CRANFIELD UNIVERSITY

Megan Barnett

# Implementation of In-Field Life Detection and Characterisation Techniques in Icy Environments

Cranfield Health

PhD
Academic Year: 2006 - 2010

Supervisors: Professor David C. Cullen
Dr. Jemma L. Wadham (Bristol Glaciology Centre, University of Bristol)

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## Ponderings on a PhD

To understand, and then to tell, any good story we want to know who, what, when, where, how and why. Microbiological populations in subglacial environments are no different. The "Who" involves the taxonomic classification of microbial population; from kingdom to strain level. The "What" are the active microbial populations that influence their environment, and maybe a better question to address is "How Much," hence the need for measurements of, and proxies for, microbial activity under *in situ* conditions. The "When" involves looking at glacial environments over daily, seasonal and longer timescales, as well as making inferences on the past and predictions about the future. The "Where" is the investigation into the differences and similarities in microbial populations in a variety of subglacial environments, initially on Earth and then beyond. Finally, The "How" explores questions like whether these microorganisms are autotrophic or heterotrophic and what functional pathways they use.

This work helps provide techniques to address the "Who" (taxonomic classification through real-time PCR), the "What" (detection of microbial populations by the multiple techniques), How Much (detection of enzymes), the "When" (the application of high-temporal resolution microbial monitoring in Greenland), the "Where" (Greenland and Engabreen including more detailed spatial distribution in Engabreen) and the "How" (detection of functional primers using real-time PCR). That just leaves the "Why", and if I could answer that then this thesis would be a very different one.

## Acknowledgements

Firstly I would like to thank my supervisors Prof. David Cullen and Dr. Jemma Wadham, and the Engineering and Physical Sciences Research Council (grant number EP/D057620/1), for providing me with the opportunity to conduct this research, during which I have travelled to fascinating places; making many friends and colleagues mildly envious. To Dave, who has honed my analytical skills, encouraged critical thinking, advised me on experimental design and most recently provided constant reminders to the scientific writing style. This process has been invaluable to my growth as a research scientist, and even though it was occasionally painful I am thankful for everything. To Jemma, thank you for your encouragement, guidance and enthusiasm for field work and for life.

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## Abstract

An emerging trend towards non-laboratory based biological and microbiological marker analysis is occurring in multiple sectors of science and industry. In the medical sector, these trends have demonstrated that conducting sample analyses away from centralised laboratories not only makes analyses quicker and more convenient (*e.g.* a home pregnancy test), but can offer services that are otherwise impractical (*e.g.* mobile laboratories to diagnose disease in the developing world). In the environmental sector, similar benefits, plus the ability to develop and test hypotheses, protocols and sampling strategies within a field campaign, are possible with in-field analyses. Icy environments in particular would benefit from *in situ* or in-field life detection as they are typically remote, and hence impart high logistical costs for repeated field campaigns and associated sample return with the implication that the efficiency of scientific return is poor. Unfortunately, most equipment and protocols developed for microbiological analyses in other sectors of science and industry are unsuitable for direct application to in-field use in icy environments because of poor compatibility with icy environment sample matrices and frequently inappropriate microbiological targets.

Hence within this work, two hypotheses were tested: that (i) microbiological detection in-field in icy environments is possible and through this (ii) unique and more efficient scientific studies can be conducted.

To allow for in-field detection, commercially available assays and detection instrumentation were identified that fulfilled the following criteria: microbiologically relevant targets, low detection minima and portable detection instrumentation. Seven techniques were identified and considered further (ATP bioluminescence, recombinant Factor C (rFC) lipopolysaccharide (LPS) detection, fluorescence cell counts, real-time PCR, immunoassays, hydrolytic enzyme detection and colorimetric respiration measurements). Initially ATP bioluminescence, rFC LPS detection and immunoassays were modified for field use by testing extraction protocols and adapting the manufacturer's assay protocols, and with initial demonstration at a glacial field site (Engabreen, Norwegian Arctic). Further field campaigns at the same field site included the in-field testing of the other techniques. Early within these field campaigns it was identified that the ATP extraction protocol was unsuitable for samples with high clay content, leading to laboratory based development of a field deployable extraction protocol for these samples. This protocol, with detection via ATP bioluminescence, along with rFC LPS detection, fluorescence cell counts and real-time PCR (a suite of four techniques), was subsequently used to collect microbiological data from

samples from Engabreen and an additional glacial field site in Western Greenland. Parallel development of bespoke fluidic devices was undertaken to adapt the ATP bioluminescence and rFC LPS detection life detection techniques into a more suitable in-field format than the commercially available assay.

Applying this suite of life detection techniques, revealed similar distribution of microorganisms in Engabreen system as found in other glacial systems; subglacial water sources have a lower microbial concentration than subaerial water samples ($3x10^2$ to $3x10^4$ cells.ml-1 compared to $4x10^3$ to $7x10^4$ cells.ml$^{-1}$) and within the ice samples the microorganisms are associated with sediment ($3x10^4$ to $1x10^6$ cells.ml$^{-1}$ in debris-rich glacial ice compared to $2x10^2$ to $1x10^3$ cells.ml$^{-1}$ for debris-poor glacial ice). Applying Principal Component Analysis to the Engabreen microbiological data obtained from the multiple techniques allowed for discrimination between the debris-poor and debris-rich ice (confidence >0.999), with glacial meltwater samples appearing intermediate to the two ice samples. Conducting analyses in the field in Western Greenland enabled a high temporal resolution record of the microbial concentration of meltwaters of Leverett Glacier to be collected for the first time, showing changes in the microbial load over diurnal and longer timescales.

The implementation of the suite of four techniques in two glacial field locations has demonstrated that life detection in the field in icy environments is possible. This improved scientific return by providing the ability to development sampling strategies and sample processing protocols and test hypotheses in the field. In addition in-field analysis provided the opportunity to produce unique datasets – the first high-time resolution study of microbial concentration in subglacial outlet streams. By using multiple, independent techniques, more robust data has been collected and used to provide new methods to understand the role of microbiology with icy environments. Beyond icy environments, these and other techniques, and knowledge learnt from their in-field application in this work can be applied to other extreme environments that would benefit from in-field analysis.

## Keywords

Cryosphere, *in situ* life detection, microorganisms, subglacial, ATP bioluminescence, real-time PCR, fluorescence cell counts

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# List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| ARDRA | Amplified ribosomal DNA restriction analysis |
| AS | Alex's Stream |
| ATP | Adenosine triphosphate |
| BCPA | Sodium bicarbonate, chloroform, phosphate and adenosine |
| BI | Basal Ice |
| BLAST | Basic local alignment search tool |
| BSA | Bovine serum albumin |
| CAD | Computer aided design |
| cDNA | complimentary DNA |
| CFU | Colony forming units |
| Ct | Cycle threshold |
| CV | Coefficient of variation |
| DAPI | 4',6-diamidino-2-phenylindole |
| DDTMA | Dodecyltrimethylammonium (bromide) |
| DGGE | Denaturing gradient gel electrophoresis |
| DIC | Dissolved inorganic carbon |
| DNA | Deoxyribonucleic acid |
| dsDNA | double stranded DNA |
| EBI | Engabreen basal ice |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EN | Englacial |
| ENDURANCE | Environmentally Non-Disturbing Under-ice Robotic ANtarctic Explorer |
| EPSRC | Engineering and physical sciences research council |
| EU | Endotoxin units |
| FAME | Fatty-acid methyl esters |
| FISH | Fluorescence *in situ* hybridisation |
| FS | Forest Stream |
| GC-MS | Gas chromatography-mass spectrometry |
| GS | Glacial bed Stream |
| GSS | Greenland Surface Sediment |
| HSD | Honestly Significant Differences |
| HDTMA | Hexadecyltrimethylammonium (chloride) |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| LAL | *Limulus* amoebocyte lysate |
| LED | Light emitting diode |
| LOC | Lab-on-a-chip |
| LPS | Lipopolysaccharide |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MW | Meltwater |
| NADH | Nicotinamide adenine dinucleotide (reduced) |
| NASBA | Nucleic acid based sequence analysis |
| NCIMB | National collection of industrial and marine bacteria |
| NVE | Norwegian Water Resources and Energy Directorate |
| PA | Phosphoric acid, urea, dimethylsulphoxide, adenosine and EDTA |
| PAM | Pulse amplitude modulation |
| PCA | Principal Component analysis |
| PCR | Polymerase chain reaction |
| qPCR | quantitative PCR |
| $R^2$ | Coefficient of determination |
| rDNA | ribosomal DNA |

| | |
|---|---|
| rFC | recombinant Factor C |
| RFU | Relative Fluorescence Units |
| RLU | Relative Light Units |
| RNA | Ribonucleic acid |
| RS | Research shaft |
| RT-PCR | Reverse transcription PCR |
| SD | Standard deviation |
| SEM | Scanning electron microscope |
| SEM-BSE | SEM-back scattered electrons |
| SERS | Surface enhanced Raman spectroscopy |
| SS | Shrubland Stream |
| ST | Spiral Tunnel |
| TCAPP | Trichloroacetic acid, paraquat and phosphate |
| TE | Tris EDTA |
| TEM | Transmission electron microscope |
| T-RFLP | Terminal restriction length polymorphism |

## Gene sequences

| | |
|---|---|
| dsrA | (bi) sulphite reductase |
| mcrA | methyl coenzyme-M reductase |
| mxaF | methanol dehydrogenase |
| nifH | nitrogenase reductase |
| nirK | nitrite reductase (copper) |
| pmoA | particulate methane monooxygenase |

# 1. Introduction to Thesis

This chapter introduces the body of work within this thesis by outlining the context within which this thesis is based, the hypotheses, aims and objectives of the thesis and the thesis structure. This chapter also explains the selection of field sites and introduces the life detection and characterisation techniques that have been applied to detect and characterise life in the field in icy environments within this work.

## 1.1. Context within Automated Biochemical Sensing of Icy Ecosystems Project

This thesis project was funded by the Engineering and Physical Sciences Research Council (EPSRC), grant number EP/D057620/1, as part of a multi-disciplinary project entitled Automatic Biogeochemical Sensing of Icy Ecosystems which runs from 2006 to 2011. It currently involves collaborators at over ten University Departments and Institutions (http://www.ggy.bris.ac.uk/icy_ecosystems). The primary aim of the Icy Ecosystems research project is to develop and collate a suite of instruments for high resolution monitoring of the cryosphere to address the lack of information on biogeochemical cycles in the cryosphere. The different collaborators are focussing on sensors and techniques to study the biology (microbiology), chemistry and physics of icy environments, all of which are developed to be compatible with in-field use. This thesis focuses on the in-field application and adaptation of techniques to detect and characterise microbial life in icy environments.

## 1.2. Project Hypotheses

There are two hypotheses that underlie all chapters of this thesis:

- That the detection of microorganisms in the field in icy environments is possible.
- Through this, unique and more efficient scientific studies can be conducted.

The case for in-field life detection in icy environments is outlined in more detail in Section 2.2, however it can be summarised under three main arguments: the ability to perform multiple cycles of in-field analysis within a single campaign, sample storage and transportation issues and opportunities for intelligence-led sampling. Together the first two arguments allow for the completion of unique experiments and more efficient scientific studies as they allow for the in-field development and testing of hypotheses, sampling strategies and processing protocols without restrictions on sample size and number as the samples would otherwise be predetermined before analysis. Also they reduce logistical costs through reduction of sample transport and storage. Finally, an intelligence-led choice of

samples can be made when samples need to be returned to institutional laboratories for analysis which is not presently compatible with in-field use, *i.e.* conducting preliminary in-field microbiological analysis can be used to select the most appropriate size and distribution of samples.

## 1.3. Project Aims

- To test the hypotheses that the detection of microorganisms in the field in icy environments is possible and through this, unique and more efficient scientific studies can be conducted.
- To establish a suite of techniques to detect and characterise microbial populations in icy environments.
- To adapt these techniques for use in the field in icy environments.
- To use one, or more, relevant field sites to demonstrate the above.

## 1.4. Project Objectives

To apply life detection and characterisation techniques in the field and to successfully analyse glacial samples, several objectives needed to be met.

### *1.4.1. Techniques for in-field life detection and characterisation*

- Identify a number of complementary techniques that have the potential to detect and characterise life at the low target concentrations that are typical of glacial environments.
- Identify suitable commercially available assay reagents, instrumentation and consumables and any additional equipment needed to apply these techniques and that are compatible with field deployment.
- Consider and test modifications to assay reagents, assay protocols, instrumentation and consumables that may be necessary for in-field use.

### *1.4.2. Sample processing*

- Identify suitable published protocols and commercially available kits for the extraction of targets of each technique and that are compatible with field deployment.
- Test these protocols and any adaptations that are necessary for use in-field in institutional laboratories and in the field.

- Develop suitable target extraction protocols if the published and commercially available ones are unsuccessful.

#### *1.4.3. Field testing*

- Identify field sites in icy environments appropriate to field testing of techniques.
- Test the target extraction, sampling strategies and the analytical techniques at these sites, making necessary in-field adaptations to protocols.

#### *1.4.4. Bespoke analytical devices*

- Consider a method to integrate a number of life detection techniques into a single system.
- Test the system by integrating commercially available assay reagents that were identified as suitable for in-field detection of life in icy environments.

## 1.5. Thesis Structure

The structure of this thesis is as a series of draft and submitted papers for peer-reviewed journals, and together they produce a coherent single body of work. Therefore, each chapter has an individual abstract and sections covering the introduction, materials and methods, results, discussions, conclusions and references. Figure 1-1 shows the connections between the different chapters and links them with the objectives; some objectives are covered in more than one chapter.

A brief summary of each chapter follows.

Chapter 2 “Towards in-field life detection” reviews the current techniques that have been use to detect and characterise life in icy environments (irrespective of use in-field or in institutional laboratories) and where those techniques have been used in the field, in icy environments or otherwise. This demonstrates the potential techniques that could be applied and highlights some of the advantages of in-field life detection.

