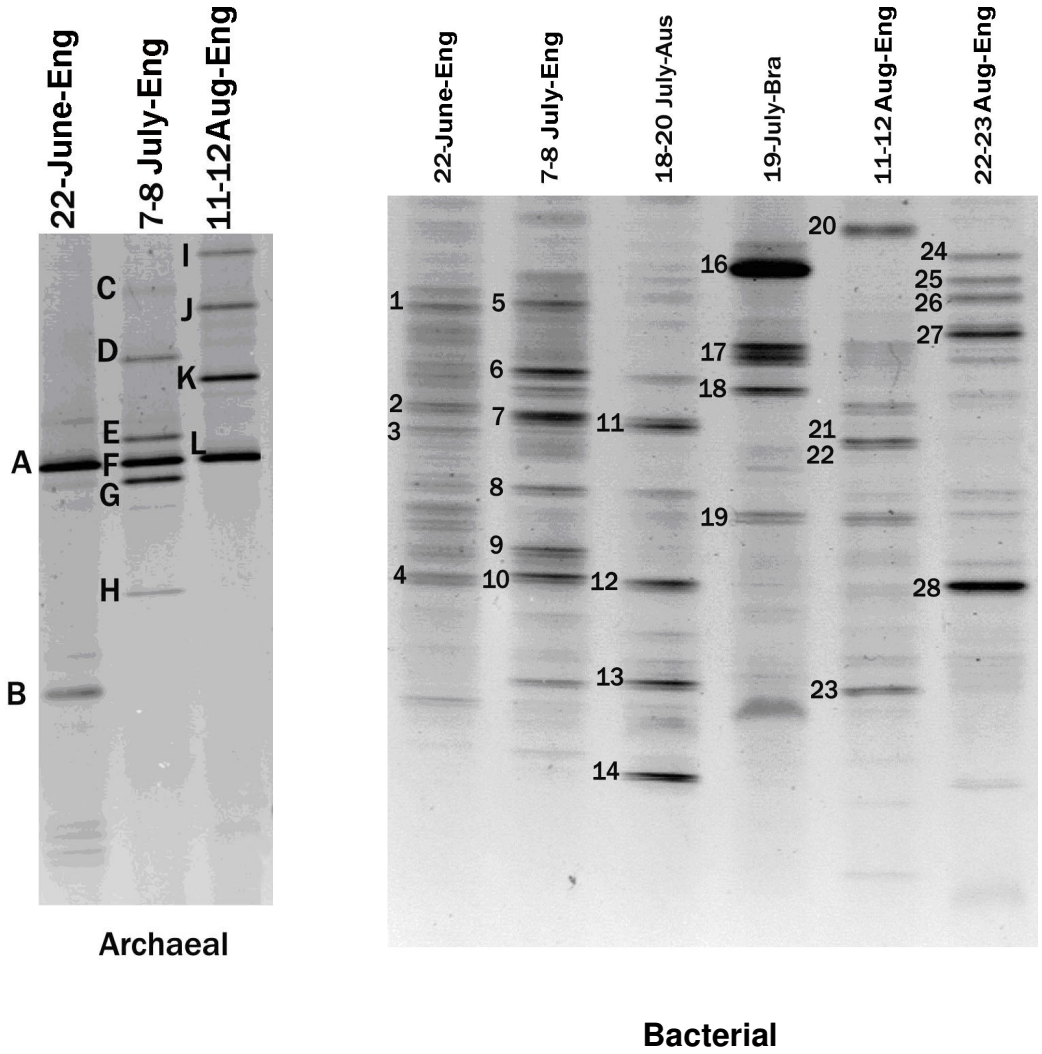

Figure 10: Bands of 16s rDNA from a DGGE gel. Archaeal DNA was looked for in only three samples and bacterial DNA was extracted from seven samples.

Table 5: Microbial results from the DGGE analysis.

Site	Band	Organism	% Match	Habitat
The Entrance, Australia	11	***Pedobacter***	74	
	12	***Caulobacter***	97	soil, drinking water, freshwater
	13	***Spirosoma***	100	desert endolithic community
	14	unsequencable		
Sao Carlos, Brazil	17,18	***Cytophagales str. (2)***	89,81	
	19	uncultured or glacier bacterium	94	groundwater or subglacial sediments.
	16	unsequencable		
Sidmouth, England	A,D,E,F,H,L	uncultured crenarchaeote (6)	98,82,83,86,73,85	soil
	B	***Methanosarcina***	81	strict anaerobes in sewers or intestines
	G	Uncultured archaeon	83	rice patty soil
	I	Uncultured archaeon	83	hydrothermal vents on east Pacific rise
	K	Uncultured crenarchaeote	83	hydrothermal mounds
	C,J	Unsequencable		
	1,5	***Cytophagales***	64,70	
	2	***Tuber borshii*** symbiont	61	ectomychorrhizal fungus in soil
	3	uncultured ***Cytophaga***	70	soil, water
	4	***Brevundimonas***	100	soil, water
	10	***Caulobacter***	100	soil, drinking water, freshwater
	6,21,22	***Sphingobacterium-like sp.(3)***	84,81,87	
	9	***Flavobacterium***	72	freshwater, soil
	26	***Pedobacter***	89	
	20	***Cytophaga***	75	soil, water
	24	***Zoogloea ramigera/Duganella zoogloeoides***	96	freshwater, sewage
	25	***Herbaspirillium***	72	soil, plants (N_2 fixer)
	28	***Chitnophaga***	85	lake
	7,8,23,27	Unsequencable		

3.5 ACGT

The culturing experiments produced a variety of microorganisms, but only certain characteristics of the identified organisms were focused on. These characteristics were the G+C %, the optimum growing temperature, metabolism and pathogenicity for the organisms cultured. Most of the microbes cultured were organisms common to soil and/or freshwater (*Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptomyces* and *Arthrobacter*). Some Psychrophiles were also cultured. For specific information on the microbes cultures at each site see Table 6.

G+C%:

For the purpose of this study the guanine+cytosine% (G+C%) was used as a relative indicator of an organism's resistance to radiation damage to its DNA. Organisms with a higher G+C% content tend to be more resistant than organisms with low G+C%. The G+C% for each cultured organism was catalogued with the results ranging from just above 30% to just above 70%. The average G+C% for all cultured microbes was just above 60%. Approximately half of the microbes cultured possessed a G+C% that fell within the 60-65% range (Figure 11).

Optimum Growing Temperature:

Optimum growing temperatures for the cultured microbes were obtained from literature searches as indicated in Table 6. The optimum growth temperatures ranged from 15˚C to above 35˚C, with the most microbes having an optimum growth temperature in the 25˚-30˚C range (Figure 12).

Pathogenicity:

A main focus of this study was to determine the prevalence of pathogens associated with the water samples. Surprisingly, almost half of the organisms cultured (44%) were considered

known pathogens for plants, animals or fungi (Figure 13). 56% of the microbes were either non-pathogens or of unknown pathogenicity. The breakdown between plant/fungi and human and animal pathogens is nearly equal, with 13 plant/fungi pathogens and 12 recorded human and animal pathogens.

Aerobicity:

77% of the microbes cultured were aerobes, 19% were facultative anaerobes, and 4% had unknown respiratory metabolisms (Figure 14). The culturing conditions performed by ACGT Inc. were under aerobic conditions so no anaerobic organisms were cultured.

Table 6: Compilation of the cultured bacteria from ACGT, Inc. "LB1" and "LB2" mediums represent the two LB culturing experiments and "S" represents samples cultured on Starkey medium. % matches were taken from NCBI database comparisons.

Site	Organism	% Match	Media	G+C mol %	Optimum Temp.ºC	Aerobicity	Habitat	Special:
South Africa	***Pseudomonas fluorescens*** [1]	100	LB1	59.4-61.3	25º-30º	Aerobe	soil, water	commonly assoc. with food spoilage
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Uncultured bacterium clone	100	S					
	Uncultured bacterium clone	100	S					
The Entrance, Australia	***Arthrobactor nitroguaiacolicus*** [1]	99	LB1	59-70	25-30º	Aerobe		
	Pseudomonas putida [1]	99	LB1	62.5	25-30º	Aerobe	soil, water	
	Pseudomonas putida [1]	99	LB1	62.5	25-30º	Aerobe	soil, water	
	Curtobacterium flaccumfaciens [1]	99	LB1	68.3-73.7	24-27º	Aerobe		causes vascular wilt in some crops
	Enterobacter sakazakii [1]	96	S	57	30º	fac. anaerobic	environment, foods	
	Pseudomonas sp. [1]	99	S	58-70	25-30º	Aerobe		
Condor, Brazil	***Pseudomonas tolaasii*** [1]	99	LB1	60.8-61.3	25º	Aerobe		mushroom pathogen
	Pseudomonas tolaasii [1]	99	LB1	60.8-61.3	25º	Aerobe		mushroom pathogen
	Pseudomonas tolaasii [1]	99	LB1	60.8-61.3	25º	Aerobe		mushroom pathogen
	Pseudomonas tolaasii [1]	99	S	60.8-61.3	25º	Aerobe		mushroom pathogen
	Pseudomonas tolaasii [1]	99	S	60.8-61.3	25º	Aerobe		mushroom pathogen
	Pseudomonas putida [1]	99	LB1	62.5	25-30º	Aerobe	soil, water	
Sao Carlos, Brazil	***Agrobacterium larrymoorei*** [1]	99	LB1	57-63	25-28º	Aerobe	soil	causes proliferating tumor cells in plants