Chapter 3 “ATP field development”, Chapter 4 “LPS field development” and Chapter 5 “application of multiple in-field techniques” describe the adaptation of selected techniques for in-field application and the development of sample processing, sampling strategies and procedures applied to a glacial field site, Engabreen in Northern Norway. Chapters 3 and 4 develop and test two specific techniques for in-field life detection; ATP bioluminescence and recombinant Factor C (rFC) lipopolysaccharide (LPS) detection assay, respectively, and

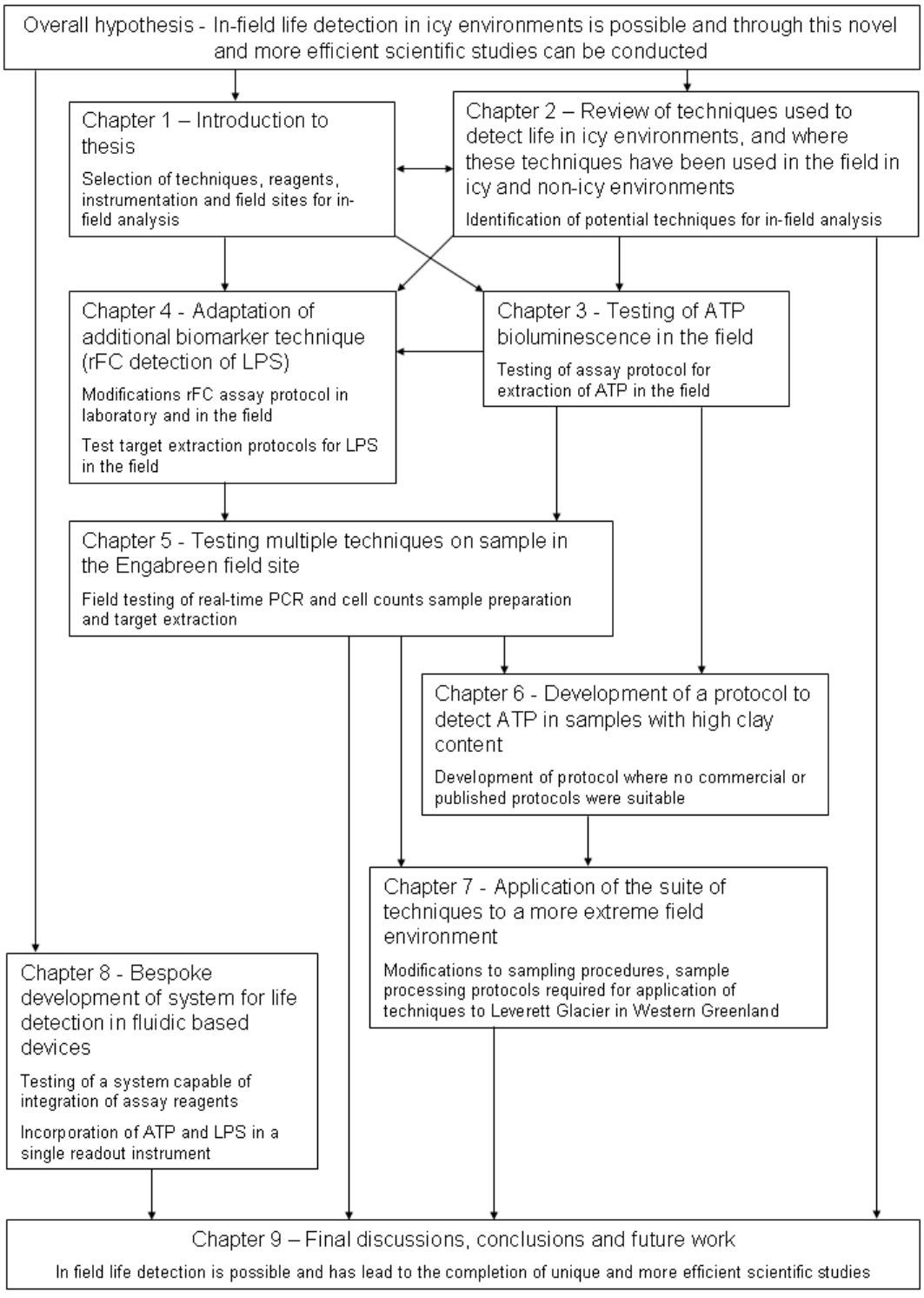


*Figure 1-1. Flow diagram demonstrating the connection of the chapters and the thesis objectives.*

demonstrate the adaptations necessary for in-field deployment specific to these techniques. They are also used to expand knowledge of generic in-field microbiological detection issues

within an icy environment context and to establish sampling strategies that are applied to other life detection techniques and in an additional field location in later chapters. Chapter 5 applies these two techniques, plus real-time PCR and cell counts to systematic analysis of glacial and water samples from Engabreen in November 2008 and April 2009.

Chapter 6 “ATP extraction protocol” addresses laboratory based development of a sample processing protocol for ATP extraction. This was developed for samples with high clay content and low biomass, and stems from problems encountered measuring ATP in the clay-rich ice and water samples in the Engabreen field site described in Chapters 3 and 5.

Chapter 7 “Greenland field detection” applies the sample processing methods and sampling strategies developed during the Engabreen field campaigns, the four life detection techniques used on the Engabreen samples in Chapter 5 and the ATP extraction protocol developed in Chapter 6 to the analysis of subglacial meltwaters from a second field site (the Leverett Glacier in South Western Greenland).

Chapter 8 “Bespoke fluidic devices” describes and demonstrates the laboratory based development of a system for the integration of multiple life detection techniques into a single format, and has been tested on ATP bioluminescence and rFC assay.

Chapter 9 contains the overall discussion, conclusions and future work of the body of work within Chapters 2 to 8 and therefore is not in the style of a peer-review publication.

Appendix A contains photographs depicting various aspects of the field work.

Appendix B contains the optimisation of the real-time PCR primers and calculation of their amplification efficiency.

## 1.6. Selection and Description of Field Sites

Two Arctic field sites were used during this project: Engabreen in Northern Norway (note a translation from the Norwegian of Engabreen is “the Enga glacier” and therefore throughout the thesis the term “Engabreen” is used rather than “Engabreen Glacier”) and the Leverett Glacier in South Western Greenland. Both field sites were used as they provided locations suitable for the development and testing of equipment and protocols required in this project. An overview of the field sites through selected photographs in included as Appendix A.

Engabreen is an outlet glacier of the Svartisen Icecap which straddles the Arctic Circle in Northern Norway. A unique aspect of Engabreen is the Svartisen Subglacial Laboratory, a facility run by the Norwegian Water Resources and Energy Directorate (NVE) which

provides access to pristine subglacial samples and provides laboratory space, electricity and running water within 100 m of the glacier access point. Engabreen was an ideal location to explore the challenges involved in equipment and reagent transportation and to test the equipment, techniques, sampling strategies and sample processing protocols in the field on glacial samples, as through the Svartisen Subglacial Laboratory basic laboratory facilities are available on-site. The field development and testing was conducted during three one-week field campaigns in November 2007, November 2008 and March 2009. One additional field campaign was conducted in April 2006 prior to the start of this thesis; some data from this is included in Chapter 3.

The Leverett Glacier is a land terminating glacier draining the western margin of the Greenland Ice Sheet. A field camp was established during summer 2009 on land 1 km from the snout of Leverett Glacier to allow for easy access to the glacier surface and the subglacial river that drains the glacier. All microbial analyses were conducted in a dedicated laboratory field-tent. The Leverett field camp allowed for the testing of the techniques used in Engabreen in more extreme field conditions, including challenges of non-temperature controlled laboratory and in-field storage of samples (up to 8 days) and reagents (up to 9 weeks). As the Leverett field campaign was significantly longer than Engabreen field campaigns, multiple cycles of analysis could be conducted, hence allowing the advantages of in-field development of hypotheses, sampling strategies and protocols, as outlined in the case for in-field analyses, to be tested.

## 1.7. Selection of techniques for the thesis

During the course of this thesis seven life detection and / or characterisation techniques were identified as suitable for in-field studies, developed in the laboratory and tested at the Engabreen field site. The seven techniques had to comply with the following four criteria: low, lower limit of detection, commercially available assay reagents, portable (*i.e.* relatively robust and light) detection instrumentation and field deployable target extraction techniques. These seven techniques are listed below along with their targets, the basic premise of the technique, the manufacturer stated detection limit of the assay (where appropriate) and the detection instrumentation.

**ATP bioluminescence**

ATP is used as a measure of generic extant life as it is found in every living cell. In this thesis ATP was detected using an ATP bioluminescence assay which relies on the enzyme and

substrate responsible for the reaction which emits light in the tails of fireflies. As light emitted by this reaction is proportional to ATP concentration, purified enzyme and substrates are used to quantify ATP concentration in a sample. The assay kit used in this study can detect ATP down to $10^{-12}$ M, which is equivalent to $3x10^3$ to $4x10^4$ cells.ml$^{-1}$ (Fairbanks *et al.* 1984). Detection of the luminescent endpoint of this ATP bioluminescence assay was conducted using a handheld luminometer.

**Recombinant Factor C (rFC) lipopolysaccharide (LPS) detection assay**

LPS is a significant component of the cell wall of Gram-negative bacteria and can cause toxic shock in animals. The rFC assay is based on the LAL (*Limulus* amoebocyte lysate) assay. This uses a defence mechanism in horseshoe crabs (*Limulus*) where LPS activates a clotting cascade. The LPS sensitive enzyme (Factor C) has been isolated from the hemolymph of horseshoe crabs and a recombinant form produced. A commercial assay has been developed where this rFC cleaves a fluorescence substrate in the presence of LPS. The lower limit of sensitive of the rFC assay is 0.01 EU.ml$^{-1}$ which approximates $10^2$ Gram-negative bacterial cells.ml$^{-1}$ (Mueller *et al.* 2004 and La Ferla *et al.* 2004). Detection of the fluorescence product of the assay was conducted using a handheld fluorometer.

**Real-time PCR**

The quantitative or relative concentration of a nucleic acid sequence can be detected by simultaneous amplification and detection of that sequence using real-time PCR. The target nucleic acid sequences are selected by using a pair of oligonucleotide primers which correspond to the ends of the sequence of interest. In this thesis DNA sequences for taxonomic and functional pathways were detected in relative concentrations. Targets and the development of real-time PCR conditions are given in Appendix B. Detection was conducted using a small laboratory thermal-cycler with an integrated real-time PCR readout unit.

**Fluorescent cell counts**

Standard technique for enumerating total bacterial concentration. Cells were stained using a generic nucleic acid stain and manually counted by a fluorescence microscope that was manufactured for field use.

### Immunoassays

Enzyme-linked immunosorbent assay (ELISA) format was used to detect GroEL (a bulk protein) and *Staphylococcus aureus* (to test for contamination) as examples of a protein and a microbial target. Detection of the colour change was using a manual optical absorbance strip reader[*].

### Hydrolytic enzyme activity

The presence of a target enzyme in a sample cleaves the substrate releasing a fluorescent product (Marx *et al.* 2001). The enzymes acid phosphatase and sulfatase were tested. Detection of the fluorescence product was with a handheld fluorometer used in the rFC assay.

### Colorimetric respiration measurements

Respiration is measured by changes in $CO_2$ concentration that are detected by a pH sensitive dye incorporated into agar. The sample and dye were sealed, and changes in the colour were detected using a manual strip reader used to read the immunoassays.

The first four techniques were systematically applied to in-field detection within ice and water samples from Engabreen and surrounding environment and in subglacial and supraglacial water samples of the Leverett Glacier. Immunoassays were not pursued as neither target could be detected in any of the glacial or periglacial samples tested, and immunoassays were more complex to conduct than the ATP and LPS detection techniques trialled during the same field campaign. The hydrolytic enzyme activity and colorimetric respiration measurements were successful but not pursued due to time constraints; they were only tested during the final, March 2009, Engabreen field campaign.

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[*] BioTek Manual Microplate reader EL301E was used, the details are included here as the instrument is not described further in subsequent chapters

# 2. Towards In-Field Life Detection and Characterisation in Icy Environments

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## Abstract

The historic view of icy environments has been predominantly lifeless; this standpoint has been overturned in the past 20 years as studies have started to reveal the fundamental importance of microbial life as an integral part of the cryosphere. Our present knowledge of the distribution, taxonomy and metabolic activity of microbial life in icy environments has been learnt primarily from laboratory-based analyses of collected field-samples. To date, only a few life detection and characterisation techniques have been used in the field in icy environments. Examples include the detection of biomarkers (such as adenosine triphosphate (ATP)) and metabolism measurements (through the uptake of radio-labelled isotopes). On-going improvements in technology mean smaller and more robust life detection and characterisation systems are being built, making them suitable for in-field use. It is the view of the authors that the ability to perform life detection and characterisation in the field, in icy environments, is expected to improve the efficiency of data generation and scientific studies.

Twenty five techniques are reviewed here that have been used to detect and characterise microbial life in icy environments. These include microscope techniques, analysis of cell physiology, detection of biomarkers, analysis of nucleic acids and chemical and physical based sensors. Of these techniques, only nine have been reported to have been used in icy field environments and a further three in non-icy field applications. A further review follows of the established and emerging technologies in other areas of science and industry to show the future potential of in-field life detection and characterisation in icy environments. Of the

current techniques molecular based technologies (including examples such as qPCR), developments of miniaturised fluorescent microscopes and Raman spectroscopy-based technologies look to be the most immediately promising due to the current level of technology development and applicability of the technique. Furthermore, we consider potential icy environments that will benefit from in-field life detection, such as the forthcoming exploration of Antarctic Subglacial Lake Ellsworth.

In conclusion, using technological developments in other scientific and industrial sectors as resources for in-field life detection technologies, the range of techniques that can be applied to icy environments should increase in the future. In turn, these in-field studies and *in situ* technology development should lead to an increase in the abundance and diversity of information available on life in icy environments, as well as the ability to explore new icy environments that are currently difficult to access.

## Keywords

*In situ* analysis, icy environments, cryosphere, psychrophiles, portable technology, in-field

## 2.1. Introduction

Much of the Earth's biosphere[†] is perennially or seasonally cold (Laybourn-Parry 2009). Over the past twenty or so years there is a realisation that throughout the cold biosphere, life, in particular microorganisms, is prevalent and capable of metabolism (Price & Sowers 2004). Within the cold biosphere, icy environments[‡] typified by the large ice sheets of Antarctica and Greenland, polar regions, perennial alpine-type snow and glaciers, permafrost and sea ice, are all of great topical interest. The interest arises for a variety of reasons which include: (i) climate change covering historical, current and future changes, (ii) study of life in extreme conditions and (iii) survival of life on early Earth and elsewhere in the Solar System and beyond – for example Mars, Europa and extra-solar planets.

In the past, icy environments have reacted quickly and dramatically to changes in external forcing (Zachos *et al.* 2001). The mechanisms for these changes are not fully understood

[†] This ignores the emerging idea of a deep hot biosphere being a significant component of the Earth biosphere.

[‡] Throughout this document, readers can interchange the use of the terms "icy environment" and "cryosphere" although for consistency, the term "icy environment" will be used here.

however there is an increasing realisation of the potential for a significant microbial contribution (Laybourn-Parry 2009). Therefore only by studying the biological influences on the cryosphere, and integrating these with physical and chemical influences, can the behaviour of icy environments be more fully understood.