	Agrobacterium larrymoorei [1]	99	LB1	57-63	25-28º	Aerobe	soil	causes proliferating tumor cells in plants
	Arthrobacter ramosus [1]	98	LB1	62.2	25-30º	Aerobe	soil	
	Staphylococcus sp. [1]	99	LB1	36-43	~37º	fac. anaerobic		some are highly pathogenic
	Pseudomonas tolaasii [1]	99	S	60.8-61.3	25º	Aerobe		mushroom pathogen
	Pseudomonas lurida [1]	100	S	58-70%	25º	Aerobe		
	Pseudomonas lurida [1]	99	LB2	58-70%	25º	Aerobe		
	Pseudomonas lurida [1]	98	LB2	58-70%	25º	Aerobe		
	Pseudomonas putida [1]	99	LB2	62.5	25-30º	Aerobe	soil, water	
	Pseudomonas fluorescens [1]	99	LB2	59.4-61.3	25º-30º	Aerobe	soil, water	commonly assoc. with food spoilage
China	***Variovorax sp.***	99	LB2	62-70		Aerobe	soil, water	
	Acidovorax sp.	98	LB2	68.8-69.4		Aerobe	soil, water	
	Leifsonia xyli	99	LB2	66-73		Aerobe	soil	plant pathogen
	Acinetobacter sp.	99	LB2	38-47	30º-35º	Aerobe	soil, water, foods	can cause urinary tract infections in humans
	Agrobacterium larrymoorei [1]	98	LB2	57-63	25-28º	Aerobe	soil	causes proliferating tumor cells in plants
	Agrobacterium larrymoorei [1]	99	LB2	57-63	25-28º	Aerobe	soil	causes proliferating tumor cells in plants
	Agrobacterium larrymoorei [1]	98	LB2	57-63	25-28º	Aerobe	soil	causes proliferating tumor cells in plants
	Pantoea ananatis	99	LB2	53.6-56.4		fac. anaerobic	soil, water, plants	some strains are pathogenic
Sidmouth, England	***Staphylococcus epidermidis*** [1]	100	LB1	30-37	30-37º	fac. anaerobic	human skin	very common source of infections in humans
	Pseudomonas libaniensis [1]	99	LB1	58	30º	Aerobe	fresh water	isolated from Lebanese spring
	Pseudomonas synxantha [1]	99	LB1	61	20º	Aerobe		
	Streptomyces tauricus [1]	99	LB1	69-78	25-35º	Aerobe	soil	pathogenic for animals and man
	Streptomyces tauricus [1]	99	LB1	69-78	25-35º	Aerobe	soil	pathogenic for animals and man
	Streptomyces tauricus [1]	98	LB1	69-78	25-35º	Aerobe	soil	pathogenic for animals and man
	Streptomyces tauricus [1]	98	LB1	69-78	25-35º	Aerobe	soil	pathogenic for animals and man
	Pseudomonas putida [1]	99	LB1	62.5	25-30º	Aerobe	soil, water	
	Pseudomonas putida [1]	99	LB1	62.5	25-30º	Aerobe	soil, water	
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Rahnella aquatilis [1]	99	LB1	51-56	Mesophile	fac. anaerobic	soil, fresh water	
	Pseudomonas rhodesiae [1]	99	LB1	58-60	25-30º	Aerobe	fresh water	
	Pseudomonas rhodesiae [1]	99	LB1	58-60	25-30º	Aerobe	fresh water	
	Pseudomonas rhodesiae [1]	99	LB2	58-60	25-30º	Aerobe	fresh water	
	Pseudomonas sp. [1]	100	LB1	58-70	25-30º	Aerobe		
	Pseudomonas sp. [1]	100	LB2	58-70	25-30º	Aerobe		
	Janthinobacterium lividum [1]	100	S	61-67	25	Aerobe	soil, water	
	Bacillus cereus [1]	99	S	31.7-40.1		Aerobe or fac. Anaerobic		may lead to food poisoning
	Pseudomonas poae [1]	99	S	58-70	25-30º	Aerobe	soil, grass	
	Pseudomonas poae [1]	99	S	58-70	25-30º	Aerobe	soil, grass	
	Enterobacter sakazakii [1]	96	S	57	30º	fac. anaerobic	environment, foods	may contaminate milk powder and cause infections in newborns

	Pseudomonas fluorescens [1]	99	LB2	59.4-61.3	25º-30º	Aerobe	soil, water	commonly assoc. with spoilage of foods
	Swine manure bacterium 37-8 [7]	99	LB2	low	24-37		manure	
Novosibirsk, Russia	***Rhodococcus fascians*** [1]	100	LB1	63-72	30º	Aerobe	soil, dung	some strains are pathogenic for man and animals
	Leifsonia rubeus (rubra) [2]	95	LB1	66	15 °	Aerobe	Antarctic pond	
	Leifsonia rubeus (rubra) [2]	95	LB1	66	15 °	Aerobe	Antarctic pond	
	Leifsonia rubeus (rubra) [2]	95	LB1	66	15 °	Aerobe	Antarctic pond	
	Sphingomonas pituitosa [4]	99	S	64.5	30(?)	Aerobe	water	
	Variovorax sp.	99	S	62-70		Aerobe	soil, water	
Cloudcroft, New Mexico	***Micrococcus luteus*** [1]	99	LB1	64-75	25-37º	Aerobe	mammalian skin, soil, water	some strains may be opportunistic pathogens
	Williamsia murale [3]	100	LB1	64±8	30 °	Aerobe		isolated from children's day care center, Finland
	Williamsia murale [3]	100	LB1	64±8	30 °	Aerobe		isolated from children's day care center, Finland
	Williamsia murale [3]	100	LB1	64±8	30 °	Aerobe		isolated from children's day care center, Finland
	Uncultured alpha proteobacterium	97	S					clone AKYH1296
	Sphingomonas faenia [5]	99	S	63.1	25º-30º	Aerobe	soil, manure	
	Pseudoxanthomonas mexicana	99	LB2			Aerobe		
	Pseudoxanthomonas mexicana	99	LB2			Aerobe		
	Staphylococcus hominis [1]	99	LB2	30-36	30-40º	fac. anaerobic	human skin	may be assoc. with a variety of human infections
	Sphingopyxis alaskensis [6]	100	LB2	65	37º	Aerobe	arctic marine	

1. Bergey's Manual of Systematic Bacteriology, 1984 or 2005.
2. Reddy et al. (2003)
3. Kampfer et al. (1999)
4. Ewald et al. (2001)
5. Busse et al. (2003)
6. Vancanneyt et al. (2001)
7. Whitehead et al. (2004)

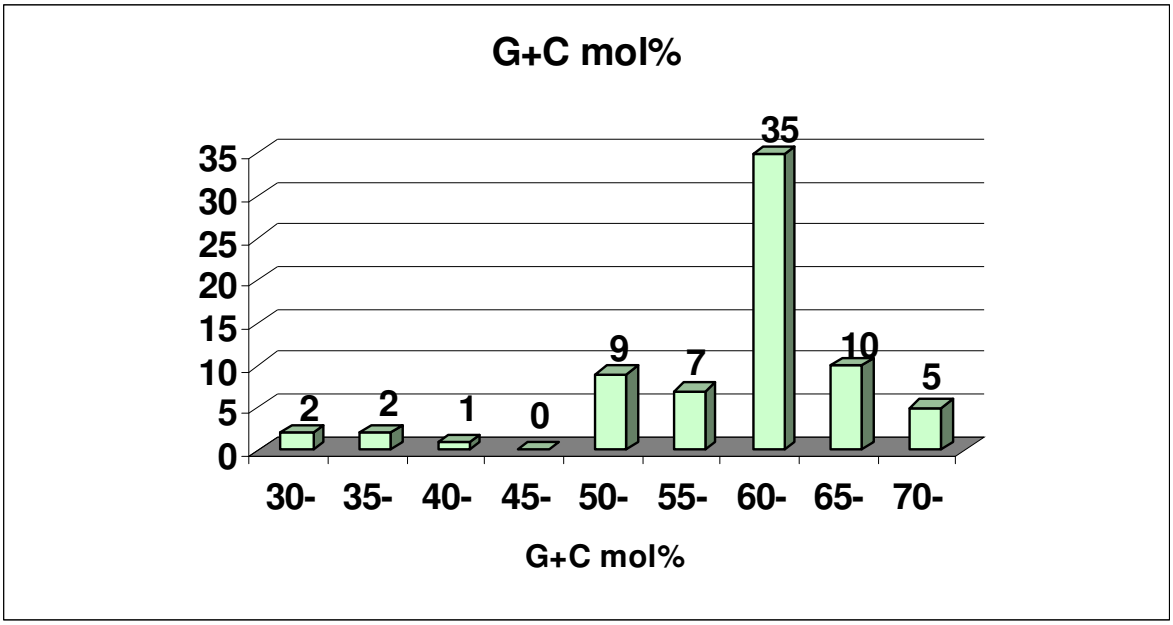

Figure 11: Guanine + Cytosine DNA content for the microbes analyzed. There were six organisms with unknown G+C mol%.

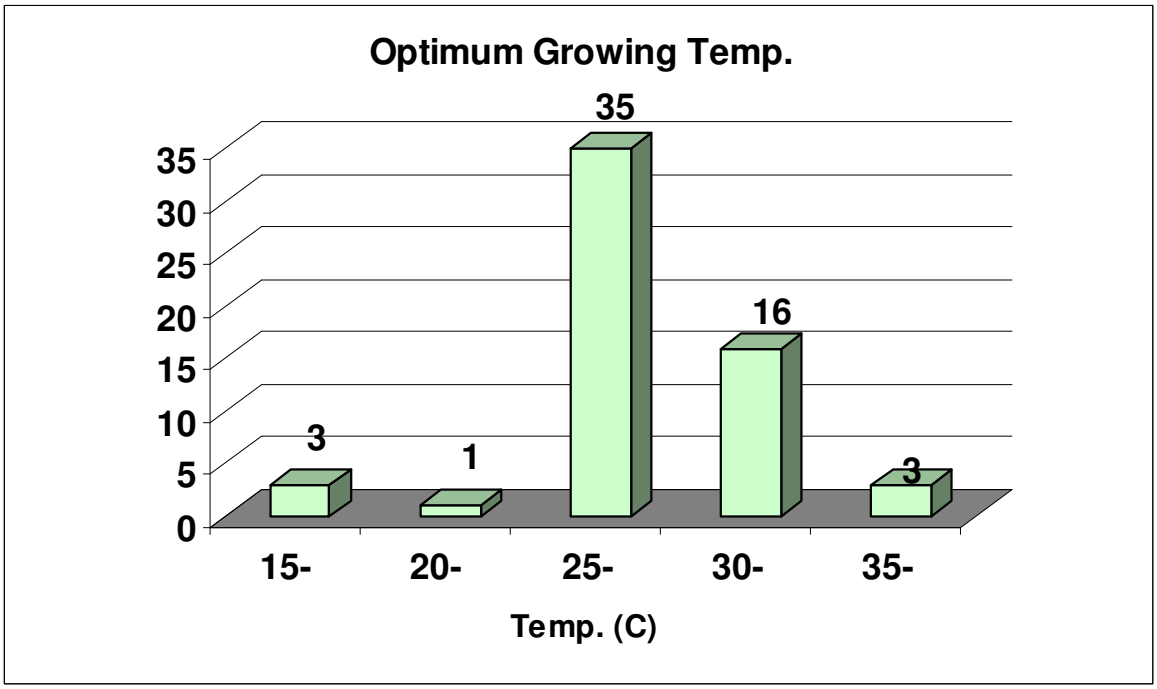

Figure 12: Breakdown of the optimum growing temperatures for the cultured microbes. Nine microbes cultured possessed an unknown optimum growing temperature.