To understand the role of microorganisms in icy environments, techniques are needed to study the presence, abundance, diversity and activity of microorganisms, *i.e.* to detect and characterise life. The first technique that attempted to study microbial life from icy environments was culturing, where samples from icy environments were exposed to an artificial culture media (McLean 1918). These cultures were grown at temperatures that were much higher than those found in the field, as it was assumed at that time that any native microorganisms must be dormant. It was not until 1960s that the first bacteria from icy environments were studied under *in situ* temperature conditions and were shown to be capable of growth, hence being an intricate part of their environment (Straka & Stokes 1960). Culturing samples from icy environments is still an important part of microbial analysis, however is now complimented by culture independent methods such as DNA analysis, microscopy and biomarker detection. These techniques can be used to provide a wide variety of microbiological information on samples collected in the field and analysed in institutional laboratories.

An alternative to transporting samples to institutional laboratories is to transport appropriate equipment and reagents to the field to conduct the analytical techniques nearer the sampling locations. Within the context of this review, we have three sub-locations within in-field analysis: in-field laboratories *i.e.* minimal sample transportation is required, the sampling site, *i.e.* without the need for any sample transportation; and *in situ*, where the target is measured in its original position. Being able to analyse samples in the field should not only compliment techniques already being conducted on returned samples, but bring additional benefits including novel scientific studies.

Objectives of this review are:

- To state the case for the in-field use of life detection and characterisation techniques in icy environments.
- To review the current techniques used to detect and characterise microbial life in icy environments.
- To review examples of analyses where steps have been made towards in-field analysis.

- To identify potential analytical techniques being developed or implemented in all areas of science and industry that could be appropriate for in-field measurements in icy environments.

## 2.2. A Case for In-Field use of Life Detection and Characterisation Techniques in Icy Environments

The section is divided into three major sub-sections, each of which encompass various aspects of one or more of the following; science issues, logistical issues, reduction of environmental impact and efficient use of resources.

### *2.2.1. Multiple cycles of in-field analysis*

If the timescale for the completion of an analytical technique is short compared to the length of a given field campaign, then multiple cycles of analysis can be completed. Thus, the analytical results gained from the early cycles permit for the development, refinement and testing of hypotheses, sampling strategies and sample processing protocols during the later cycles. In addition, multiple cycles of analysis allow for the rapid exploitation of opportunistic or serendipitous discoveries.

Refinement of sampling strategies and sample processing protocols in the field is of particular importance with novel scientific studies, *e.g.* testing new techniques and / or analysis of established techniques in new environments and on new sample types. The unknown aspects of novel studies limit the ability to define the final sampling and sample processing protocols prior to a field campaign. Therefore an ability to perform multiple cycles of in-field analyses allows the initial analysis cycles to be used to refine and finalise the sampling strategies and sample processing protocols. The remaining cycles can then be used to perform the scientific study.

During data analysis, hypotheses are frequently refined and new ones formed; often requiring the collection and analysis of additional samples. By conducting multiple cycles of analysis in the field, the hypotheses that are developed after earlier cycles of analysis can be tested within the same field campaign, improving the efficiency of data collection regarding scientific return for a given resource usage.

Due to the instability of some icy environments, re-sampling of the same environments may not be possible if delayed to a second field campaign, *i.e.* there will be a significant environmental change over a timescale of months to years. The ability to perform multiple cycles of in-field analysis optimises the scientific return from that particular field site as the

hypotheses can be development and tested in the field reducing the requirement or number of additional field campaigns.

### *2.2.2. Sample storage and transportation issues*

Conducting analyses in remote in-field locations reduces the turnover time between sample collection and final analyses. Hence, reducing the physical stresses that samples undergo prior to analysis. This sample perturbation could contribute to the degradation of targets, changes in the physiological state of cells and / or changes in the structure of the microbial community, and may require additional investigations into quantifying these effects. By reducing turnover time these changes are limited and, in addition, unstable targets may be measured.

In-field analysis can increase temporal and spatial resolution of sampling, as sample size and number may otherwise be limited because of the logistical costs involved in sample return. This is of particular importance in icy environments as large samples may be required for analysis due to the low biomass and hence target concentration in some icy environmental samples.

For those techniques which are completed *in situ* (*e.g.* incubation experiments), contextual parameters (*e.g.* water activity, light levels, temperature and other chemical and physical parameters) are preserved. As these parameters can impart a significant influence on microbial behaviour and the simulation of all of these conditions is difficult to reproduce in institutional laboratories, *in situ* analysis allows for the inclusion of the influence of these parameters.

Furthermore, samples for microbial analysis from icy environments are typically transported and stored cold (<4°C) or frozen. When comparing to the transportation of equipment suitable for in-field analysis, this specialised transportation to institutional laboratories can be costly. Hence reducing this requirement by analysing samples in the field can substantially reduce logistical costs.

### *2.2.3. Intelligence-led sampling*

For techniques that are currently not suitable for in-field deployment, preliminary sample analysis can be conducted in the field to enable the most appropriate choice of samples for subsequent analysis in institutional laboratory. Typically this would involve the use of in-field analysis to assess the overall biological context for sampling; including the assessment of microbial abundances and their spatial and temporal distribution. This would lead to an intelligence-led choice of samples and help clarify the most appropriate number and size of

samples. Such preliminary in-field analyses can have two additional benefits: to enable the quantification of sample degradation of certain targets during transportation by conducting identical analyses pre and post transportation; and to ensure a basic level of scientific return in case of sample damage during transportation.

## 2.3. Current Institutional Laboratory Techniques to Detect and Characterise Life in Icy Environments

The following section reviews the current techniques used to detect and characterise microbial life in samples from icy environments. This will provide the basis from which to consider further the potential for in-field analysis. Frequently complimentary techniques are used to study microbial life to increase confidence in the interpretation of results (Cowan *et al.* 2002, Pearce 2003, Gilichinky *et al.* 2007). For simplicity, in this work we consider the techniques individually. Table 2-1 summaries the advantages and disadvantages of the techniques and their current status regarding in-field analysis.

### *2.3.1. Microscopy*

Early studies of microbial life in icy environments involved classification of bacterial cultures using microscopes at visible wavelengths (McLean 1918, Darling & Siple 1941, Marshall & Ohye 1966). These microscopes have also been used to make inferences on activity, through the motility of cells and evidence of dividing cells. The use of fluorescent stains and the development of electron microscopes increase the scope and the detail that can be gathered using microscopy and therefore remains an important tool in microbial investigations.

#### *2.3.1.1. Fluorescence microscopy*

Some compounds produced by microorganisms will naturally fluoresce under certain excitation wavelengths, for example NADH and chlorophyll. By selecting appropriate excitation and emission wavelengths with a fluorescence microscope, these compounds can be identified. Kaštovská *et al.* (2007) used the autofluorescent property of chlorophyll to enumerate concentrations of cyanobacteria and eukaryotic microalgae from two Arctic glaciers. In addition, as the chlorophyll from cyanobacteria and microalgae differ in their maximum excitation and emission wavelengths the cells can be readily distinguished.

The majority of fluorescence microscope techniques involve the addition of nucleic acid binding dyes. These dyes either only fluoresce if bound to the target molecule, or the unbound dye is removed prior to analysis. Several fluorescent dyes have been used to enumerate bacterial concentrations in icy environments: 4',6-diamidino-2-phenylindole - DAPI (Collins

*et al.* 2008, Kaštovská *et al.* 2007), acridine orange (Sharp *et al*. 1999) and the SYBR® (Invitrogen, Corporation, CA, USA) group of stains (Pearce *et al.* 2007). All of these dyes bind to double-stranded DNA and some also bind to RNA (acridine orange and DAPI) and readily travel through membranes thus identifying live and dead bacteria and viruses (Pearce *et al.* 2007). Other than preceding examples of direct microbial enumeration, microscope analysis using fluorescent stains has been applied to samples from icy environments to estimate biomass through measurements of cell size (Säwström *et al.* 2002), estimate microbial activity by counting dividing and recently divided cells (Fabiano & Danovaro 1998, Sharp *et al.* 1999) and differentiate between live and dead cells using different stains (Sheridan *et al.* 2003, Zhang *et al.* 2008).

As opposed to generic staining of all DNA, domain to species taxonomic identification of microorganisms is possible using fluorescence microscopy; fluorescence *in situ* hybridisation (FISH) (Amann *et al.* 1990). FISH uses short nucleotide sequence (oligonucleotide) probes that will bind/hybridize to the complimentary DNA or RNA sequence if present in the target cell. During synthesis the probes are either tagged with fluorescent dye, which allows for direct visualisation, or tagged with an antigen, to which antibodies conjugated to fluorescent dyes will bind. Brinkmeyer *et al.* (2003) used established domain and group specific oligonucleotide probes with FISH to compare assemblages of Arctic and Antarctic pack ice. Pearce (2003) used generic probes for archaea and bacteria and four probes for different classes of bacteria to analyse community structure in an Antarctic oligotrophic lake, and was able to assign between 49.4 and 84.0% of hybridized cells to known bacterial groups.

In addition to the identification and enumeration of bacteria, fluorescence microscopy has been used to study the behaviour of microorganisms within ice. The partitioning behaviour of microorganisms has been studied in model (Mader *et al.* 2006) and real samples (Junge *et al.* 2001). Thus, confirming the hypothesis that bacteria are most frequently associated with sediments, and concentrated in liquid veins within the sea ice (Price 2000).

#### *2.3.1.2. Electron microscopy*

The development of electron microscopy, both transmission and scanning, allows microorganisms to be studies in finer detail than with light microscopes. A transmission electron microscope (TEM) directs a beam of electrons at an ultra thin sample. At magnifications suitable for identifying microorganisms the beam of electrons is absorbed by the sample and the resulting image shows contrast that is sensitive to sample thickness and

material composition. The higher resolution achievable with TEM has allowed for the enumeration of virus-like particles in an Antarctic island freshwater lake (Wilson *et al.* 2000). To enhance the contrast in samples de los Ríos *et al.* (2004) stained samples for TEM (with lead citrate) to determine the ultrastructure of microorganisms, the interactions of living and degraded cells, and their relationship to biofilms in Antarctic rocks.

Scanning electron microscopes (SEM) create images by using interactions between an electron beam and electrons at the surface of a sample. Tung *et al.* (2006) used standard SEM to create topographic images of carbon coated microorganisms in Greenland basal ice, the detailed topography was then used to identify types of microorganisms and their association with clay minerals in melted basal ice. Back scattered electrons are collected from SEM (SEM-BSE) as they provide information on the chemical composition of imaged surface. SEM-BSE has been used to study endolithic communities in Antarctic rocks, revealing details of bioweathering (Ascaso & Wierzchos 2003) and characterising biogenic magnetite (Wierzchos *et al.* 2003).

The electron microscopy techniques described above require dry samples. As living cells have high water content, samples require treatment before imaging. This is generally completed by replacing the water with an organic solvent before embedding in resin, however this can result in a modification in the structure of the biological material. De los Ríos *et al.* (2007) studied Antarctic biofilms using low temperature SEM (*i.e.* shock freezing samples then analysing the samples cold) as this is reported to cause fewer changes to biological material than drying with an organic solvent thus more representative of the original sample.

### *2.3.2. Cell physiology & biochemistry*

#### *2.3.2.1. Cell culture*

Culturing can be attempted on any type of sample, normally by exposing the sample to a nutrient rich media, however media are frequently diluted or specially designed due to low nutrient composition of the microorganisms in their natural environment (Christner *et al.* 2000, Junge *et al.* 2002, Foght *et al.* 2004, Liu *et al.* 2009). By incubating the samples at different temperatures culturable psychrophilic and psychrotolerant isolates have been identified (Christner *et al.* 2000).

As these isolated microorganisms are viable and exhibit adaptations to cold conditions (Margesin *et al.* 2007), they are frequently used in model systems to study the behaviour of microorganisms relevant to icy environments (Mader *et al.* 2006). Viable, pure cultures of

microorganisms are required by some techniques for further identification, such as nucleic acid sequencing (Xiang *et al.* 2005, Miteva *et al.* 2005), ARDRA (Miteva *et al.* 2005) or proteomic studies (Goodchild *et al.* 2004).

#### *2.3.2.2. Use of labelled substrates*

To assess rates of metabolism under given conditions, the uptake of labelled substrates can be measured in a closed system over a given incubation time. Specific parameters, *e.g.* temperature and light levels, have been controlled in laboratory experiments (Rivkina *et al.* 2000, Sala *et al.* 2005), or the sample is returned to collection point hence incubating under *in situ* conditions (Mock & Gradinger 1999, Laybourn-Parry *et al.* 2004, Hodson *et al.* 2007, Anesio *et al.* 2009). These substrates can be labelled with fluorophores (Sala *et al.* 2005) or more frequently radiogenic isotopes (Rivkina *et al.* 2000, Säwström *et al.* 2002, Mock 2002, Laybourn-Parry *et al.* 2004, Kirchman *et al.* 2005). To assess microbial metabolism in samples from icy environments, the uptake of amino acids (leucine – Sala *et al.* 2005, Hodson *et al.* 2007), nucleosides (thymidine – Laybourn-Parry *et al.* 2004), sodium bicarbonate (Mock & Gradinger 1999, Anesio *et al.* 2007) and acetate (Rivkina *et al.* 2000) have been measured. These studies have revealed active microbial populations in all studies, with the inclusion of labelled acetate down to -20°C (Rivkina *et al.* 2000).

#### *2.3.2.3. Lipid analysis*

The total lipid content of a sample has been used as a proxy for microbial load in Antarctic mineral soils and was assessed through gravimetric measurement after extraction (Smith *et al.* 2006). By studying the details of the lipids present in a sample, microbial biomass can be characterised, most commonly by analysis of phospholipids (a major component of microbial membranes). Phospholipid analysis is completed by the formation of fatty acid methyl esters (FAME), and then their separation by gas-liquid chromatography. As microbial species have characteristic phospholipids analysing pure cultures can lead to inference of relationships based on the similarities and differences of FAME profiles (Mergaert *et al.* 2001, Van Trappen *et al.* 2002). Additionally, FAME profiles of the total community can be constructed to compare microbial assemblages (Kaštovská *et al.* 2007).

In addition to total lipid content and phospholipid analysis, samples can be characterised by using various lipids as biomarkers. Jungblut *et al.* (2009) performed lipid biomarker analysis in meltwater ponds on the McMurdo Ice Shelf; studying fatty acids, wax esters, hopanols, sterols, hydrocarbons and ether-linked components. Detection of lipids involves several stages

of extraction and separation before detection with gas chromatography-mass spectrometry (GC-MS). Some of these lipids are characteristic of extant microbial groups, such as ester-linked components in archaea, and some are stable products of microbial degradation and hence used to detect extinct life, such as hydrocarbons and steranes.

#### *2.3.2.4. Detection of biomarkers*

Methods for the detection of biomarkers described within this section are based on the use of assay reagents extracted, purified and/or modified from organisms. In all cases, the assay reagents molecularly recognise the biomarker of interest and produce a detectable response to the recognition.