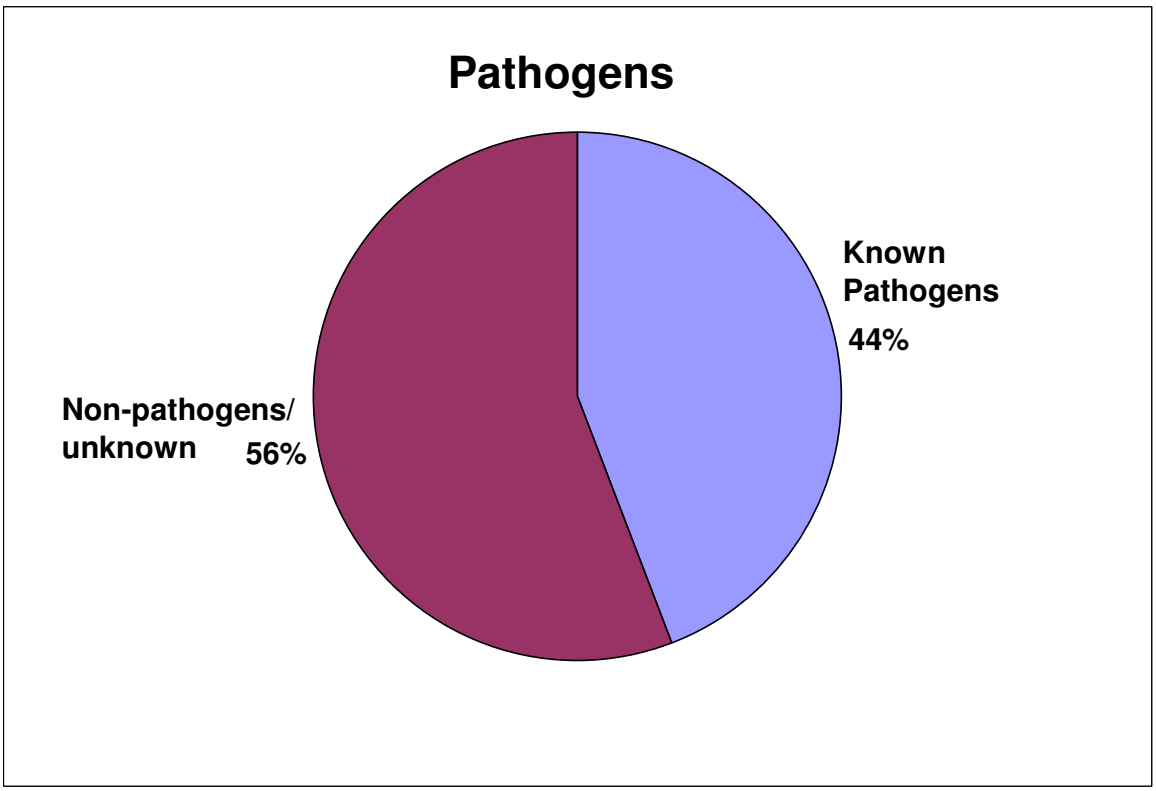

Figure 13: Pathogenicity of the cultured microbes.

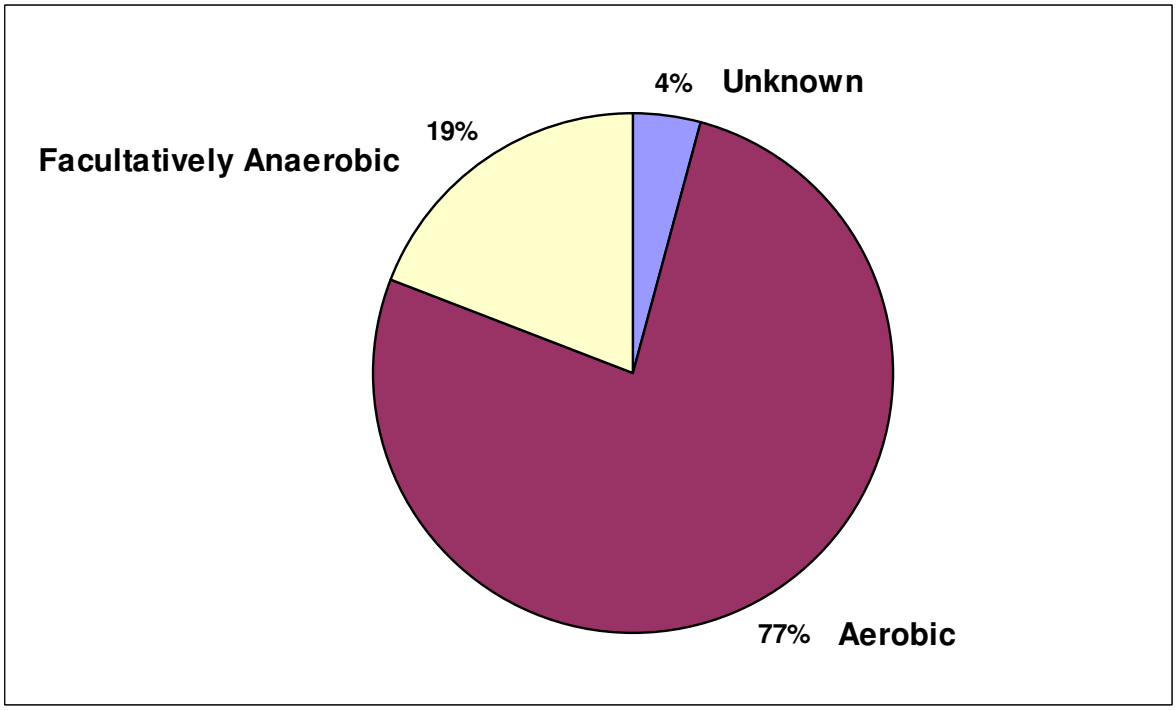

Figure 14: Chart indicates the metabolic tendency of the cultured microorganisms.

CHAPTER 4: DISCUSSION

4.1 Discussion

Chemically, most of the rain water sampled contained the nutrients considered important for biologic function, albeit usually in limited amounts. Nitrogen, sulfur, potassium, magnesium and calcium are certainly available for uptake. Iron was only detected in one of the samples. As the most abundant metal in the atmosphere, iron is mostly in particulate form or bonded with other chemical species such as oxygen or sulfate and not in a mobile form (Kieber *et al*., 2005). Any dissolved iron in the water would mostly form iron oxides and precipitate. Consequently most iron would have been filtered out of the sample prior to the chemical analysis. Although no phosphate was detected in any of the rainwater samples during the anion analysis, the cation analysis for phosphorus can also be used as a relative indicator of phosphate. This is because any phosphorus detected with the ICAP is probably in the form of phosphate. However, phosphorus was only detected in one sample of the ICAP analysis. This paucity of phosphate in rain water samples is not unusual. Previous studies show that phosphate, if detected in rain water samples, is usually in low concentrations, generally less than 1ppm (Chen et al., 1985; Callaway et al., 1991; Ahn, 1999; Migon et al., 1999; Pollman et al., 2002). This lack of available phosphate in rainwater could be a significant limiting factor for microbes since phosphate is an important nutrient for organism function (Chapelle, 2000). Of the micronutrients needed by microorganisms, manganese, copper and molybdenum were tested for. Low concentrations of Cr, V, Mo, Cu, and Mn are not surprising since these elements are not abundant in the atmosphere either as an aqueous component in rain water or in the solid form of particulate matter (Coqliati et al, 2002; Migliavacca et al. 2005).

Exactly 2/3 of the samples tested for sulfate and nitrate showed anion levels significantly higher, some times dramatically higher, than the total cation counts. This charge imbalance suggests the presence of a major cation that was not targeted, such as NH_4^+ which has been detected in significant amounts in other studies (Ceron et al, 2002; Zunkel et al., 2003; Migliavacca et al., 2004).

Out of all the sample locations, Condor, Brazil posted the highest levels of Na^+ and Cloudcroft, NM posted the highest levels of Ca^{2+}. In the two samples (15-Jul-Condor-Brazil and 4-Jun-Cloudcroft-NM) where the cation concentrations greatly overshadowed the anions, both cases possessed anomalously large cation concentrations. However, large levels of Na^+ in Condor, which is close to the coast and large levels of Ca^{2+} in New Mexico are not unexpected. The high Ca^{2+} concentrations in New Mexico were likely from local terriginous sources. Limestone is ubiquitous in the area and White Sands Nat. Park, which is composed of gypsum sands, is only ~80km away. Additionally, the prevailing arid conditions could lead to substantial dust contribution to the atmosphere. If the origin of the high Ca^{2+} ions has significant contribution from gypsum sands though, we would expect to see higher levels of sulfate in the samples. But sulfate levels for 4-Jun-Cloudcroft-NM, which had the highest recorded levels of Ca^{2+} of any sample, did not possess markedly high levels of sulfate. From this result we can reasonably conclude that most of Ca^{2+} contribution originates from limestone dust at this location. A test for bicarbonate concentrations would possibly confirm this.

Analysis for elemental sulfur within the rainwater samples did not find a pervading amount of elemental sulfur in all the samples. It is unclear why some samples tested positive for S_8 and others did not, even at the same location. The relatively short time period (three months) set aside for sample collection does not allow for speculation into seasonal affects on rainwater chemistry. But some explanation must account for random fluctuations, as in the case of

Australia where some samples tested positive for elemental sulfur and other samples showed no presence of elemental sulfur over the collection period. Perhaps the occurrence of S_8 in a sample is related to both a close proximity to a sulfur source and the prevailing wind direction that carries the sulfur. This implies that S_8 is either quickly dispersed in the atmosphere or is quickly removed by either dry or wet deposition. S_8 is actually a sufficiently stable molecule (Steudal et al., 2003). The chemical conversion of S_8 to other sulfur species is not favored on the appreciable time scales of atmospheric residence times. Elemental sulfur, itself, has a very low solubility in water and a tendency to flocculate (Mcguire, et al., 2000). However, the relatively low reactivity of sulfur can be altered by the presence of ferric iron in solution. The oxidation of elemental sulfur is accelerated in the presence of ferric iron in acidic conditions (Druschel, et al., 2002) and Fe is one of the most abundant trace metals found in rainwater in both dissolved and particulate forms (Kieber et al., 2005).

One reason for looking for S_8 specifically in the samples is the possibility that some organisms might use S_8 as a shield to protect themselves from UV damage or as an energy-converting pigment as suggested by Schulze-Makuch et al (2004). In microbial studies in acid mine drainage, Druschel et al (2002) suggest that microbial utilization of elemental sulfur may be limited by a reaction involving the S_8 ring, and not single sulfur atoms in solution. Druschel et al (2002) feel this emphasizes the importance of extracellular material as either a surfactant, to import an S_8 ring inside the cell, or to enable a reaction to transform S_8 into a more soluble and easily transported species. As such, organisms that use this strategy to utilize S_8 may accumulate significant amounts of sulfur on their outer membranes in environmental conditions where elemental sulfur is particularly abundant. Any benefit an organism gains in regards to UV protection may be coincidental but nevertheless beneficial.

While elemental sulfur is not prevalent in the atmosphere, sulfur does exist in the atmosphere in other, more reactive forms that organisms can utilize. Reduced sulfur compounds, such as H_2S, dimethyl sulfide, and dimethyl disulfide will be oxidized to SO_2 by photochemical reactions (Junge and Werby, 1958; Song et al., 2007). SO_2 is in turn converted to sulfuric acid (Seinfeld and Pandis, 1998) and sulfate (Junge and Werby, 1958) by photochemical processes. Of the samples analyzed for sulfate none possessed markedly high levels of sulfate. All were in a range typical for water samples (Zunkel et al, 2002; Ceron et al, 2002; Migliavacca et al, 2005; Shaheen et al, 2005).

The microbial analysis began with the PLFA studies and a DGGE analysis to obtain a general idea about the diversity of the microbial communities in the rain water samples and also the relative populations of major groups of organisms present. The samples analyzed for the microbial community breakdown using information from PLFA studies were largely dominated by Proteobacteria, a large family of Gram negative bacteria that are dominantly facultative and obligate anaerobes (Bergey's, 2005). This is not surprising given that Proteobacteria represent 1/3 of all known bacteria (Bergey's, 2005) and are ubiquitous. The relatively low percentage of Firmicutes (0-5.6%) within the community is not surprising either. This Gram+ group of organisms generally possesses a low G+C%. Since organisms with higher G+C% are more resistant to UV damage it is possible that the higher UV radiation found at higher altitudes reduced their numbers and lowered their survivability. The four samples that failed to detect any Firmicutes were also the four samples with the lowest available biomass. It is probable that Firmicutes exist in these samples as well, albeit in amounts that were below detection levels. The DGGE analysis failed to produce any Firmicutes in any of the samples however. The reason for this is likely because the relatively low percentage of Firmicutes within the samples provided insufficient DNA to register in the DGGE analysis.

Detecting anaerobic metal reducers and SRB/actinomycetes in the samples was surprising even if in very low amounts. The appearance and survivability of these organisms is a mystery as is the question of their source. It is interesting to note that the same samples tested positive in both categories, with the exception of 19-Jul-Bra, which was only positive for anaerobic metal reducers. This strong correlation indicates a similar source for these two groups. However, it is hard to imagine a significant source for these organisms that could contribute enough of a population to register in the analysis. Since the SRBs are grouped with the actinomycetes, the hint of having SRBs in the samples may be misleading. It is possible that all, or at least the overwhelming majority, of the organisms in this category are in fact actinobacteria. The DGGE analysis did not show significant SRB among the identified bands and since actinobacteria are Gram +, dominantly aerobic soil organisms with a high G+C DNA content, their presence is more likely than the appearance of SRBs in the samples. But the presence of sulfur reducing bacteria cannot be completely ruled out. Although SRBs generally require the complete lack of oxygen and a reducing environment, they have been known to circulate in aerated waters, albeit most likely in a resting state. Nevertheless the presence of SRBs in the samples should be very limited and since the two groups, SRBs and actinomycetes, are not separable in a PLFA analysis, it is assumed that the segment of the population represents actinomycetes only. As with the SRB, the presence of anaerobic metal reducers in the PLFA analysis were not represented in the DGGE analysis, but can still not be completely ruled out. The low percentages of metal reducers indicated in the PLFA analysis could provide insufficient amounts of DNA to be detected in the DGGE.

The eukaryotes were detected at appreciable levels (11-39%) in most of the samples of the PLFA analysis. Eukaryotes, and fungal spores specifically, should be a component in all rain water samples according to previous studies (Fuzzi et al, 1997; Griffin et al, 2001; 2003; 2006;

Kellogg et al, 2004; Jaenicke, 2005; Schlesinger et al, 2006). What is surprising is that three of the samples showed no detectable eukaryotic population representation, but there may be local phenomena that could explain these results. All three samples that tested negative were the Australia samples. It is not likely that this is a coincidence. The three samples from Australia however, were also the three samples with the lowest population levels. Again, it is possible that there was simply insufficient amounts of the required PLFA for minor community members to register in the analysis.

There was a surprising lack of similarities and overlap between samples, even at the same location. The three samples from England that underwent the Archaeal DGGE analysis all tested strongly positive for an uncultured *Crenarchaeote* common in soil. Of the six samples of the bacterial DGGE analysis however, there were no ubiquitous organisms to be found prevalent in every sample, but there was some overlap. Of these six samples, three samples detected positive for *Cytophagales*, two for *Pedobacter*, two for *Caulobacte*r, and two for *Sphingobacterium*. However, with the exception of both Sphingobacteria found in two samples from England, pairing organisms were not found at the same location. And several other identified organisms were found solely at one location. The factors that would lead an organism to have a significant presence in one sample, but not in another are not understood. Perhaps, for organisms with limited habitat and close proximity to the collection point, there might accumulate a significant population of site-specific organisms in the sample if the weather conditions favored their transport and deposition before atmospheric dispersion could limit their numbers in a sample. It is still surprising that ubiquitous and plentiful bacteria common in soil are not found in all samples. The presence of *Caulobacter* in two of the samples was an interesting result. This genus of Gram negative, strictly aerobic organisms generally live in dilute aquatic environments where phosphorus is a limiting nutrient (Bergey's, 2004). Rainwater is, in general, such an

environment and members of *Caulobacter* could have a survival advantage over many other species.

It is interesting to note that the PLFA analysis indicated that only half of the samples tested were stressed or starving. This indicates that some microbial populations were apparently comfortable in rainwater, even after being in cold storage (4˚C) for months. Location did not appear to be a direct factor in the levels of stress or starvation in the samples. The PLFA analysis showed a strong correlation between higher microbial population densities and stress. The samples with the four highest population densities all showed some stress and the sample with the highest population density (11-12-Aug-Eng), with ~$1.5x10^6$ cells/ml, had very high stress levels. This stress is likely the result of overcrowding and limited resources as the four samples with the lowest population densities showed no signs of stress. However it should also be considered that all of the samples that showed signs of stress also possessed low levels of anaerobic metal reducers and four of the five samples with stress also possessed low levels of SRB/actinomycetes. Such organisms should be stressed since they are not in their optimum environment. This possibility is not conclusive, though, since samples with lower population densities may simply possess anaerobic constituents in amounts that are below detection limits and the associated stress of those microbes would also be below detection limits.

Starvation showed a similar trend to *stress* among samples of higher microbial population densities; that is, samples with higher population densities also demonstrated significant levels of starvation. One exception however was the 10-11-July-Aus sample that showed relatively high levels of starvation despite having a relatively low population density of $1.9x10^4$ cells/ml. Despite this one anomalous sample there is a strong correlation between higher population density and stress/starvation. One interpretation of this is simply that more microbes are competing for limited resources and showing signs of starvation. It is also probable that some

microbial growth occurred during storage, which could put stress on the entire population, but it is not possible to determine if and how much growth occurred. Significant growth would, however, skew the analysis and subsequent interpretation. When the rainwater chemistries of the samples with higher population densities were compared with those of lower population densities, there did not appear to be a correlation between stress/starvation and the variable rainwater chemistries. Higher or lower levels of sulfate and nitrate were apparently independent of population counts.

Previous studies involving microbial population estimates for cloud water have ranged from 1500 to 355,000 bacteria/ml (Sattler et al., 2001; Bauer et al., 2002; Amato et al., 2005). The PLFA biomass analysis of the samples represented cell counts ranging from $1.1x10^4$ cells/ml to $1.5x10^6$ cells/ml. Four samples had cells counts higher than previous studies with the highest approximately three times higher. These higher results are likely due to a number of contributing factors. First, precipitation will accumulate particulates (and microbes) as it falls in a process called "washout" (Anthes, 1992), thereby leading to higher bacterial counts than in samples collected directly from clouds. Also, bacterial counts near the land surface are generally higher than atmospheric counts (Tong and Lighthart, 1999). This creates a circumstance inherent with ground collection techniques that allows for an unknown amount of near surface microbial contribution. Another inescapable possibility is the likelihood of significant growth and reproduction after collection and during storage, but before the analysis could be performed. Since the samples were refrigerated for 4-6 months before analysis could occur, significant growth could have occurred during this period of time. Even though the samples were stored at temperatures between 1˚ and 4˚C, it has been demonstrated that growth and metabolism does occur in some organisms at temperatures at or below 0˚C (Psenner et al., 1998; Sattler et al., 2001; Christner, 2002; Junge et al, 2006). Some species, such as *Rahnella aquatilis*, a

pychrotolerant species found multiple times in the England and South Africa samples, is capable of growth at 4˚C (Bergey's, 2005). *Leifsonia rubeus*, a psychrophilic organism found in three of the samples from China, grows between 0 and 22˚C (Reddy, et al, 2003). *Janthinobacterium lividum*, cultured from an England sample, has also been observed to grow at temperatures as low as 2˚C (Bergey's, 2005).

In general, the DGGE and culturing results did not correlate well. Because bands of DNA excised and analyzed during the DGGE analysis only represent organisms within the community that make up greater that 1-2% of the community, there is an inherent bias towards more prolific organisms. Less numerous, but no less important, microbes are unavoidably overlooked by this procedure. Another limitation of this method is the relative small size of the DNA strand sequenced (276bp and smaller), which lowered the matching potential for most sequences and in some cases the DNA was 'unsequencable'. Though identification of microbes to the species level was problematic, sequences could be matched to the genus level in most cases. In this capacity the DGGE experiment was capable of demonstrating a general overview of the largest groups of organisms within the population.

A better understanding of the base microbial population provided by a PLFA and DGGE analysis is a helpful introduction to devising culturing experiments. One advantage of culturing over a nonspecific DGGE analysis is that specific organisms can be identified with high confidence. Although this study chose to use conventional methods of culturing the biologic component of the samples, this created an unavoidable bias in the microbes cultured. Microbes with greater numbers have a greater chance of being selected at the expense of other organisms with limited numbers. Also, the culturing media used will favor certain microbes, often generalists, over microbes with more restrictive nutritional needs. As an example, the PLFA analysis detected the presence of low levels of anaerobic metal reducers and possibly SRBs in

the bulk population of some samples. Additionally, the DGGE analysis showed that organisms of the genus *Methanosarcina*, which are strict anaerobes, were present in some of the samples. All of these groups of organisms would not have been cultured using the methods that were applied for this study because the culturing was performed under aerobic conditions. The DGGE information from this study produced similar results, in terms of the genera represented, as found in other studies that utilized 16S rRNA genes to identify the biological constituents of rain water (Griffin et al, 2003; Amato et al., 2005; Amato et al, 2006; Griffin et al, 2006; Ahern, et al, 2007).