Adenosine triphosphate (ATP) is present in all known cells and, due to its rapid hydrolysis in the natural environment, it represents extant life. ATP can be detected with a one-step assay using extracts of the tails of fireflies that contain an enzyme (luciferase) and substrate (luciferin) that in the presence of ATP cause bioluminescence. The assay, using purified firefly extract, emits light in proportion to ATP concentration; the light is then detected by a sensitive luminometer. Cowan *et al.* (2002) used ATP bioluminescence to estimate microbial biomass in the Antarctic Dry Valleys, the range of ATP values were equivalent to $10^5$ and $10^9$ cells $gram^{-1}$ sediment.

Lipopolysaccharides (LPS) are an important component of the cell wall of Gram-negative bacteria and can be detected using the *Limulus* amoebocyte lysate (LAL) assay. The LAL assay uses extracts of the horseshoe crab's (*Limulus*) hemolymph, which is sensitive to LPS in nanogram concentrations (Bang 1956). Commercialised LAL assays have various detectable endpoints, depending on the product, and include turbidity and colorimetric. The LAL assay has been used to estimate Gram-negative microbial biomass in the refrozen zone of Subglacial Lake Vostok ice core showing Gram-negative bacteria constitute about 36% of total bacterial population (estimated from direct microbial enumeration with fluorescent dyes – Karl *et al.* 1999). A recently commercialised LAL-based assay (PyroGene® rFC endotoxin detection system, Cambrex Corporation, USA) has been used to detect LPS in subglacial samples (author's unpublished data). The LPS sensitive protein from the LAL assay is conjugated to a fluorescent dye (Ding & Ho 2001). The substrate cleaves in the presence of LPS, releasing the fluorescent product in proportion to LPS concentration.

Immunoassays rely in the specificity of antibodies (either natural or synthetic) to detect a particular target. Wood *et al.* (2008) quantified two toxic chemicals produced by

cyanobacteria in Antarctic microbial mats by two different immunoassays, an enzyme-linked immunosorbent assay (ELISA) and a protein phosphatase inhibition assay. These immunoassays provide a standard format in which to set up and analyse the target, allowing for the development of new and target specific assays.

#### *2.3.2.5. Techniques for assessing biogeochemical cycling*

Isotopic composition in microbially important chemicals can be detected via gas chromatography and / or mass spectrometry, and their ratios are used to infer information about microbial processing. Microorganisms preferentially absorb lighter isotopes therefore compounds of biological origin will have isotope ratios distinct from abiotic material of the same source. There are abiotic methods to enrich samples in light isotopes, however these processes fractionate isotopes by different magnitudes and the elements are affected differently. Therefore detection of isotopic ratios from different elements can allow for differentiation between biotic and abiotic origins of the isotopic shift. Wadham *et al.* (2004) measured the isotopic ratios of sulphur and oxygen in $SO_4$ dissolved in glacial meltwater, and the carbon isotope ratios in dissolved inorganic carbon (DIC) to elucidate source of $CO_2$ in subglacial upwelling water. The enrichment in the lighter isotope in all elements implied microbially mediated processes at the glacier bed, specifically sulphate reduction and microbial oxidation. Also shifts in nitrogen isotope ratios in meltwaters from a glacier in Svalbard suggest an excess of microbial nitrate (Wynn *et al.* 2007). Light carbon isotope ratios in methane in permafrosts have been used to confirm a biological origin of the methane, which Rivkina *et al.* (2007) inferred to be most likely due to the presence of active methanogenic archaea.

Measuring concentrations and fluxes of microbially important chemicals can be used to indicate microbial influence in a system. Due to the studies completed on icy environments they have been divided into three different techniques for the purpose of this review: oxygen concentrations, gas fluxes and nutrient concentrations.

The oxygen concentration can be used to measure the rate of community respiration and photosynthesis. Three different oxygen sensors have been used to detect oxygen concentrations in icy environments: microelectrodes, dissolved oxygen meters and micro-optodes. Oxygen reacts with the cathode of microelectrodes producing an electrical signal proportional to oxygen concentration, and hence consumes oxygen at the tip. McMinn *et al.* (2000) and Trenerry *et al.* (2002) deployed oxygen microelectrodes under sea ice to measure

the structure of diffuse boundary layer, as changes in photosynthetic activity can induce changes in the structure of this layer. Both dissolved oxygen meters and micro-optodes rely on the luminescence quenching property of oxygen on certain chemical dyes, hence do not consume oxygen at their tip. Dissolved oxygen meters have been used to measure changes in dissolved oxygen concentration in dark incubation experiments to measure community respiration in cryoconite holes (Hodson *et al.* 2007, Anesio *et al.* 2009). Micro-optodes have been deployed within restricted brine channels in sea ice mesocosms to detect changes in oxygen concentrations (Mock *et al.* 2002) indicating most of oxygen production in the sea ice mesocosm was from diatoms.

Greenhouse gas fluxes can be measured by creating a sealed headspace around the area of interest and measuring changes in gases in the headspace. Sommerkorn (2008) used an infrared based gas-exchange instrument to measure $CO_2$ flux to calculate respiration rates in Siberian permafrosts. Using this technique to investigate the response of changing temperature and water table on $CO_2$ flux to the atmosphere indicates that the cold and wet permafrost were most sensitive to external changes of the types of permafrost tested. Along with $CO_2$ flux measurement of $N_2O$ flux, by collecting discreet samples and later analysing by gas chromatography, has been used to study contribution of nitrifiers and denitrifiers to $N_2O$ production in High Arctic soils, suggesting that bacterial denitrification is the dominant process occurring in these soils (Ma *et al.* 2007).

Characterising nutrient concentration in the water in one part of a system can be used to infer processes in otherwise unreachable areas of the system. Measurements of nutrients, via established laboratory methods, in samples from subglacial meltwaters have been used to infer microbial metabolism in subglacial environments, *e.g.* sulphide oxidation (Sharp *et al.* 1999, Bottrell & Tranter 2002). Hodson *et al.* (2005) studied nitrogen and phosphorus, amongst other chemical and nutrient species of two Arctic glaciers. The imbalance of nutrients was used to propose that microbially mediated processes are operative at the glacier bed.

### *2.3.3. Molecular biology*

The majority of molecular techniques used to study life in icy environments concentrate on the analysis of nucleic acids, targeting both DNA and RNA.

Note that fluorescent *in situ* hybridisation (FISH), where oligonucleotide probes bind to specific sections of nucleic acids to classify microorganisms has been described with the microscopy techniques in Section 2.3.1.1.

#### *2.3.3.1. Nucleic acid amplification*

All subsequent nucleic acid techniques described here require amplification of a selected section of the DNA or RNA sequence. Although multiple methods for amplification of nucleic acids exist, polymerase chain reaction (PCR) has, to date, been used to amplify medium to long chain nucleotide sequences in icy environments exclusively of other techniques. The amplification of oligonucleotide sequences has been conducted by rolling-circle amplification for subsequent analysis with DNA microarrays (Yergeau *et al.* 2007).

PCR amplifies a specific sequence of DNA through multiple three-stage amplification cycles, which theoretically doubles the amount of DNA per cycle. The sequence is selected by designing a pair of oligonucleotide primers that corresponds to the ends of sequence of interest. PCR amplifies DNA however RNA can be studied by first transcribing the RNA to its complimentary DNA (cDNA) by reverse transcription PCR (RT-PCR), and then amplifying this DNA as described previously (Yergeau & Kowalchuk 2008).

Standard PCR cannot be used as a stand alone technique for nucleic acid analysis as the PCR products need to be analysed, thus is generally the first step for further nucleic acid analysis. However inclusion of fluorescent product in the standard PCR protocol and thermal cyclers with fluorescence detection capabilities allow for simultaneous amplification and measurement of PCR product (real-time PCR). The DNA is either detected by the inclusion of a generic fluorescent dye that binds to all DNA (most commonly SYBR® green) of a sequence specific fluorescent oligonucleotide probe during amplification (Shipley 2006). Real-time PCR can be used to quantify the DNA copy number of unknown samples by including a dilution series of target DNA of known concentration (real-time qPCR). Segawa *et al.* (2005) used species specific primer pairs, with real-time qPCR to show an increase in microbial load of snow in Japanese mountains. Yergeau & Kowalchuk (2008) used real-time qPCR with universal primer pairs and primer pairs for functional genes to study the effect of freeze-thaw cycling on Antarctic microbial soil communities showing different responses of bacteria and fungi.

*2.3.3.2. Nucleic acid characterisation*

This section covers the methods used to analyse the products of nucleic acid amplification, *i.e.* DNA. This DNA can be simultaneously amplified and detected by real-time PCR which has been covered in the previous section. Some of the techniques described in this section do not require prior amplification of DNA, however due to the low microbial concentration in samples from icy environments nucleic acid amplification has been conducted prior to analysis on these samples.

**Gel electrophoresis**

Gel electrophoresis separates nucleic acid fragments according to their size by applying an electric current along a gel. Running PCR products on a gel is the simplest method to confirm amplification of nucleic acids of the appropriate size by including a nucleic acid ladder of DNA of known molecular weights in the gel. Gel electrophoresis has also been used to purify the nucleic acid sequence prior to further analysis (Smith *et al.* 2006). Denaturing gradient gel electrophoresis (DGGE) is used to further separate similar sized DNA fragments, as small sequence changes are sensitive to the increasing denaturing conditions in the gel, and cause melting of the DNA fragments which impedes progress. Mosier *et al.* (2007) used DGGE on a depth profile in perennial lake-ice samples from Antarctic Lake Vida where changes in microbial assemblages were reflected in changes in the DGGE banding patterns, as bands in the same positions in all samples are likely to represent phylotypes common to all the assemblages. These bands can then be used in further identification of phylotype, *e.g.* extracting DNA from gel and sequencing (Wartiainen *et al.* 2003).

**Clone libraries**

The creation of a clone library from a sample allows for the analysis of nucleic acids from the entire community and avoids issues of selectivity by culturing. Firstly, the DNA region of interest is amplified from extracted community DNA. The PCR products, containing the complete range of sequences, are then inserted into host bacteria which will produce colonies containing the required DNA sequence for further analysis. These host bacteria can be grown rapidly. The inserted sequence of DNA is then extracted and purified prior to further analysis, for example through restriction enzymes (Sjöling & Cowan 2003, Taton *et al.* 2003) or sequencing (Alekhina *et al.* 2007).

**Restriction enzymes**

Restriction enzymes cut PCR products at a specific short nucleotide sequence. Differences in the location of these restriction sites create restriction fragments from the original amplified product, and have been used to characterise microbial assemblages. Terminal restriction fragment length polymorphism (T-RFLP) fluorescently labels one of the PCR primers and then the amplification products are subjected to one or more restriction enzymes (two in Bhatia *et al.* 2006). The resulting mixture of DNA fragments are separated on a gel and imaged fluorescently, hence only the bands with terminal ends are visible. The diversity of both 16S rDNA and rRNA has been measured this way in Antarctic Subglacial Lake and high Arctic (Priscu *et al.* 1999 and Bhatia *et al*. 2006, respectively). The number of distinct bands gives a measure of the diversity of the environment and by comparing the position of the bands two or more microbial assemblages can be easily compared. Bands in the same position on the fluorescence images are generally interpreted to represent microorganisms common to assemblages.

Amplified ribosomal DNA restriction analysis (ARDRA) exposes amplified DNA from a pure DNA sequence from cultures or clone library to restriction enzymes, producing a unique profile for that microorganism. The unique set of DNA fragments created by the restriction enzymes is subsequently separated on a gel, where each fragment creates an individual band. The distribution of bands is then compared and can be used to assess the diversity of the cultures or clones from a particular library (Zeng *et al.* 2007), and are frequently used to identify the number of unique microorganisms in that community which are then analysed further using DNA sequencing (Sjöling & Cowan 2003, Taton *et al.* 2003, Miteva *et al.* 2005).

**DNA sequencing**

DNA sequencing provides the most accurate method for the phylogenetic classification of microbial species. DNA sequencing has been carried out on cultured isolates (Bowman *et al.* 1997, Shi *et al.* 1997, Ravenschlag *et al.* 1999, Christner *et al.* 2001, Brinkmeyer *et al.* 2003, Xiang *et al.* 2005) and on clone libraries (Alekhina *et al.* 2007, Liu *et al.* 2009), and have been applied to samples from sea ice, glacial ice, subglacial lake ice and glacial meltwaters. These sequences are then compared to databases of known sequences (BLAST database) and the similarities between the new and known species can be calculated and visualised by phylogenetic trees. This allows for the affiliation of sequences to existing species or

identifying their closest relatives. By studying bacterial assemblages from different icy environments, common species and phylotypes can be identified, leading to studies into the nature of psychrophilic and psychrotolerant species (Christner *et al.* 2001, Sjöling & Cowan 2003).

**DNA microarrays**

DNA microarrays allow for the simultaneous detection of multiple oligonucleotide sequences in a sample by exposing the sample to an array of micrometer scale oligonucleotide probes immobilised to a surface. If present, the complimentary nucleic acid sequence will bind to the corresponding probe, and the array is then fluorescently imaged; either directly if the probe is pre-labelled with fluorophores or by the addition of a fluorescent dye that binds to double-stranded DNA. Yergeau *et al.* (2007) used a DNA microarray with >24000 probes that targeted functional pathways, specifically comparing genes involved in nitrogen and carbon cycling along an Antarctic transect. Using the microarray data, the location and vegetation cover affect both carbon and nitrogen cycle related gene distributions.

*2.3.3.3. Whole genome analysis*

Sequencing the whole genome of an organism allows for the association of metabolic pathways to functional genes and analysis of particular sequences encoding for proteins in that organism. This can lead to insights into the adaptations made by that organism, *e.g.* adaptations made for psychrophilic lifestyle (Methé *et al.* 2005). To date, four psychrophilic bacteria and one archaean have had their complete genome sequenced and published, only one of these bacteria, and the archaean, have been isolated from icy environments. The bacterium was *Psychromonas ingrahamii* 37 from Arctic sea ice (Riley *et al.* 2008). The psychrophilic archaean was *Methanococcoides burtonii* DSM 6242 and was isolated from an Arctic lake (Allen *et al.* 2009).The other psychrophiles were isolated from deep sea, shallow Arctic sea or marine sediments (Hou *et al.* 2004, Médigue *et al.* 2005, Methé *et al.* 2005).

*2.3.3.4. Proteomics*

Studying a large number of proteins produced by a single organism is used to understand how that organism reacts under certain conditions. Proteomic studies have been completed on three microorganisms, isolated from icy environments, to investigate methods for cold adaptation; two bacteria isolated from Siberian permafrosts (Bakermans *et al.* 2007, Zheng *et al.* 2007) and on one archaean isolated from an Arctic Lake (Goodchild *et al.* 2004, 2005).

The instrumentation used to study the proteome in these microorganisms involves different methods to identify the individual proteins, but each involves four stages; (i) extraction of proteins from cellular material, (ii) separation of the protein mixture into individual proteins, generally using a chromatographic technique, (iii) characterisation of individual proteins, through mass spectrometry-based techniques, and (iv) identification of these proteins by comparison with databases (Zheng *et al.* 2007) or proteins predicted from the genome, where available (Bakermans *et al.* 2007). Proteomic studies have revealed that temperatures of 4°C and below drastically alter the proteome of an organism and the individual organisms produce different proteins below this temperature hence use different strategies for cold adaptation (Bakermans *et al.* 2007, Zheng *et al.* 2007).

### *2.3.4. Physical sensors*

#### *2.3.4.1. Raman spectroscopy*

Raman spectroscopy is a technique that collects vibrational bond information at optical frequencies. It is a well established technique used in chemical analysis to fingerprint particular molecules using the distribution of peaks from the detected Raman scattering. Raman spectroscopy has been used to fingerprint molecules to identify pigments in lichen and cyanobacteria in Antarctic rocks (Edwards *et al.* 2003) and in paleolakes (Edwards *et al.* 2004). The combination of these studies had lead to a database of Raman spectra including 18 pigments used in extreme Antarctic habitats (Edwards *et al*. 2005).

The intensity of Raman scattering can be increased if the target of interest is attached or optically close to a rough metal surface, through surface enhanced Raman spectroscopy (SERS). SERS has been used to compare cell membranes sea ice bacteria, which exhibited a narrower range of SERS spectra when compared to mesophilic bacteria (Laucks *et al.* 2005).

#### *2.3.4.2. Autofluorescence*

Quantifying photosynthetic biomass provides information on the primary productivity of a system. Photosynthetic biomass can be estimated by detecting fluorescence emitted from photosynthetic pigments within cells. The autofluorescence of photosynthetic organisms can be detected under microscope (Kaštovská *et al.* 2007 Section 2.3.1.1) or by pulse amplitude modulation (PAM) fluorometry. PAM fluorometry relies on supply of weak, modulated light pulses (measuring light), that allows chlorophyll fluorescence to be monitored in living

*Table 2-1. Summary of techniques used to detect and characterise life in icy environments highlighting the advantages, disadvantages and, where applicable, when and on what type of samples they have been used for in-field analysis. Many of the advantages and disadvantages are relative to other techniques with the same target,* e.g. *techniques for nucleic acid analysis.*

| Technique | Advantages | Disadvantages | In-field status |
|---|---|---|---|
| Direct cell counts | Easily compared between environments, sample type, etc. | Time consuming; operator dependent | Icy environments (Arctic soils and subglacial ice and meltwater): Nadeau *et al.* 2008, Authors |
| FISH | High specificity; information on community structure; flexibility through choice of probe | Time consuming and operator dependent; limitations to hybridisation efficiency thus can cause false negatives | |
| SEM | Direct visualisation allows for microorganisms to be seen in environmental context | Time consuming sample preparation; samples have to be dried which can modify structure | |
| TEM | Very high resolution to ~0.5 nm | Technical sample preparation | |
| Cell culture | No electronic instrumentation required; viable microorganisms for further investigations | Only captures small percentage of community; microbial growth is time consuming at low temperatures | Icy environments (Antarctica): McLean 1918 |
| Labelled substrate measurements | Allows for measurements of *in situ* activity | Long term experiments; frequently requires hazardous materials (radiolabelled substrates); natural environments are not a closed system | Icy environments (Cryoconite holes): Hodson *et al.* 2007, Anesio *et al.* 2009 |
| Lipid analysis | Represents active community | Time consuming sample preparation | |
| Immunoassay | Specific, sensitive, some are quantitative without the need for electronic instrumentation (although with low precision) | Targets have to be known, need to have specific antibodies to develop assays | Icy environments: Authors |
| ATP bioluminescence assay | Rapid assay (15s); simple application; commercially available detection technology and reagents | Single target; no universal extraction procedures; difficult to separate the contribution of biomass and activity to ATP concentration | Icy environments (Antarctic soils and subglacial ice and meltwater) : Cowan *et al.* 2002, Authors |
| LAL and LAL-based assay | Low lowest limit of detection (~$10^2$ cells $ml^{-1}$) | Single target; standard assay fairly complex; no universal extraction procedures | Icy environments (subglacial ice and meltwater): Authors |
| Stable isotope analysis | Information on metabolism | Detection generally requires specialised instruments and dedicated laboratories | |

| | | | |
|---|---|---|---|
| Measurement of oxygen concentrations | Small, portable detection instrumentation readily available | Single target; abiotic influences on oxygen concentration | Icy environments (beneath sea ice and cryoconite holes): Trenerry *et al.* 2002, Hodson *et al.* 2007, Anesio *et al.* 2009 |
| Measurement of gas fluxes | Automated commercial instrumentation available capable of continuous measurements | Possible abiotic influences | Icy environments (Siberian permafrost): Sommerkorn 2008 |
| Measurement of nutrient concentrations | Established detection protocols | Requires different instruments for types of nutrient species | Icy environments (subglacial meltwater): some nutrient species Hodson *et al.* 2005 |
| Real-time PCR | Flexibility of target sequence; sensitive to ~10 DNA copies; analyses whole community; PCR products never exposed; processing multiple samples at once; flexible applications | Need to know targets prior to analysis; cannot be used to precisely classify microorganisms | Icy environments (subglacial ice and meltwater): Authors. Other sectors: Schaad *et al.* 2002, Tomlinson *et al.* 2005, Krause *et al.* 2006, Rasmussen *et al* 2008 |
| RT-PCR | Information on active community | RNA less stable than DNA therefore harder to extract | |
| Gel electrophoresis | High throughput; gives pure PCR products | Qualitative only or pseudo quantitative | |
| T-RFLP | Used on whole environmental sample; community analysis; high throughput | Community needs low diversity; no information about what is there | |
| ARDRA | Used to fingerprint bacteria; high throughput | Need pure cultures or clone libraries, no universal method | |
| DNA microarray | Multiple analytes per run (> 100000) | High cost | |
| DNA sequencing | Most accurate method for genetic identification | Requires pure DNA extract, high cost per sample | |
| Whole genome analysis | Complete genetic information to link pathways and taxonomy; understanding pathways | Only be completed on culturable organisms, expensive | |
| Raman spectroscopy | Accurate; non-destructive | Instrument requires training; problems with interfering targets; low sensitivity | Other sectors: Wood *et al.* 2005, Yan and Vo-Dinh 2007, Luo and Lin 2008 |
| SERS | High sensitivity, to single (bio)molecule detection | Sensitive to surface chemistry; currently no universal preparation techniques | Other sectors: Wood *et al.* 2005, Zhang *et al.* 2005 |
| Autofluorescence measurements | Non-destructive | Requires target to fluorescence *e.g.* chlorophyll | Icy environments (Arctic soils and sea ice): Nadeau *et al.* 2008, Trenerry *et al.* 2002 |

organisms, without photosynthesis occurring. McMinn *et al.* (2003) used a custom built monitoring PAM fluorometer on a robotic arm, for studying photosynthetic activity under Antarctic sea ice and was able to measure diurnal changes and demonstrated rapid adaptation by sea ice microalgae to changing light levels.

#### *2.3.5. Summary of current techniques*

The techniques discussed above study the presence, abundance, diversity and activity of microorganisms. A varied range of techniques have been applied to study microbial life in icy environments, from standard molecular microbiology techniques, to the detection of microbially-induced changes in their chemical and physical environment. These techniques have mainly been applied to the large polar land masses in Antarctica, northern America, northern Asia and Greenland, but also include analysis of samples from the smaller polar islands and the high altitude areas in Europe, Asia, North America and New Zealand. The types of samples from icy environments that life detection and characterisation techniques have been successfully applied to are also varied. These include snow, surface and basal ice from valley glaciers and ice sheets, permafrosts and sea ice.

Some techniques have been applied to an extensive variety of locations and sample types, whereas other techniques just once in icy environments, or used by one research group. The uneven distribution in sample locations, types of samples and techniques applied to those samples shows that life detection and characterisation in icy environments is still in its infancy.

Conducting life detection and characterisation techniques have revealed that microorganisms are an active part of icy environments (Hodson *et al.* 2008), frequently with a higher diversity and biomass than was previously believed (Cowan *et al.* 2002, Sjöling and Cowan 2003). As the relationship between microorganisms and their environment is only starting to be understood there are still many opportunities and need to study microorganisms in icy environments (Laybourn-Parry 2009).

### 2.4. Life Detection and Characterisation of Techniques Conducted in the Field

In this section we consider examples where the life detection and characterisation techniques described in the previous section have (i) already been used in the field within icy environments and / or (ii) been used in-field in non-icy environments with potential relevance to future deployment in-field in icy environments. Examples of the latter situation include applications in medical, food, security and defence, environmental and astrobiology sectors.

Of the twenty five techniques applied to life detection in icy environments reviewed in the previous section, eleven have been used in-field in peer review literature. Eight of these techniques have been used to detect and characterise life in icy environments and a further three techniques have been used elsewhere in other scientific and industrial sectors. Also included in this section are three techniques that have been used in the field in icy environments by the authors (authors' unpublished data), one of which has been applied to in-field life detection elsewhere in science and industry. These techniques are summarised in the final column of Table 2-1.

### *2.4.1. In-field life detection and characterisation in icy environments*

Those techniques already used to detect and characterise life in icy environments have already overcome the challenges posed by in-field analysis in these usually remote environments. The eleven techniques used so far constitute direct cell counts, cell culture, labelled substrate measurements, ELISA immunoassay, ATP bioluminescence assay, LAL-based assay, gas concentration measurements, gas flux measurements, nutrient flux measurements, real-time PCR and autofluorescence measurements (Table 2-1). The techniques are discussed by examining the technology, analytical reagents, experimental design and sample preparation techniques that make in-field life detection using these techniques possible. More broad issues, involving the type of facilities where sample analysis is conducted and the benefits highlighted in these studies, are also discussed.

As life detection and characterisation in icy environments is a niche science, there are limited funding opportunities for bespoke instrumentation development for in-field analysis in such environments. Using commercially available instrumentation removes the need for major development and reduces the cost and timescale of pre-field development and testing. All in-field life detection studies identified for this section of the review have used commercially available instrumentation except Nadeau *et al.* (2008) where a prototype microscope was tested. This prototype microscope, along with commercially available dyes for direct cell counts and autofluorescence measurements, was being developed for future missions to Mars and hence tested in an Arctic Martian analogue site. The commercial systems used for the other studies range from complete portable life detection systems, used directly, to custom-built systems predominantly using commercially available parts. Nine types of commercially available instruments, without modification for field use, have been transported and used in the field. The instruments and applications are as follows. (i) A fluorescent microscope was used with commercially available dyes for direct cell counts in Arctic subglacial ice and

meltwaters (authors' unpublished data). (ii) Liquid scintillation counters were required to measure the concentration of radiolabelled substrates that have been taken up by microorganisms to measure net primary productivity in Arctic sea ice and Arctic cryoconite holes (Mock & Gradinger 1999, Mock 2002, Hodson *et al.* 2007, Anesio *et al.* 2009). (iii) Portable luminometers and ATP bioluminescence assay reagents were used to quantify ATP concentration as a proxy for generic biomass in Antarctic Dry Valley soils and Arctic subglacial meltwaters (Cowan *et al.* 2002, authors' unpublished data). (iv) A portable fluorometer was used to measure the fluorescence endpoint of a LAL-based assay to detect LPS as a proxy for Gram-negative bacteria in Arctic subglacial samples (authors' unpublished data). (v) A portable absorbance reader was used to detect difference in colorimetric endpoint of ELISA assay for bulk protein GroEL (authors' unpublished data). (vi) Optical absorbance instrumentation was transported to the field to allow for the quantification of nutrients for the calculation of nutrient fluxes in Arctic glacial meltwaters (Hodson *et al.* 2005). (vii) Dissolved oxygen meters were used for the quantification of dissolved oxygen to measure community respiration in Arctic cryoconite holes (Hodson *et al.* 2007, Anesio *et al.* 2009). (viii) A small real-time PCR thermal cycler was used to detect relative changes in DNA, targeting taxonomic identification and specific metabolic pathways in Arctic subglacial ice and meltwaters (authors' unpublished data). (iv) Finally, a gas exchange instrument was used to measure gas fluxes ($CO_2$) for community respiration in Arctic permafrosts (Sommerkorn 2008). Two of the instruments used for life detection in icy environments required modifications, in both cases custom built by manufacturer or manufacturer-licensed company. A custom built PAM fluorometer was modified to enable autofluorescence measurements on algae under sea ice by changing the excitation LED and adding a waterproof case (McMinn *et al.* 2003). An oxygen microelectrode was produced to enable high resolution oxygen measurements to calculate oxygen flux across the diffuse boundary layer under sea ice and hence calculate primary productivity. Therefore the microelectrode was built with a small sensor tip diameter, rapid response time and a minimal stirring effect (McMinn *et al.* 2000, Trenerry *et al.* 2002).

Frequently the reagents needed to perform life detection and characterisation techniques in institutional laboratories require cold storage either at +4ºC, -20ºC or occasionally colder. These include the typical analytical reagents for direct cell counts, ATP bioluminescence assay, LAL assay and real-time PCR. Cold storage can pose a problem during transportation to, and storage at, a given field site. As transportation tends to be of limited duration and

reagent volumes are small, transportation in insulated boxes with cool blocks or dry ice, hand carried to the field is generally sufficient. If no facilities are already available for cold storage at the chosen field site then in-field storage, possibly up to several months, can be a significant issue. Three different approaches have been made to solve this problem (i) transportation of portable domestic freezer (authors' unpublished data), (ii) modification of stock solutions to increase the shelf life *e.g.* fluorescent dyes are more thermally stable at higher concentrations (Nadeau *et al.* 2008) and (iii) identification and use of alternative reagents that do not require cold temperature storage, *e.g.* DNA extraction kits (authors' unpublished data).

Certain scientific studies necessitate equipment, additional to the analytical instrumentation, for experimental set up. In the studies identified for in-field applications in icy environments this equipment had been required to isolate sub-samples for *in situ* incubation with labelled substrates, and to deploy detection instrumentation to allow for access to the sampling site. In a simple example of experimental set up, glass bottles were used to isolate debris and water from cryoconite holes, and wrapped in aluminium foil to incubate samples in the dark to calculate community respiration (Hodson *et al.* 2007, Anesio *et al.* 2009). A more intricate system was developed to isolate sections of a sea ice core to calculate *in situ* algal primary production within sea ice. The core sections were cut, isolated with labelled substrate in Petri dishes, replaced in original position in the core and then the core was returned to the borehole (Mock & Gradinger 1999, Mock 2002 Figure 2-1). To allow for direct access to the base of sea ice to conduct *in situ* measurements, a robotic arm was developed to deploy a PAM fluorometer (McMinn *et al.* 2003) and an oxygen microelectrode (Trenerry *et al.* 2002) to measure photosynthetic activity and algal productivity respectively. The robotic arm allowed for *in situ* measurements to be conducted away from the influence of the access hole which was required to deploy the instruments (Figure 2-1).