In theory, the advantage of 16S rRNA gene sequencing directly from environmental samples enables a greater scope of the microbial community of the samples to be presented. Most of the microbes cultured in this study were organisms common to soil and fresh water (*Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptomyces* and *Arthrobacter*), as were most of the organisms resulting from the DGGE analysis (*Pedobacter, Caulobacter, Cytophagales).* This is not unexpected since dust born aloft into the atmosphere can host a large number of bacteria on its surface and within the grain itself. This dust can also act as a nucleation point for cloud droplets that are subsequently removed with rain. But some results are surprising, like *Sphingopyxis alaskensis* (100%match), an arctic marine organism (Vancanneyt, et al. 2001). It was found in a Cloudcroft, New Mexico sample. Another example is *Leifsonia rubeus (rubra)* (95% match), an organism found in Antarctic melt-water ponds (Reddy, et al. 2003), but cultured from the samples of Novosibirsk, Russia. Though microbes of marine origin probably exist in all rainwater samples in some amounts, the survival of marine organisms in significant numbers is not expected to be high during atmospheric transport over longer distances and greater residence times. Further, since the culturing media (LB and Starkey's) used in this project did not favor marine conditions, their presence in a culture is not expected to high.

Perhaps marine microbes exist in rainwater in much greater concentrations than is expected. Both terrestrial and marine organisms are certainly capable of traveling anywhere on the surface of the earth via the atmosphere.

A surprising result was that only about 5% of the organisms cultured were spore-forming bacteria. This is contrary to previous culturing studies of atmospheric samples that recorded much higher percentages of spore-forming bacteria in their samples (Griffin *et al*, 2001, 2003, and 2004). However, these studies cultured their bacteria on different media (R2A) and this may have favored the growth of spore-formers. Also Griffin's studies collected bacteria from higher altitudes (up to 20,000m), and the harsher conditions associated with increasing attitude may favor such adaptive species. Certainly some organisms possess a greater likelihood of enduring extended forays in the atmosphere. Spore formers are obvious candidates, but their strategy for coping with harsh conditions is to 'hibernate' until conditions are more favorable.

The average G+C% content of DNA from cultured isolates was fairly high (above 60%). This is possibly due to the potential of microbes with higher G+C% to be more resistant to the higher UV radiation levels at higher altitudes. This result is exactly what is expected.

When considering the pathogenicity of the identified organisms, the DGGE analysis did not positively identify any pathogens. The culturing experiments however evinced a surprisingly high percentage of organisms that are known pathogens (44%). But the culturing techniques used in this experiment could be sufficiently biased to give a false representation of pathogen occurrence. The breakdown between plant and human and animal pathogens is nearly equal, with thirteen plant/fungi pathogens and 12 recorded human and animal pathogens. This high percentage of animal pathogens may be the result of the culturing conditions which were dominantly performed at 37˚C.

The majority of the organisms cultured possess optimum growing temperatures between 25˚C and 30˚C, with the next greatest number of microbes favoring temperatures between 30˚C and 35˚C. These conditions are much warmer than temperatures found at higher altitudes. Again, we may be observing a culturing bias due to most of the culturing taking place at 37ºC.

In an effort to determine the possible effects of meteorological conditions on chemical and biological results at sample locations, the weather conditions during collection were obtained at the Cloudcroft, NM and Sidmouth, England sites. Location definitely played a dominant role influencing the chemical constituents in rain water collected globally. At the Sidmouth, England site, weather conditions were taken from meteorological archives collected at Exeter, England, which is located approximately 16km west of Sidmouth. The Sidmouth site presented an interesting situation whereby the collection site was located immediately on the coast. Wind conditions at this location were landward winds from the westerly, southwesterly or southerly directions for all samples (Met, 2004). This prevailing wind situation is not uncommon in coastal areas where warm air rising from land masses pulls air in from the ocean (Anthes, 1992). Because of this wind pattern, samples were expected to have higher indications of marine chemical contributions. Any air movement over land and subsequent terriginous contributions were expected to be limited, although anthropogenic sources could still be high. On the whole, cation concentrations were somewhat low compared to samples from other locations. One interpretation is simply that terriginous dust contributions that alter the atmospheric chemistry are very low, since winds arriving at this location progressed dominantly over the Atlantic Ocean with few significant landmasses available. However, sodium levels at this site were not exceptional, suggesting a low marine contribution that is contrary to expectations. Perhaps frequent rains minimize the propensity of chemical accumulation in the atmosphere at this location. The biological constituents at this site were dominated by organisms common to soil

and freshwater habitats, though. One notable observation is the relative abundance of biomass in the samples collected at this site. Of the four Sidmouth, England samples that had a PLFA analysis performed on them, three of these samples possessed the highest biomass readings of any sample tested.

The Cloudcroft, NM site was sufficiently inland to produce negligible chemical contributions from marine sources, as evidenced by the relatively low Na^+ concentrations. Weather histories (W.U.I., 2004) were taken from weather station archives at Alamogordo, NM, ~20 km west of Cloudcroft. The prevailing weather conditions on the date the samples were collected at the Cloudcroft, New Mexico were studied. For all samples collected at this location, winds were westerly or southerly in nature. The 4-June sample possessed the highest ion concentrations of all the samples at that location and it should be noted that on this date was recorded the highest wind levels; with a mean wind speed of 15kph and maximum sustained winds of 55kph for the day of June 4, 2004 at that location (Figure 15). The previous day of June 3, 2004 proved to be an even windier day with gusts rising to 80kph (Figure 16).

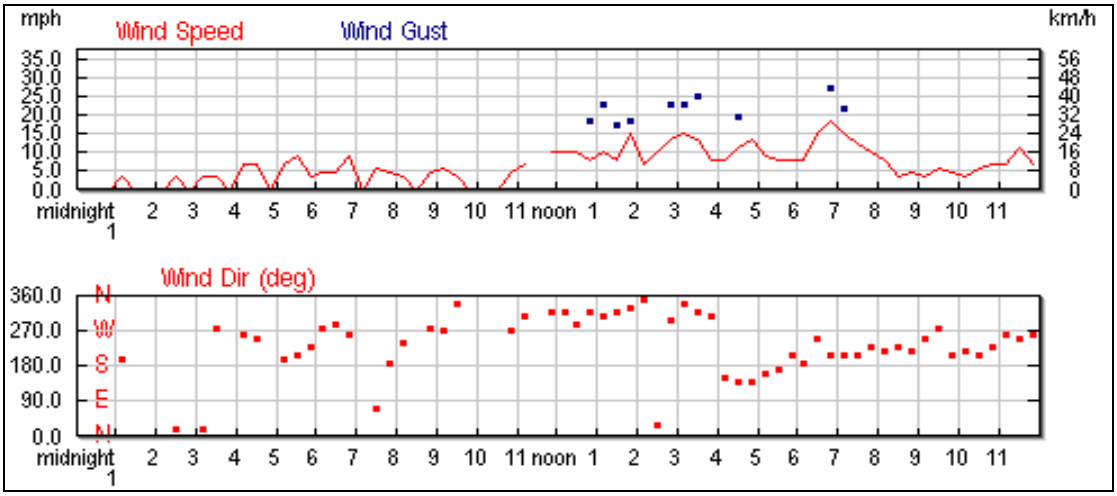

Figure 15: Wind data collected from Alamogordo, New Mexico – June 4, 2004 (W.U.I., 2004)

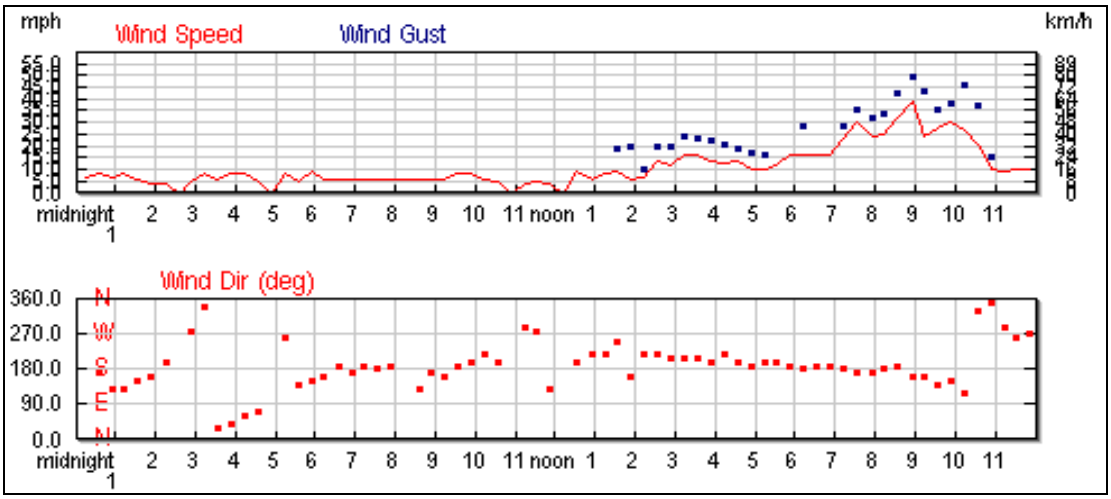

Figure 16: Wind data collected from Alamogordo, New Mexico – June 3, 2004 (W.U.I., 2004)

It is likely that these higher wind speeds whipped up significantly more dust from the arid landscape that ultimately made a considerable contribution to the rain water chemistry on June 4, 2004. The source of the anomalously high Ca^{2+} levels in the New Mexico samples are open to some interpretation. The two most likely sources of the elevated Ca^{+} levels are from gypsum and limestone dust. A potentially significantly source of gypsum does exist ~50km southwest of the collection site at White Sands National Monument. However, if gypsum dust made a significant contribution to the chemistry of the rainwater we would expect to observe comparatively significant levels of sulfate in the samples, but sulfate levels in the samples were not elevated. So limestone dust appears to be the most likely source of the high Ca^{2+} measurements.

At inland collection sites such as from Fujin, China, which had no detectable sodium in any of the samples from that location, marine contributions are expected to be consistently low over the entire collection period. But situations exist that allow for variable weather patterns in a region. At Condor, Brazil, some samples were very low in sodium compared to other samples at the same location. It is probable that the rain contributing to the lower Na samples did not travel

in from the coast but arrived from landward breezes, but meteorological data at this location could not be obtained. However, if this were true, one would expect higher concentrations of other cations more commonly associated with a terrestrial origin, but dust levels in the atmosphere are a function many factors, including, but not limited to, vegetation cover, anthropogenic activity, wind conditions, and amount of previous rainfall and the duration between rain events. These contributing factors could produce a situation whereby a relatively small amount of dust is prevalent in the atmosphere during precipitation, particularly in regions subject to frequent precipitation events (Rastogi and Sarin, 2007).

Since dissolved particulate matter is a major contributor to varied rain water chemistries, the chemical signatures of the water reflect its source and in turn should indicate the types of microbes within the rainwater. One would not expect to see an abundance of marine microbes from rain approaching from inland areas. And in opposition, one would expect to see greater concentrations of plant and agricultural related microbes from rain systems that progress over land.

However, no clear biological connection could be made between rainwater samples dominated by marine chemistry and the associated observed biota. This possibly indicates that atmospheric dust, and the microbes in association with that dust, experience enough migration and mixing on a regional or global scale to offset, or blur, origin distinctions. Another possibility could be a limited propensity for marine organisms to be borne aloft or limited survivability of marine organisms in response to atmospheric conditions. Other possibilities for the occurrence of limited marine organisms in the observations is simply a culturing bias that favors terrestrial organisms. Most culturing media for marine microbes is essentially seawater with peptone and yeast extract added (Vazquez *et al.*, 2004), which is functionally very different from the LB and Starkey's media used in the culturing experiments of this study.

There were some procedures that could have been utilized to provide additional information on both the chemical as well as the biological data procured. One such procedure is the use of field blanks, which were not collected at any of the sites. These field blanks would have been composed of pure DI water in the same sample containers that were used to store the rain water. These "closed" blanks could be used to confirm any possible chemical contribution from the sample containers themselves. It would also be desirable to collect "open" field blanks of pure DI water that would be open to the air during the same time period as the other collection samples, but in such a way that rainwater would be prohibited from entering. These "open" blanks could be used to discern any possible chemical contribution associated with wind blown dust that might enter a sample container during a rain event. Both "open" and "closed" field blanks would then be subject to the same storage conditions as the rainwater samples, so that the pertinent effects of storage duration and conditions on the samples could be discerned and quantified. These blanks could also have been useful during the biological analysis too. It would have been helpful to gain an understanding into possible microbial contribution outside of the rainwater itself, such as from near surface wind blown particulates.

CHAPTER 5: CONCLUSIONS

To determine the habitability of the atmosphere for microorganisms, one must consider the needs of the organism. Are nutrients available? Are temperatures favorable? Are other environmental conditions, such as moisture availability and radiation, within tolerable limits? The answers to these questions are undeniably "yes" for many organisms. Nutrients are available, albeit in limited amounts. Temperatures are cold, but not too cold for metabolism. Moisture is limited but obtainable. Radiation levels are hazardous and damaging, but manageable.

These implications are clear. Microorganisms have adapted to survive and even exploit atmospheric conditions; encouraging precipitation and altering rainwater chemistry. The methods and means by which microorganism interact with the atmosphere is a source of academic interest. The ability of this study to enhance our understanding of these processes was limited, but the scope and magnitude of these enigmatic interactions needs to be further studied.

From this study there does not appear to be any clear connection between the biological components found in the rainwater and the chemistry of the associated rainwater. Although the biological analysis performed in this study indicates a dominance of terriginous organisms in rain water, the biologic variability associated within each sample is sufficiently diverse to reduce any noticeable trend with the highly variable chemical nature or rainwater. Samples that were chemically dominated by marine influence were still prolific with terriginous microbes. Perhaps this is a product of near surface contamination of samples or a bias in culturing experiments. Or perhaps terriginous organisms are so prolific and widespread in the atmosphere and have sufficiently long residence times, that sufficient mixing takes place on a global level to minimize the influence of any localized chemical variably.

The potential of the atmosphere as an avenue for microbial transport is relatively unexplored and not completely understood. Obviously some microbes can survive prolifically in the atmosphere, and have developed strategies to enhance survival rates. More investigation is also needed to determine the diversity and interaction of airborne organisms and more testing is needed to understand the metabolic and chemical reactions taking place in the atmosphere and their relation to the specific microbes involved. Further knowledge in these areas will help us determine whether the microbes found in the atmosphere are merely transients or have evolved specific adaptations to exploit this environmental niche.

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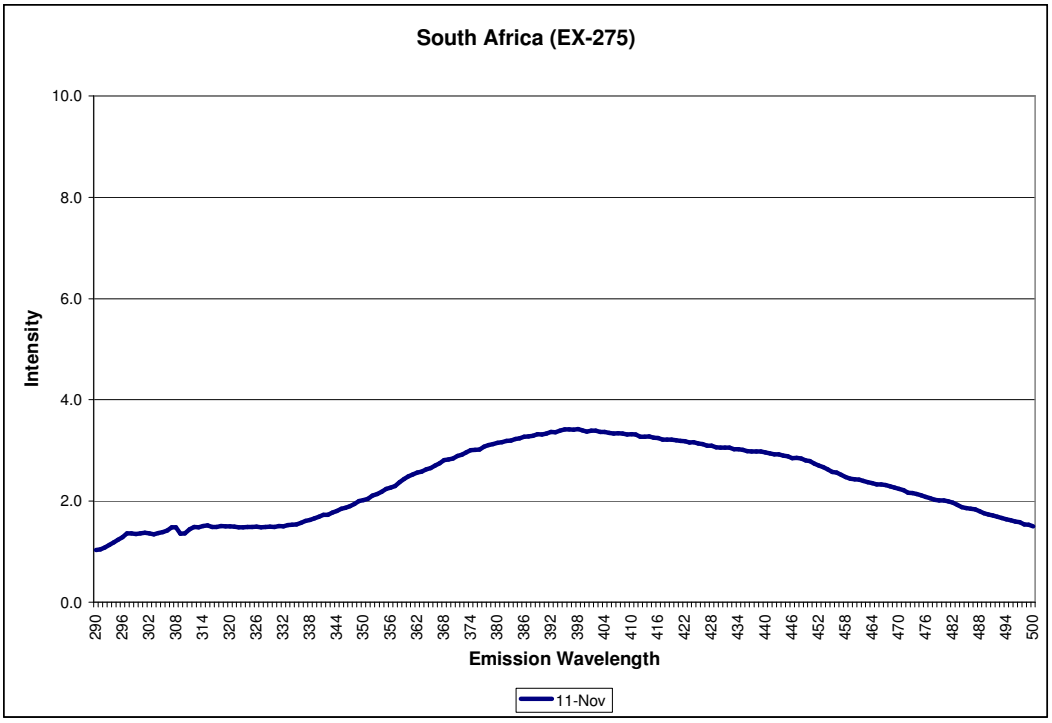
APPENDIX A: ICS

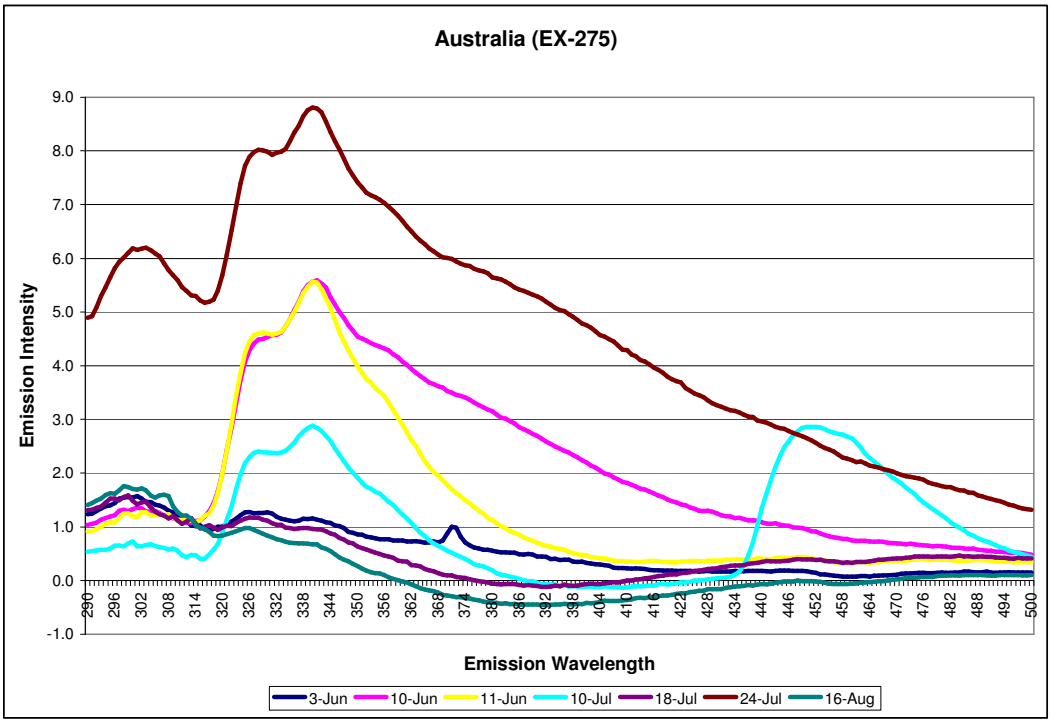
Table A1: ISC anion results, including standards, blanks and duplicates. Detection limits for nitrate and sulfate are approximately 8 µeq/L (1.0ppm) and 10 µeq/L (0.5ppm) respectively.