To analyse samples with most techniques, the target needs to be in a fluid, normally water or air. The exceptions studied here are some of the physical sensors, *i.e.* autofluorescence with PAM fluorometer or fluorescent microscope, and the measurement of Raman spectra. As the sample of interest in icy environments can be liquid, gas or solid, either as ice or sediment, the direct analysis of these samples is not compatible with the majority of techniques. Therefore, sample processing is required prior to analysis, and can involve different levels of extraction, concentration, purification and / or preservation of the target. From the techniques studied for in-field analysis in icy environments commercially available kits, with a different intended

purpose (*e.g.* ATP extraction solution for soils) (Cowan *et al.* 2002), or established laboratory protocols have been used (Hodson *et al.* 2005, Hodson *et al.* 2007, Anesio *et al.* 2009). In some cases protocols have been adapted to be more field suitable, often limiting the equipment required for field deployment *e.g.* vigorous shaking instead of using vortex mixer (author' unpublished data).

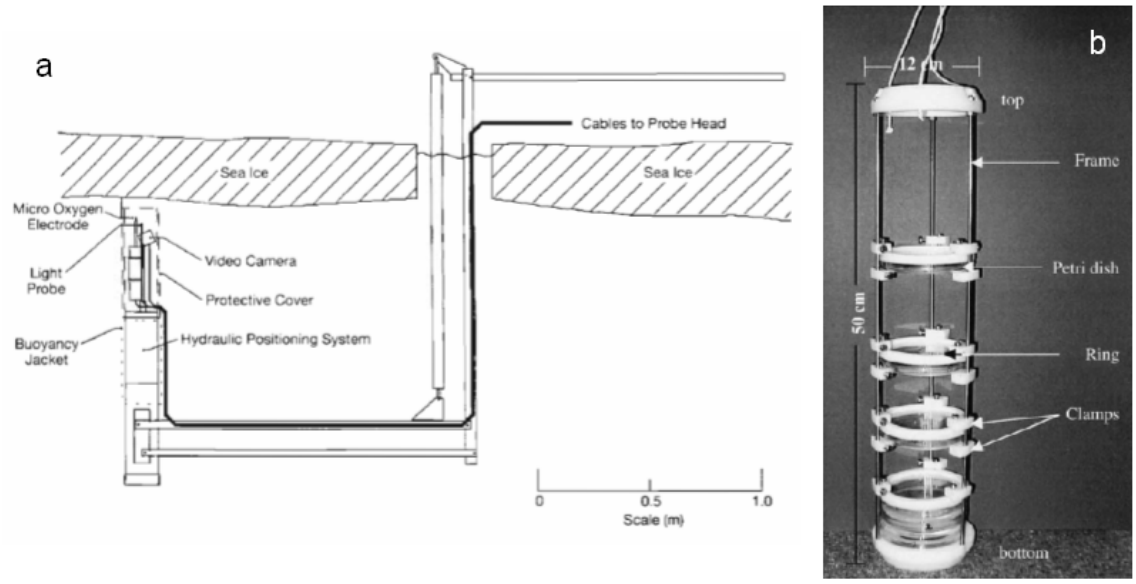


*Figure 2-1. Experimental set-up for in situ sea ice experiments. a) Robotic arm to position micro electrodes from McMinn et al. (2000), b) Equipment to contain sealed Petri-dishes to all for in situ incubation of sections of sea ice from Mock (2002).*

To perform complete cycles of life detection and characterisation in-field, data analysis and interpretation are required. For the various techniques demonstrated to date for in-field application within icy environments, the following examples demonstrate a number of these issues. ATP bioluminescence is an example of a technique involving biochemical reactions that are both temperature dependent and typical performed under standard temperature conditions. The in-field implementation of temperature controlled equipment or environment adds complexity, mass and power requirements to instrumentation. Therefore, for the example of ATP bioluminescence measurements, commercial portable luminometers have been developed that contain ambient temperature sensors and software algorithms that compensate for a variable reaction temperature, giving standardised measurement values. For accurate data, calibration of measurement instruments and analytical reagents are required. As some of these instruments and analytical reagents can exhibit significant drift over the timescale of a typical field campaign they have to be calibrated in the field. An example of instrument

calibration include oxygen microelectrodes, where the endpoints for calibration are produced by saturating a suitable water sample with air, and deoxygenating water by the addition of sodium nitrite. Therefore only minimal reagents and a small low powered pump were required for in-field calibration (McMinn *et al.* 2000). Some of the analytical reagents used in techniques reviewed here produce a response proportional to the target concentration; *e.g.* ATP bioluminescence assay and real-time PCR. The performance of these analytical reagents can vary between production batches and exhibit 'drift' during storage. To correct for these changes standards need to be assayed along with samples. In addition analysis a set of standards allows for the construction of a calibration curve for that particular assay, therefore allowing for quantification of that target.

The final consideration worth addressing within the context of this review is where the in-field analysis has been conducted. Environmental influences such as wind and rain make working conditions difficult and can damage the analytical equipment. To address these issues, dedicated laboratories have been set up on land (Anesio *et al.* 2009, authors' unpublished data) or on ship (Mock & Gradinger 1999, Mock 2002). The authors have conducted in-field analysis in two types of laboratory facilities; in prefabricated structures located within underground tunnels under Svartisen Icecap, Norwegian Arctic and in a tent in Western Greenland on land one kilometre from snout of the glacier being studied. The prefabricated structures were operated by the Norwegian Water Resources and Energy Directorate (NVE) and were equipped with mains electricity and running water, and provided a constant temperature in which to conduct the in-field life detection assays. However, all equipment and materials, including that for sterilisation, were transported to the field. The tent laboratory in Western Greenland provided protection of equipment and personnel from sun, wind and rain. In this situation electricity was provided by a generator and water was collected from meltwater streams on the glacier surface. During this particular field campaign the tent laboratory temperature varied from +7ºC to +37ºC depending on weather and time of day, meaning it was best to perform certain analytical techniques at particular times of day (especially fluorescent cell counts during cooler times). These examples indicate the field laboratory is a balance between the ideal environment in which to conduct analyses and the logistical issues of transporting equipment.

Some of the advantages of in-field analyses have been highlighted by these studies. Sommerkorn (2008) conducted community respiration measurements with seven study areas, in three locations, totalling over 600 measurements, therefore improving spatial resolution

and increasing confidence in results due to a large number of repeats made possible by in-field analysis. *In situ* measurements by Trenerry *et al.* (2002) allowed for high temporal resolution measurements of primary productivity, where a set of oxygen concentration measurements were made at nearly sixty time periods in fewer than 12 hours. Several studies have highlighted issues of target instability during transportation and storage, and overcome this by in-field analysis. Firstly, pre-field testing of the stability of chemical species related to nutrient cycling negated the possibility of accurate measurement of $NO_3^-$ and $PO_4^{3-}$ in institutional laboratories (Hodson *et al.* 2005). Transporting equipment to the field allowed for the measurement of these chemical species, which were required to analyse the complete nutrient budget for the glaciers studied. Secondly, analysis of microbial metabolism by incubation using labelled substrates requires analysis as soon as possible after the end of incubation. As many icy environments are remote this means to acquire accurate data it is preferential to conduct these analyses in the field (Hodson *et al.* 2007, Anesio *et al.* 2009).

Reviewing the in-field life detection and characterisation techniques currently conducted in icy environments shows that a variety of techniques have been conducted on different sample types including rocks, sediments, soils, subglacial meltwaters, sea ice and water and debris from cryoconite holes. All of these studies have been conducted in high latitude regions, possibly due to their remote locations meaning sample transportation is more of an issue than from high altitude studies, *e.g.* in the Alps. Commercially available detection instrumentation is used exclusively for in-field measurements in icy environments, and have been used either directly (*e.g.* Cowan *et al.* 2002) or with adaptations made by the manufacturer (*e.g.* McMinn *et al.* 2000). With five studies to date, the *in situ* incubation with labelled substrates could be considered to be the only established in-field technique. The authors hypothesise this is due to the difficulty of recreating the natural environment in the laboratory, necessitating that incubation experiments are conducted in the field if all environmental influences are to be included. The remaining techniques have only been used in one or two studies so far, where the authors have conducted systematic study using four techniques in the field in Norwegian Arctic and in South Western Greenland (authors' unpublished data). The eleven techniques already conducted in icy environments provide a good basis when considering in-field microbial analysis in any icy environment. In addition, they are an important resource for how other microbiological techniques could be conducted in the field as together these studies show that considerations need to be made to the detection instrumentation, sample preparation

methods, experimental design, reagent storage requirements, instrument and reagent calibration and the location for conducting analyses.

### *2.4.2. Relevant in-field life detection instrumentation from other disciplines*

In this section we review the techniques used to detect and characterise life in icy environments described in Section 2.3, which have been used in the field in industrial or other scientific sectors but not yet in-field in icy environments. Also included are any significant field developments from other sectors in the techniques that have already used in the field in icy environments. Techniques for in-field analysis in other sectors show the potential for the near future of in-field life detection in icy environments as some of challenges of in-field analysis have already been overcome (*e.g.* logistics of transportation of equipment, reagents and consumables).

The techniques used to study life in icy environments, described in Section 2.3, are adapted from techniques primarily developed for similar applications in industrial and other scientific sectors. Although the research aims in these other sectors frequently differ from the research aims when studying icy environments, the accurate detection and characterisation of microorganisms is essential for all these applications. We have identified five sectors as potential sources of life detection and characterisation technologies with relevance to icy environments; medical, food, security and defence, environmental and astrobiology. Generic microbial detection is important in the food and medical sectors and for astrobiology applications as generic biomarkers (*e.g.* ATP and bulk proteins) are used as a measure of biological contamination (Davidson *et al.* 1999, Sims *et al.* 2005, Lipscomb *et al.* 2006). The characterisation of species and strains is important in different sectors with various applications *e.g.*: to diagnose some diseases in the medical industry (Jass *et al.* 2003), to identify potential agents of bio-warfare in the defence industry (Sapsford *et al.* 2008) and to detect faecal indicating bacteria in recreational waters in the environmental sector (Noble & Weisberg 2005). All of these applications can and have been conducted near the sample site, which could be the patient bedside, the frontline in a war zone or a busy seaside resort. The development of instrumentation for astrobiological applications is of particular interest to icy environmental applications for two reasons. Firstly, the instruments are highly suitable as they are built robustly to detect microbiologically relevant targets at low concentrations. Secondly, during development these instruments are frequently tested in Martian analogue sites, some of which are icy environments. These sites include Svalbard, Norway (Steele *et al.* 2007), Canadian High Arctic (Nadeau *et al.* 2008) and Antarctic Dry Valleys (Edwards *et al.* 2003).

The portable instrumentation for the detection and characterisation of life that has been developed in other sectors of science and industry are now predominantly commercial available (Figure 2-2).

Microscopes are an essential requirement in the diagnosis of many diseases, however the microscopes required for these tasks are frequently large, delicate and expensive. The CyScope® TB and CyScope® Malaria (Partec GmbH, Germany Figure 2-2) have been designed specifically for field use, focussing on diagnosis of diseases in developing countries with a rudimentary healthcare system. These systems include a fluorescence microscope with disposable glass slides preloaded with reagents to detect tuberculosis or malarial biomarkers in the field. The construction of this field portable microscope provides flexibility as filters are easily interchangeable, opening up the range of techniques for in-field microbial enumeration using autofluorescence and dyes, including those already used for microbial enumeration in icy environments.

*Figure 2-2. Field portable microbiological life detection technology. Starting in top left and moving clockwise: Endosafe®-PTS™ with cartridge (Charles River Laboratories International Inc., MA, USA), CyScope® (Partec GmbH, Germany), LIGHTENING MPV® (BioControl Systems Inc., WA, USA) and MiniOpticon™ (Bio-Rad Laboratories, CA, USA).*

Portable immunoassays are typically applied to the medical (von Lode 2005) and environmental industries (Van Emon & Gerlach 1995). Immunoassays lend themselves to field deployment as some can be packaged as lateral flow immunodiagnostic devices. These devices are simple to use and frequently require only simple detection instrumentation (Faulstich *et al.* 2008), if any *e.g.* pregnancy tests. Several companies produce a variety of immunodiagnostic test strips for rapid medical diagnosis and testing water quality, including Merck KGaA (Germany), Oxoid Limited (UK), DuPont™ (DE, USA), Global Water Instrumentation Inc. (CA, USA) and Industrial Test Systems Inc. (SC, USA). The test strips produced for environmental monitoring could have direct applications as they include tests for parameters relevant to icy environments, and include iron concentration, nitrate and nitrite, hardness, total bacteria, phosphate and sulphate. Due to the limited precision of these test strips, their immediate use is probably restricted to intelligence-led sampling with later analysis in institutional laboratories.

The development of Endosafe®-PTS™ (Charles River Laboratories International Inc., MA, USA Figure 2-2), includes an instrument and cartridges for incubation during and detection of the optical output of the LAL assay. The Endosafe®-PTS™ primary application is in medical industry, as testing for LPS is a requirement by the Food and Drug Administration (USA) due it potentially toxic effect (Gee *et al.* 2008). The Endosafe®-PTS™ system has also been used for the assessment of microbial research in the international space station and in and around hot springs in Svalbard as a Martian analogue site (Steele *et al.* 2004).

The *in situ* detection of microbially relevant chemicals is used to analyse potential contamination in water bodies, and instrumentation for continual *in situ* detection of some chemical species is now commercially available. The NAS-3X (EnviroTech Instruments LLC, VA, USA) nutrient analyser is an example of such an instrument, and can be used to detect nitrate, phosphate, silicate and ammonium. This system uses optical measurement to analyse the different nutrients, and includes data logger, programmable sampling regime, with the conduction of routine blank measurements calibration standards. The application of this type of system would allow for the in-field, automated monitoring of some of the chemical species already studied to infer microbiological activity, by changes in the chemistries in subglacial meltwaters.

Detection of nucleic acids provides a flexible approach to enable for the detection of specific microorganisms or strains for a variety of applications. The development of comparatively small and robust real-time PCR systems has allowed for in-field detection of microorganisms

at different levels of taxonomic classification and activity. R.A.P.I.D.® and RAZOR® EX (Idaho Technology Inc, UT, USA) are ruggedised real-time PCR systems purposely built for field applications. Along with the instrumentation, analytical reagents have been developed that do not require cold storage. Initially these targeted the detection of pathogenic bacteria for military applications, but subsequently analytical reagents have been developed for pathogenic bacteria to assess food and water quality. Multiple small real-time PCR thermal cyclers are commercially available and include MiniOpticon™ (Bio-Rad Laboratories, CA, USA Figure 2-2), SmartCycler (Cepheid, CA, USA), ABI Prism® 7700 (Applied Biosystems Inc, CA, USA), Rotor-Gene 3000™ (Corbett Research, Australia) and LightCycler® (Roche Applied Science, Germany). Such small thermal cyclers have been used to conduct real-time PCR in the field in two different studies to detect plant pathogens (Schaad *et al.* 2002, Tomlinson *et al.* 2005), in an abattoir to screen for a specific bacteria infection in chickens (Krause *et al.* 2006) and in a near field laboratory to monitor toxic cyanobacteria in water samples (Rasmussen *et al.* 2008). These studies have demonstrated different approaches to conducting real-time PCR in the field depending on the application, *i.e.* the location of in-field analysis and the DNA extraction technique. Three of these studies involved setting up a field laboratory in buildings, whereas the other study the analysis was conducted in a vehicle (Tomlinson *et al.* 2005). Three different techniques were used to extract nucleic acids prior to real-time PCR: magnetic bead transfer (Tomlinson *et al.* 2005, Krause *et al.* 2006), macerating the sample in PCR grade sterile water (Schaad *et al.* 2002) or tissue extraction kit on a centrifuged pellet (Rasmussen *et al.* 2008). The use of real-time PCR for simultaneous amplification and identification of DNA sequences meant that results were available within one to two hours of sample collection. This lead to informed decision making, *e.g.* the rapid identification of the strain of plant pathogen led to the application of suitable treatment (Tomlinson *et al.* 2005).

Raman spectroscopy has a wide variety of applications for the identification of chemical and biochemical substances. Portable Raman spectrometers are currently commercially available and include ReporteR (DeltaNu, WY, USA), InPhotote™ (InPhotonics Inc, MA, USA) and MiniRam™ (Pacer International Ltd, UK). The Raman spectra of biomarkers have been characterised for the in-field identification of cyanobacteria (Wood *et al.* 2005), microbial contamination in food (Luo & Lin 2008) and pathogenic microorganisms (Yan & Vo-Dinh 2007). The sensitivity of Raman spectroscopy can be significantly improved with SERS (surface enhanced Raman spectroscopy) by associating the target with a rough metal surface.

Several portable SERS systems have been developed that allow for the detection of microbial biomarkers (Zhang *et al.* 2005) or whole cells (Wood *et al.* 2005). These portable systems include methods for the preparation of samples to allow for interactions with the metal surface; where with microbiological applications the bacteria were suspended in a liquid with metal colloids (Wood *et al.* 2005, Luo & Lin 2008). As the Raman spectra from SERS are sensitive the configuration of the metal surface, spectra may have to be produced for each type of colloid and occasionally between batches of colloids (Kahraman *et al.* 2007).

The techniques used to detect and characterise life in the field show the potential for in-field life detection, with relevance to icy environments. Where not only can the technology developed elsewhere be used in icy environments, but knowledge gained during development may also prove to be transferable, *e.g.* stable storage of analytical reagents at higher and variable temperatures. All of the above techniques have been tested in the field, thus ensuring that all stages of sample preparation and analysis are field suitable. There is expected to be a certain level of further instrument testing and modification and protocol refinement before these techniques can applied to icy environments in the field. These primarily involve the testing of instruments to ensure they function in simulated field conditions, and to develop detection methods for targets relevant to icy environments. Pre-field testing of analytical instrument are required to ensure they work effectively at ambient field temperatures, and should be tested to ensure they are robust enough to survive freeze-thaw cycles and transportation to remote field locations. Although the analytical instrument may be suitable for use in-field, frequently the precise targets and sample types from non-icy environments are likely to be different to those in icy environments, therefore pre-field development of sample preparation and processing techniques are required to ensure that appropriate chemicals are transported to the field. Many of these developments can be, and to a certain extent already have been, completed in institutional laboratories; such as the selection of primer pairs to target relevant DNA sequences (Yergeau *et al.* 2008), the fingerprinting of microorganisms by collection their Raman spectra (Edwards *et al.* 2003), or the development of sample processing methods for sterile extraction of samples (Christner *et al.* 2000). Many detection techniques are being developed to be compatible with in-field detection, however they have not yet been tested in the field, these are discussed in the following section.

## 2.5. The Future of In-Field Life Detection in Icy Environments

### *2.5.1. Technology*

Increasing demand and improvements in technology have advanced the miniaturisation of analytical equipment. This has lead to a boom in the development of in-field diagnostic devices for wide ranging microbiological applications. The technology developments discussed here include techniques that have been used to study samples from icy environments in institutional laboratories and relevant techniques that have not yet applied to icy environment samples. The integration of sample preparation, multiple analytical techniques and readout instrumentation has lead to the development of fully automated systems and systems capable of remote deployment are discussed and include some specific examples.

The traditional method to measure stable isotopes uses an isotope ratio mass spectrometer with reference gases. Due to mass, size and their fragile nature these systems are currently not feasible for-in field analysis. Although portable mass spectrometers are available (*e.g.* MS-200, Kore Technology Ltd, UK), currently they do not have the high precision necessary for accurate stable isotope analysis. An alternative method for the detection of stable isotope ratios of water, using a portable laser gas analyser, has been assessed by Lis *et al.* (2008). This gas analyser uses off-axis integrated cavity output spectroscopy to detect gas concentrations, where highly reflective mirrors increase the effective adsorption pathlength and the vaporised sample is analysed spectroscopically. The precision of this gas analyser is similar to the sensitivity of an isotope ratio mass spectrometer, *e.g.* for $\delta^{18}O$ obtained ±0.16‰ compared to typical range of ±0.10‰ to ±0.40‰ for isotope ratio mass spectrometers. Along with the portability of this gas analyser, there are additional advantages of minimal sample preparation, lower initial and running costs and simultaneous measurements of $\delta^{18}D$ and $\delta^{18}O$. The main disadvantage of the gas analyser is the reduced flexibility of analytical performance as it is currently only possible to analyse $H_2O$, $CO_2$ or $CH_4$ and the individual instruments are generally dedicated to the absorption wavelengths a single measurement gas.

Raman spectroscopy and in particular SERS has a potential important future in the identification of biomarkers and whole bacteria (reviewed by Hudson & Chumanov 2009), including applications in icy environments. This future potential is due to current availability of commercial portable devices, the ability to detect unknown samples, high potential sensitivity and it does not necessitate the destruction of samples. Currently the wide application of SERS, including applications in icy environments, is limited by several factors:

reproducibility of colloid formation, standardisation of protocols for production of spectra (*i.e.* colloid types), the number of microbially relevant spectra and data processing required to apply SERS to mixed samples. The production of colloids for SERS are becoming more uniform (Tantra *et al.* 2007) but until standard protocols exist for the study of particular sample types individual studies cannot be compared (Kahraman *et al.* 2007). Except for one study comparing SERS of psychrotolerant and mesophilic bacteria (Laucks *et al.* 2005) the majority of the microorganisms characterised using SERS are pathogens (Jarvis & Goodacre 2004, Premasiri *et al.* 2005, Zhang *et al.* 2005, Patel *et al.* 2008, Green *et al.* 2009). Therefore more analyses of microorganisms relevant to icy environments would be required prior to widespread applications. Improvements in the reproducibility of SERS spectra by colloid formation and development of methods to identify individual bacteria in mixed samples are needed before field portable Raman spectrometers and SERS systems fulfil their potential. Before applying to icy environments, more Raman spectra for microorganisms relevant to icy environments are needed (Edwards *et al.* 2005).

Developments in nucleic acid analysis has revolutionised how microorganisms can be analysed, making it faster, cheaper and more accurate than traditional culture dependent methods. As technologies improve, a demand for genetic analysis away from institutionalised laboratories has developed, with applications in medical, food, veterinary, security and defence, forensic and environmental sectors. This demand is starting to be met with the commercialisation of machines like R.A.P.I.D.® and RAZOR® EX (Idaho Technology Inc, UT, USA), with field deployable instrumentation and reagents for real-time PCR based analysis. These systems were originally developed for the analysis of microorganisms for use in the security and defence sector, *e.g. Bacillus anthracis* and *Escherichia coli*, but now include reagents for detection of food and water pathogens, *e.g. Campylobacter* and *Cryptosporidium*. Several additional nucleic acid detection technologies are being developed with the aim of making them field deployable. These technologies are based on real-time PCR (Higgins *et al.* 2003, Wang *et al.* 2006 Xiang *et al.* 2007), real-time nucleic acid based sequence analysis (NASBA) (Baeumner *et al.* 2003, Smith *et al.* 2007), capillary electrophoresis (Xu *et al.* 2005, Liu *et al.* 2007) and biotin labelling during PCR (LaGier *et al.* 2007, Germano *et al.* 2009). NASBA is an isothermal technique predominantly used for the amplification of RNA through the cyclic formation of complimentary DNA and destruction of original RNA sequence. Capillary electrophoresis separates DNA by size within electrolyte filled capillaries. Biotin labelling allows for specific capture of labelled PCR products to a

biotin-binding protein coated on a solid support, *e.g.* avidin or streptavidin. In all of these nucleic acid analysis techniques the biochemical recognition of the target needs to be converted to a detectable output: through the use of fluorescent dyes (Demchemko 2005, Altschuh *et al.* 2006), coupling to an electrode transducer for electrochemical detection (Lee & Hsing 2005, Wang *et al.* 2006) or by changes in magnetoresistance (Graham *et al.* 2004, Germano *et al.* 2009). Some of these nucleic acid detection techniques have already been developed into hand-held instruments for field use (Higgins *et al.* 2003, LaGier *et al.* 2007, Smith *et al.* 2007). These instruments indicate the potential for future developments of in-field nucleic acid analysis, but still require developments for suitability for applications in icy environments, mostly in terms of suitable target and sample preparation techniques.

Increasing improvements in micromachining of channels and miniaturisation of pumps and valves has lead to an expansion in developments of lab-on-a-chip (LOC) assays; combining one or more laboratory processes into mm to cm sided chips. These LOC assays have the potential to miniaturise analytical equipment, increase throughput, reduce costs and reduce power requirements for chemical and biochemical analysis, lending them to in-field analysis. To date, LOC assays relevant to microbiological analysis include systems to conduct PCR (Zhang & Ozdemir 2009), ATP bioluminescence (Eltoukhy *et al.* 2006) and microarrays for DNA and protein analysis (Lee & Hsing 2005, Rivas *et al.* 2008). Currently the field portability of LOC technology is restricted by the need to improve microfluidic control, as the readout machinery is frequently bench-top scale or larger (Hindson *et al.* 2005, Lee & Hsing 2005, Diamond *et al.* 2008). These improvements are likely to come from the development of novel methods for fluid movement (Ichimura *et al.* 2000) and miniaturised valves (Weibel *et al.* 2005) and pumps (Wu *et al.* 2005). Along with general technological improvements, additional considerations are necessary to apply LOC technologies in icy environments, as the systems are likely to have to work at low temperatures or internal temperature control for microfluidic handling should be included.

The ideal endpoint of the development of these LOC assays is the production of small instruments that integrate sample extraction, analysis and data interpretation. Integrated systems have been developed to detect particular targets in clinical samples for point of care clinical testing. Commercial systems include i-STAT® (Abbott Point of Care Inc., NJ, USA) and Triage® MeterPro (BioSite Inc, CA, USA). The i-STAT® is a hand-held blood analyser, with interchangeable cartridges that detect particular analytes and concentrate on different aspects of blood, *e.g.* blood gases, chemistries or cardiac markers. The Triage® MeterPro

performs multiplexed immunoassays with cartridges on specific sample types (*e.g.* urine or whole blood), with most of the targets concentrating on markers for cardiovascular disease. As these sample types are clinical and therefore different to those studied in icy environments (*e.g.* dilute water and soil), both target identification and extraction techniques would need to be developed to be applicable to these sample types. A faster solution could be to develop separate sample preparation and detection equipment (*e.g.* Lee & Tai 1999, Breadmore *et al.* 2003, Chen & Cui 2009), however the current focus for these sample preparation systems is still on clinical samples. Some systems have been assessed for the preparation of environmentally relevant microorganisms including electrical lysis in microfluidic channels for subsequent DNA analysis (Hall *et al.* 2006).

There is an increasing interest in the monitoring of environmental parameters without the need for human intervention, either due to long time periods for data collection or the environment of interest is inhospitable, dangerous or inaccessible. This can be addressed by the deployment of autonomous vehicles and probes or fixed sensor platforms and networks. Examples of development programs of autonomous vehicles with direct applications to icy environments include (i) ongoing development of bespoke probe for the exploration of subglacial Lake Ellsworth (Siegert *et al.* 2007) and (ii) testing of autonomous underwater vehicle (ENDURANCE) for exploration of lakes in Antarctica[†]. A probe is being developed to search for life in Antarctic Subglacial Lake Ellsworth, by making physical and chemical measurements of the lake during deployment and collecting water and sediment samples for later microbiological analysis. ENDURANCE (Environmentally Non-Disturbing Under-ice Robotic ANtarctic Explorer) is an automated underwater vehicle, designed to collect 3-D biogeochemical data, including sensors for chlorophyll and dissolved organic matter amongst other physical and chemical parameters, and includes equipment for visualisation of benthic material. Commercial autonomous aerial vehicles have collected physical measurements from glaciers (Curry *et al.* 2004, Tschudi *et al.* 2008), and recently have been used for visual assessment of cryoconite density on glacial surface through digital imagery (Hodson *et al.* 2007). Another approach to remotely sense environments is through the deployment of sensor platforms and wireless networks. The commercially NAS-3X system currently used for remote monitoring of the chemistry of water bodies has been described in Section 2.4.2. Another commercially available instrument of note include is 6600 V2 (YSI Inc. OH, USA)

[†] http://www.evl.uic.edu/endurance/

water chemistry analyser with interchangeable optical ports, including instrumentation for fluorescence based measurements chlorophyll and dissolved oxygen. These instruments have been combined in ruggedised housing for remote monitoring of water bodies, *e.g.* SmartBuoy (Cefas Lowestoft Laboratory, UK). Some integrated systems for remote water analysis are summarised in Glasgow *et al.* (2004). However prior to deployment in icy environments confirmation of suitability of these instruments needs to be conducted, *e.g.* the confirmation of appropriate sensitivity, operation at low temperature and minimal interference from sediment load. The sensitivity and analytical range of systems such as NAS-3X are currently at the limit of those required for measurements in glacial samples (Brown 2002, Hodson *et al.* 2005). Therefore improvements in sensitivity as well as additional methods to protect sensors from high abrasive potential of the sediment, large boulders and ice blocks, without comprising analytical capability would be required before use in glacial applications.