Name	Nitrate µeq/L (±5µeq)	Sulfate µeq/L (±3µeq)
Cloudcroft, NM 04-Jun	279	32
Cloudcroft, NM 29-Jul	366	17
Russia-14-Jul	379	16
Russia-4-Aug	608	27
Australia-11-Jun	193	63
Australia-10-11-Jul	195	30
Australia-18-20-Jul	175	84
Sao-Carlos-Brazil-10-Jul	71	4
Sao-Carlos-Brazil-19-Jul	199	6
Condor-Brazil-10-Jun	81	15
Condor-Brazil-15-Jul	81	23
England-31-May	279	25
England-22-Jun	133	33
England-7-8-Jul	196	29
England-11-12-Aug	275	16
China-8-Jul	280	30
China-1-Aug	150	45
Africa-11-Nov	125	28
Africa-11-Nov-duplicate	121	28
England-31-May-duplicate	282	27

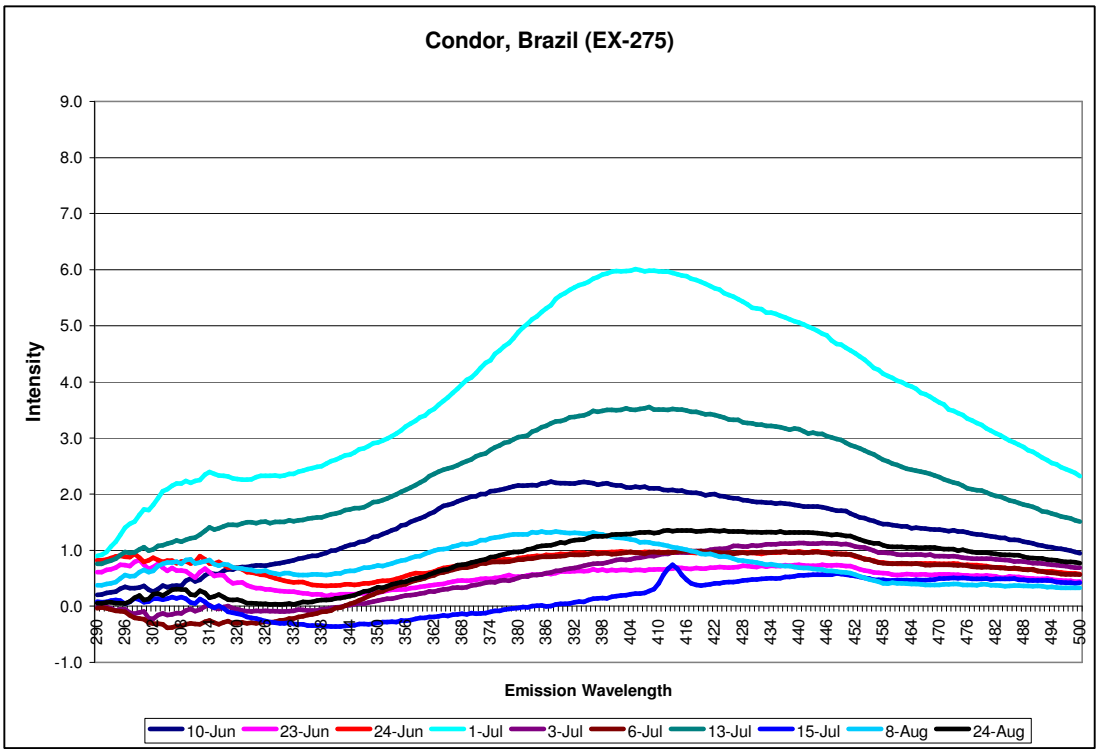
APPENDIX B: Fluorimeter



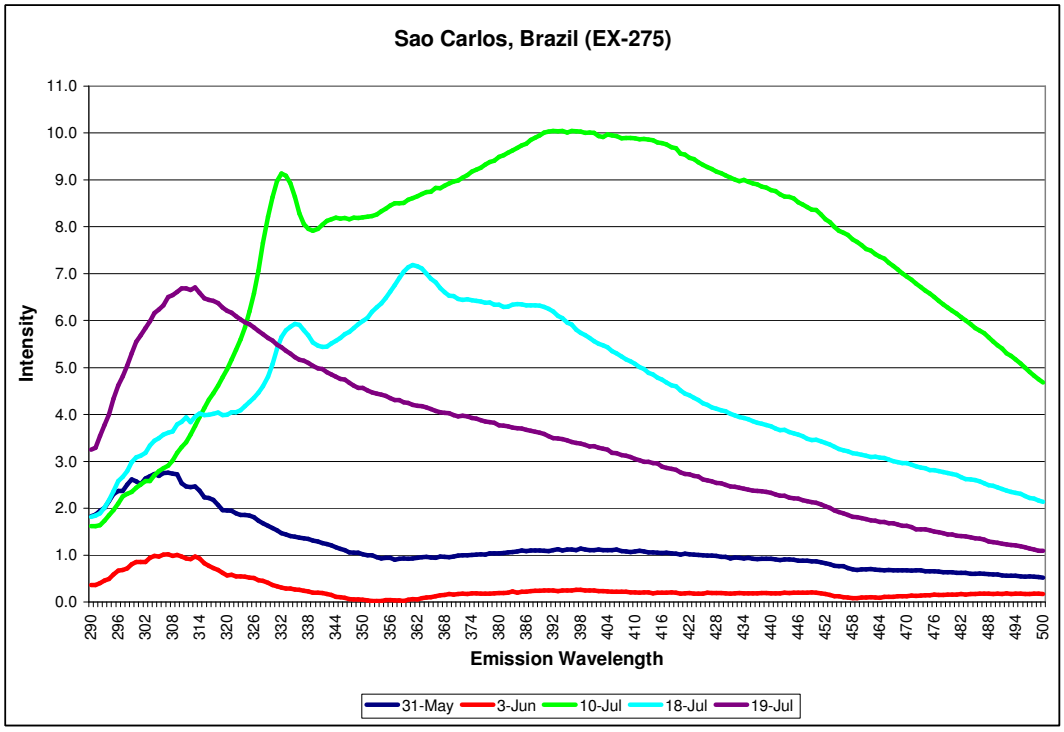
B1: South Africa



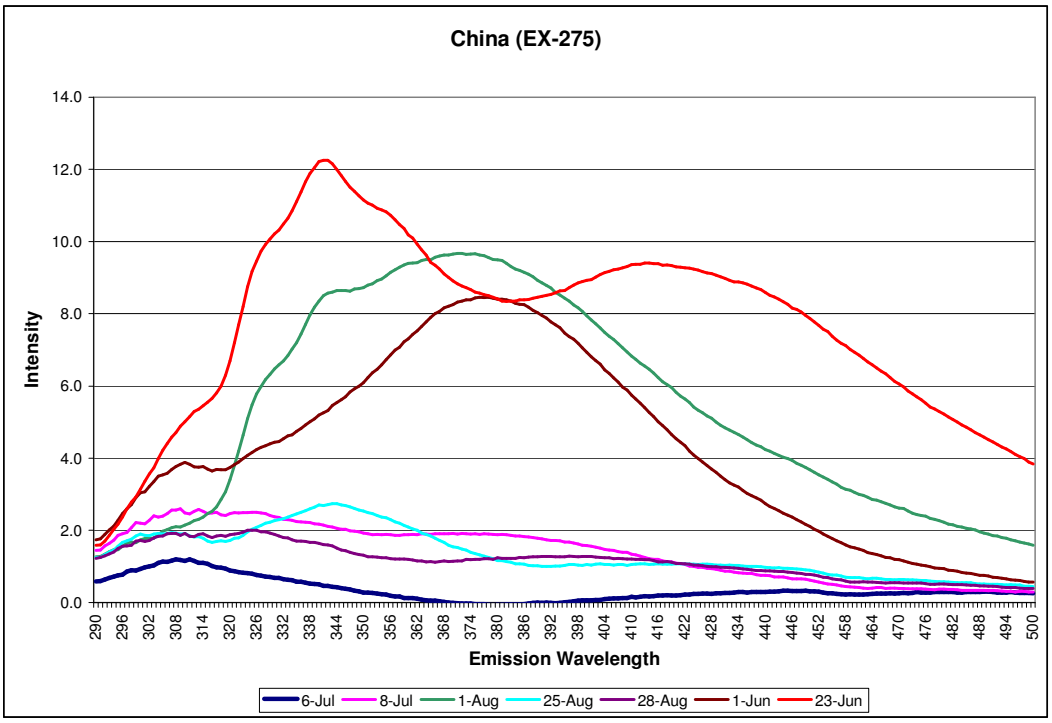
B2: Australia



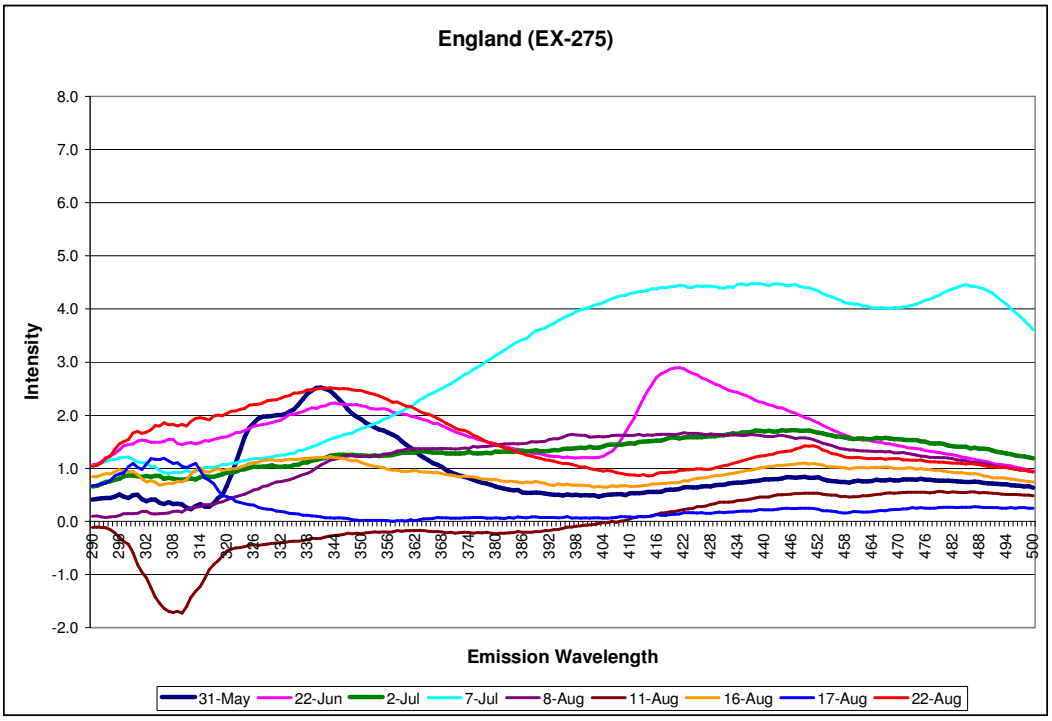
B3: Condor, Brazil



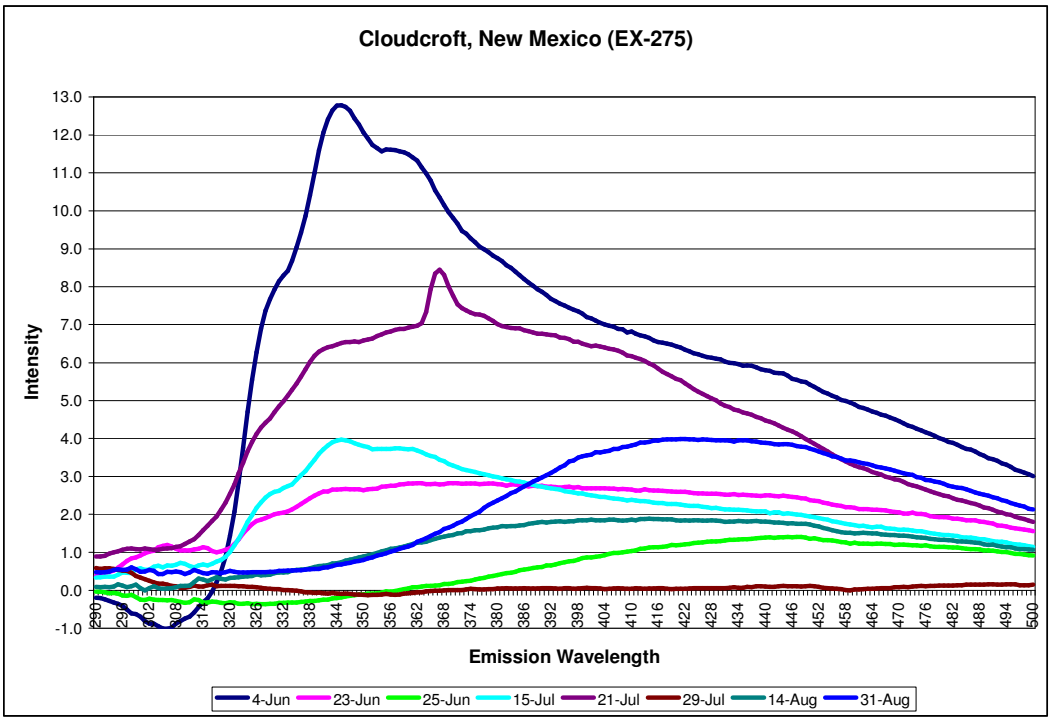
B4: Sao Carlos, Brazil



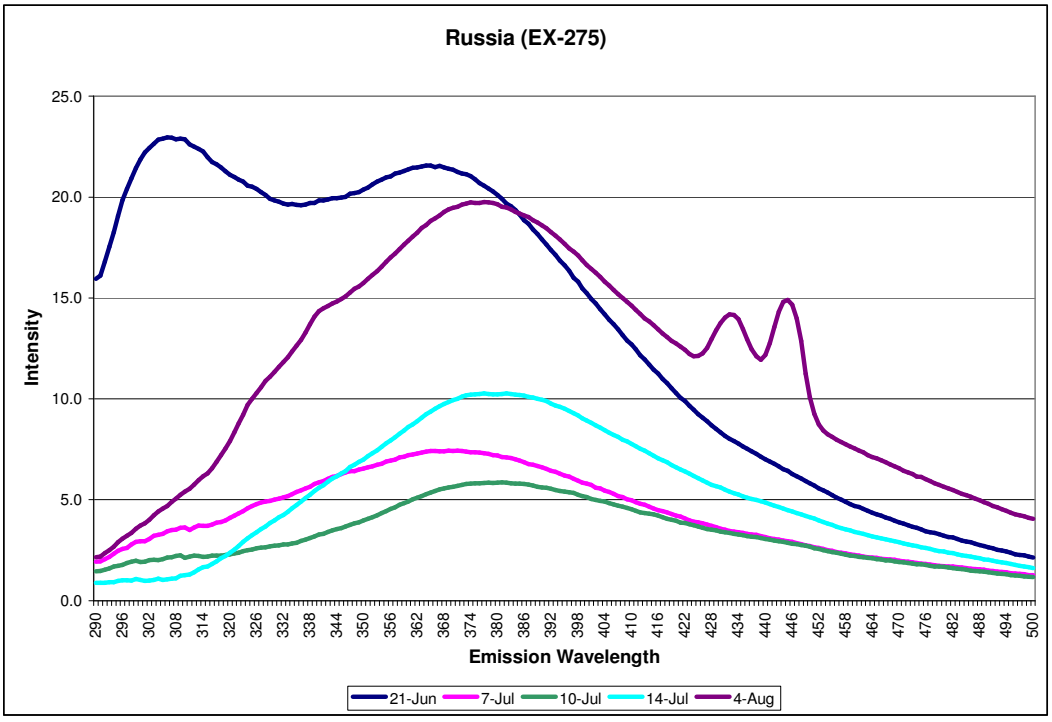
B5: Fujin, China



B6: England



B7: Cloudcroft, New Mexico



B8: Novosibirsk, Russia

APPENDIX C: MI

Table C1: PLFA community analysis performed by Microbial Insights. The numbers are in percentages found within the sample.

Sample Name	Firmicutes (TerBrSats)	Proteobacteria (Monos)	Anaerobic metal reducers (BrMonos)	SRBs/ Actinomycetes (MidBrSats)	General (Nsats)	Eukaryotes (polyenoics)
31-May-Eng	5.6	56.1	0.0	0.0	27.1	11.2
31-May-SC,Bra	0.0	52.6	0.0	0.0	26.3	21.1
22-June-Eng	2.8	30.8	0.7	2.9	23.4	39.3
7-8 July-Eng	5.0	45.5	1.1	0.5	19.4	28.5
10-July-SC,Bra	4.5	62.5	1.5	0.8	16.1	14.7
10-11 July-Aus	0.0	79.4	0.0	0.0	20.6	0.0
13-July-Con,Br	0.0	0.0	0.0	0.0	100.0	0.0
18-20 July-Aus	0.0	70.1	0.0	0.0	29.9	0.0
19-July-SC,Bra	2.5	53.8	1.0	0.0	32.5	10.1
11-12 Aug-Eng	3.3	44.3	0.7	1.2	27.4	23.1
16-17 Aug-Aus	0.0	54.4	0.0	0.0	45.6	0.0

Table C2: PLFA cell counts performed by Microbial Insights.

Sample Name	Sample Date	Cells/ml filtered
31-May-Eng	5/31/2004	1.61E+05
31-May-SC,Bra	5/31/2004	5.84E+04
22-June-Eng	6/22/2004	1.05E+06
7-8 July-Eng	7/7/2004	9.29E+05
10-July-SC,Bra	7/10/2004	5.67E+05
10-11 July-Aus	7/10/2004	1.92E+04
13-July-Con,Bra	7/13/2004	5.43E+03
18-20 July-Aus	7/18/2004	1.11E+04
19-July-SC,Bra	7/19/2004	1.30E+05
11-12 Aug-Eng	8/11/2004	1.49E+06
16-17 Aug-Aus	8/16/2004	1.63E+04

Table C3: Environmental stress and starvation indicators any number greater than 1.0 indicates stress.

Sample Name	"Starvation" Total *(cy/cis)*	"Stress" Total *(trans/cis)*
31-May-Eng	0.00	0.00
31-May-SC,Bra	0.00	0.00
22-June-Eng	0.02	0.01
7-8 July-Eng	0.21	0.02
10-July-SC,Bra	0.88	0.01
10-11 July-Aus	0.22	0.00
13-July-Con,Br	0.00	0.00
18-20 July-Aus	0.00	0.00
19-July-SC,Bra	0.00	0.04
11-12 Aug-Eng	0.13	1.05
16-17 Aug-Aus	0.00	0.00

APPENDIX D: ACGT

Table D1: Chemical components of the Modified Starkey's medium

Modified Starkey's Medium	
Elemental Sulfur	30g
Sodium lactate	3.5g
NH_4Cl	1.0g
K_2HPO_4	0.5g
$MgSO_4$-$7H_2O$	2.0g
Na_2SO_4	0.5g
$CaCl_2$-$2H_2O$	0.1g
$(NH_4)_2SO_4$-$FeSO_4$-$6H_2O$	0.05g
Yeast Extract	1.0g
Water	1.0 liter

Table D2: Chemical components of the LB medium

LB medium	
Bacto-tryptone	10g/L
Bacto-yeast extract	5g/L
NaCl	10g/L

APPENDIX E: List of Samples

Table E1: List of samples: The chart graphically displays which samples were chosen for the three experiments of ACGT Inc., Microbial Insights and the ICS anion survey.

Location	Date (2004)	Prec. (ml)	ACGT, Inc			MI, Inc		ICS
			LB1	Starkey	LB2	PLFA	DGGE	
Sao Carlos	31-May	350				SC,Bra		
England	31-May	250	Eng			Eng		D
China	1-Jun	~75						
Australia	3-Jun	80						
Sao Carlos	3-Jun	200			SC,Bra			
USA	4-Jun	10						
Australia	10-Jun	40						
Condor	10-Jun	150						
Australia	11-Jun	80						
Russia	21-Jun	5						
England	22-Jun	3000	Eng	Eng		Eng	Eng	
China	23-Jun	~130						
Condor	23-Jun	170						
USA	23-24-Jun	40						
Condor	24-Jun	330						
USA	25-Jun	40						
Condor	1-Jul	220						
England	2-Jul	300			Eng			
Condor	3-Jul	600						
China	6-Jul	~550						
Condor	6-Jul	100						
Russia	7-Jul	10						
England	7-8-Jul	2900	Eng			Eng	Eng	
China	8-Jul	~350			Chi			
Russia	10-Jul	40						
Sao Carlos	10-Jul	700	SC,Bra	SC,Bra		SC,Bra		
Australia	10-11-Jul	500				Aus		
Condor	13-Jul	150				X		

Russia	14-Jul	100	Rus-Rt	Rus					
USA	15-Jul	50							
Condor	15-Jul	300	Con,Bra	Con,Bra					
Sao Carlos	18-Jul	200							
Sao Carlos	19-Jul	1500				SC,Bra	SC,Bra		
Australia	18-20-Jul	900	Aus	Aus		Aus	Aus		
USA	21-Jul	5							
Australia	24-Jul	10							
USA	29-Jul	200	USA-Rt	USA					
China	1-Aug	~950			Chi				
Russia	4-Aug	8							
Condor	8-Aug	150							
England	8-9-Aug	500							
England	11-12-Aug	2400		Eng		Eng	Eng		
USA	14-Aug	150			USA				
England	16-Aug	100							
Australia	16-17-Aug	600				Aus			
England	17-Aug	100							
England	22-23-Aug	200	Eng				Eng		
Condor	24-Aug	150							
China	25-Aug	~750							
China	28-Aug	~750							
USA	31-Aug	50							
Africa	11-Nov	4000	Africa	Africa				D	

X-did not have sufficient PLFA for analysis

D - Duplicates

Rt -Room Temperature