The future of the routine availability of microbiological sensors for applications away from institutional laboratories shows potential. Currently the majority of the developments are for applications in medical or security and defence sectors. Where topical examples such as avian flu (Collins *et al.* 2003), anthrax spores (Zhang *et al.* 2005) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Struelens 2006) push forward the media interest in rapid on site testing abilities, and hence provide funding opportunities. The near future (next 5 years) of in-field analysis in icy environments lies with testing commercial technologies with icy environmental targets, *e.g.* stable isotope analysis, SERS and nucleic acid characterisation systems, and the in-field testing and deployment of Antarctic Subglacial Lake Ellsworth probe and components. The more distant future lies in more integrated systems, like LOC assays and miniaturisation of associated detection instrumentation, however only when flexible LOC-type systems become established in other disciplines will significant opportunities will present themselves for icy environmental applications.

### *2.5.2. Environments*

There are five major icy environments where the authors can see an immediate benefit from in-field life detection technology: permafrost, major ice sheets, non-supra glacial environments, subglacial Antarctic lakes and extraterrestrial icy environments. These icy environments are more applicable to in-field analysis as are further from established laboratory facilities, either due being geographically remote or the sample locations are difficult to access. Permafrost (26% - Steven *et al.* 2007) environment and the major ice sheets (11%) cover a significant portion of the Earth's land surface, and little is know as to

how dramatically they might react to climatic changes, making it important that their chemical, physical and biological behaviour is understood. The main drive for the exploration of glaciers is the aim to characterise the types of microorganisms that are dominant in different types of glacier, where within the subglacial system they dominate, their relationship with the bedrock and how these factors relate to behaviour of the glacier as a system. The latest inventory of Antarctic subglacial lakes has identified 145 lakes under the East and West Antarctic Ice Sheets (Siegert *et al.* 2005). These lakes are believed to provide a unique habitat for microbial life (Pearce 2009), and microorganisms have been detected in the accretion ice of Lake Vostok (Karl *et al.* 1999, Christner *et al.* 2001). There are plans to drill into Antarctic Subglacial Lake Ellsworth and deploy a bespoke probe to measure chemical and physical properties, and to return samples for additional biological analyses (Siegert *et al.* 2007). Advances in in-field life detection would allow for biological measurements to be conducted during probe deployment, protecting the scientific return in case probe malfunction. In addition, in-field life detection technology will allow for the monitoring of materials and instrumentation in order to test for contamination. The European Space Agency's forthcoming mission to Mars currently includes specific life detection technology. The landing site for this mission is unlikely to be one of the Martian poles, but future exploration of extraterrestrial icy environments (Martian poles, Europa and Enceladus) will, in part, depend on the success of this life detection mission.

## 2.6. Conclusions

A case for conducting in-field measurements in icy environments can be considered under three points: multiple cycles of in-field analyses allow for in-field hypothesis and protocol development; sample storage and transportation issues where in-field analyses allow for the conduction of novel scientific studies and reduction in logistical costs; and intelligence led sampling which can reduce improve scientific return for given resource usage.

In reviewing the current laboratory techniques that have been used for microbial analysis we have identified twenty five individual techniques that have been used to study life in samples from icy environments (Table 2-1). These are used to measure the presence, abundance, diversity and activity of microorganisms. In summary, the techniques to measure the presence and abundance were direct cell counts, electron microscopy and generic biomarker detection. To measure the diversity; FISH, cell culture, immunoassays, DNA classification techniques and Raman spectroscopy were used. Finally, to measure activity; labelled substrates, lipid analysis, RT-PCR, measurement of stable isotopes and measurement of gas fluxes were used.

Approximately half of the laboratory-based techniques used to study life in icy environments have been used in the field either in icy environments or with wider applications throughout science and industry. The application of these techniques has been possible due to the production of portable instrumentation, many of which are now commercially available. The near future of in-field detection includes the application of the techniques already used in icy environments to expand the variety of icy environments and the type of samples they have been applied to. Also the development of the modifications required to apply up and coming techniques, in particular SERS and nucleic acid classification methods.

The commercially available instruments and current on-going developments in other scientific and industrial sectors show the types of instrumentation and assays that can be combined into integrated systems for biological analysis. Unlike the commercial drivers in medical and water quality sectors, or large government support of defence industry, the funding for bespoke development of systems for extreme environments including icy environments is limited. Therefore unless there is a significant increase in the funding available, the further future of in-field life detection in icy environments will rely on the production of commercial systems with greater flexible regarding sample type and target.

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# 3. Implementing ATP Bioluminescence Measurements in Subglacial Field Environments: Engabreen

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## Abstract

Quantifying the presence of extant life is a prerequisite to understanding the role that microorganisms have in all extreme environments. Glacial and subglacial environments are of particular interest at present as the cryosphere is sensitive to external climatic influences. To aid in this understanding, in-field measurements of a biomarker for extant life have been made in a subglacial environment. Adenosine triphosphate (ATP) bioluminescence was used as a rapid, in-field method to measure extant life in subglacial, glacial and periglacial samples in the Norwegian Arctic. A commercially-available ATP detection system was used to analyse a diverse range of sample types. This system was found to be unsuitable for direct analysis of water samples as the ATP concentration was below its detection limit. Therefore, the microorganisms were concentrated by filtering onto a 0.22 µm pore size membrane and then ATP was extracted directly in a boiling buffer of Tris EDTA. Interference from the sample matrix was, where possible, corrected for by spiking the sample with a known ATP standard. ATP concentrations could be determined in four of five types of water sample. The results from both techniques revealed the highest detectable ATP concentration in a periglacial sediment sample (120 n mol ATP $kg^{-1}$), and the lowest in a periglacial water source flowing through snow covered shrubland (0.730 pM ATP). Using ATP bioluminescence assay to detect extant life in the field allowed

for the acquisition and interpretation of results in the field. This lead to in-field development and testing of hypotheses and the refinement of sampling protocols, as well as reduced logistical costs and environmental impact *e.g.* by reducing sample transport.

Keywords

Biomarkers, Engabreen, Svartisen Subglacial Laboratory, ATP bioluminescence, cryosphere

## 3.1. Introduction

Even near freezing temperatures, microorganisms provide an important contribution to geochemical weathering processes in glacial systems (Tranter *et al.* 2005). Evidence for this has been gathered from a combination of various laboratory based techniques including: direct microbial enumeration (Sharp *et al.* 1999, Mikucki & Priscu 2007), nucleic acid analysis (Skidmore *et al.* 2005, Bhatia *et al.* 2006, Mikucki & Priscu 2007), metabolism measurements on isolates (Foght *et al.* 2004), electron microscopy (Skidmore *et al.* 2000) and inference from hydrochemical analyses (Tranter *et al.* 2002, Wadham *et al.* 2004, Hodson *et al.* 2005). All of these studies have revealed that significant, active microbial populations exist at glacier beds. However the full extent of their influence upon glacial systems is still poorly understood.

To optimise the understanding of microbiological ecosystems in glacial and other extreme environments, in-field or *in situ* life detection and characterisation techniques are required. This viewpoint is based upon three assumptions:

- In-field detection allows for multiple cycles of analysis in a single field campaign which can lead to the development and testing of hypotheses and refinement of sample processing protocols and sampling strategies and therefore improving the scientific return for a given level of resources and environmental impact.
- The traditional approach of field sample collection for later analysis in established laboratories suffers from logistical issues involved in transportation, including modification of samples for shipment and which together can limit the size and number of samples, and the required sample modification can limit or compromise the type of analyses that can be conducted.
- If samples need to be returned for analyses currently only achievable in established laboratories, then completing preliminary assays in the field means the choice of

samples is intelligence-led, allowing for the optimisation of the spatial distribution and sample size of returned samples.

To achieve in-field life detection, portable microbiological techniques are needed. Technological developments have lead to the commercialisation of non-laboratory based, therefore frequently field-portable, techniques focussing on in-field detection of microbial contamination within health, food hygiene and defence industries (Aycicek *et al.* 2006, Lipscomb *et al.* 2006, Watkins *et al.* 2006). The majority of these techniques rely on the detection of biomarkers; compounds which indicate the presence of an organism or group of organisms, or activity level. A few such biomarker detection technologies have been used in the field to detect extant microbial populations in extreme environments (Cowan *et al.* 2002, Steele *et al.* 2004). However, to date, there have been no reports of *in situ* or in-field life detection measurements in a subglacial environment.

Adenosine triphosphate (ATP) is a suitable candidate to test biomarker detection techniques in subglacial field environment for three reasons. Firstly, ATP is a generic biomarker for extant life as it is present in all known active cells and it hydrolyses quickly in the natural environment (Cowan & Casanueva 2007). Secondly, ATP can be rapidly detected in a single step assay based on an enzyme-mediated bioluminescent reaction which naturally occurs in organisms such as fireflies. Commercially available assay kits have been developed to improve the efficiency and reliability of the firefly bioluminescent reaction. These kits have been further developed into single-use devices and, along with portable luminometers, allow for rapid determination of ATP levels at the sampling site. Finally, ATP detection has been previously used on environmental samples including a proglacial lake, Antarctic mineral soils and sea ice. Simmons *et al*. (1983) measured ATP in a proglacial lake water column where the ATP concentration varied between 63.4 and 222.4 $ng.l^{-1}$ and depended on depth. Cowan *et al.* (2002) used a commercial ATP bioluminescence system and previously published concentrations of ATP per cell to estimate microbial numbers in soils in the Antarctic Dry Valleys. The lowest concentration of ATP and hence cell numbers was found in permafrost samples ($5x10^5$ to $8x10^6$ $cells.g^{-1}$ or 1.2 $\mu g.kg^{-1}$) and the highest concentration associated with algal mats ($3x10^8$ to $4x10^9$ $cells.g^{-1}$ or 660 $\mu g.kg^{-1}$). The published cell ATP concentrations used varied between 0.16 and 2.25 $fg.cell^{-1}$ from Fairbanks *et al.* (1984). Dahlbäck *et al.* (1982) measured ATP concentrations in Arctic sea ice samples with the highest ATP concentration reaching 120 $ng.l^{-1}$.

Building on this previous work and the broader desire for in-field and *in situ* measurements in glacial and subglacial environments, we have applied ATP bioluminescence techniques in the field to detect ATP and hence extant microbial life in a subglacial field environment. The objectives of the study were to (i) demonstrate that in-field ATP bioluminescence measurements were possible in a glacial and subglacial context, specifically the Enga glacier (Engabreen), Norway and the associated Svartisen Subglacial Laboratory. (ii) To test two commercial ATP bioluminescence assay formats and which comprised (a) single-use, disposable ATP assay devices incorporating pre-defined sample volume collection capability and pre-dosed cell lysis and ATP bioluminescence reagents that promised ease of use and (b) a separate assay reagent kit and extraction protocol, the commercial ATP assay reagent kit requiring operator reconstitution of final assay reagents together with multiple liquid handling steps for each individual assay and along with the separate extraction protocol promised a greater flexibility for accommodation of diverse sample types. (iii) To identify any limitations to ATP bioluminescence assays in glacial samples and related environments requiring further study and development. (iv) To compare ATP assay results with an independent, established standard technique for microbial cell enumeration – specifically fluorescent cell counts. (v) Develop a view of ATP levels in Engabreen from a range of glacially relevant samples.

## 3.2. Materials and Methods

### *3.2.1. Field site and sample locations*

Samples were collected from, and in-field measurements performed at, Engabreen from 18$^{th}$ to 22$^{nd}$ April 2006 and 19$^{th}$ to 27$^{th}$ November 2007. Engabreen is an outlet glacier of the Svartisen Ice Cap in Northern Norway (66˚41'N, 13˚46'E). A unique aspect of the Engabreen site is the Svartisen Subglacial Laboratory (Lappegard *et al.* 2006); a facility owned and operated by the Norwegian Water Resources and Energy Directorate (NVE). The facility consists of tunnels drilled into the crystalline bedrock below and around the glacier (Lappegard *et al.* 2006). These contain prefabricated structures that provide accommodation and basic in-field laboratory space. The tunnel system enables direct access to pristine samples of subglacial ice via hot water excavation of "ice caves" at the glacier bed. The access point to the glacier via the bedrock tunnel is under ~200 m of ice and ~2 km from glacier snout. Other sampling sites available include the glacier itself, the bedrock tunnel system, the environment around glacier and access to subglacial meltwater not exposed to the non-glacial environment (via inlets in the bedrock tunnel roof and walls).

Samples were clustered into three different location classes: glacial samples, bedrock tunnel samples and periglacial samples.

**Glacial samples**

The basal ice samples were collected from the ice cave, where Basal Ice 1 is closest to the bed contact and Basal Ice 3 the furthest away from the contact. The glacial water samples were collected within the bedrock tunnel system from meltwater streams; Meltwater 1 through 4 in April 2006 and Meltwater 5 in November 2007. The glacial samples collected outside the bedrock tunnel system were from streams as physically close to the glacier as possible and consist of Meltwater 6 and Side Stream in November 2007. The Side Stream was flowing from the glacier but a more precise origin of the water, *e.g.* sub-, supra- or en-glacial, could not be determined.

**Ambient bedrock tunnel samples**

All the ambient bedrock tunnel samples were collected within the tunnel system and with no obvious direct influence from the glacier (*i.e.* away from any subglacial inlets) and consist of standing water from Pool 1 and Pool 2 from April 2006 and Pool 3 and Pool 4 in November 2007. Pool 1 and Pool 2 were ~ 2 km into the tunnel system. Pool 3 was in the main access tunnel ~500 m from the tunnel entrance, Pool 4 was about 1 km further into the tunnel system.

**Periglacial samples**

The periglacial samples were all external to the bedrock tunnel and glacier systems, and no obvious immediate influence from the glacier could be seen. The samples comprised Snow from April 2006 and Forest Stream, Shrubland Stream, Rock Pool and Sediment Pool from November 2007. The Rock Pool had no sediment at the base of it whereas the Sediment Pool had a layer of fine sediment.

*3.2.2. General reagents*

All consumables were sterilised via autoclave prior to shipment and again in the field laboratory. A local water source was used due to logistical problems of shipping multiple litres of laboratory grade water to the field site. Before use all local water was filtered through 0.22 µm pore size membrane (Millipore, FDR-293-050F, Fisher Scientific) and triple-autoclaved. Bioluminescent reagents were reconstituted with small volumes of sterile water that were transported to the field. The buffer used throughout the study was 100 mM Tris, 4

mM EDTA (Tris EDTA buffer) (buffer pH was not modified, therefore should have been ~9.0). All chemicals were provided by Sigma Aldrich (Poole, UK) unless otherwise stated.

### *3.2.3. ATP bioluminescence assays and materials*

The in-field analysis required a portable luminometer (Merck HY-LiTE 2) which was kindly provided by the UK distributor (VWR International Ltd., Lutterworth, UK). The luminometer has an inbuilt temperature compensation algorithm to correct for the temperature dependence of the bioluminescent assay (between +5°C and +35°C), and bioluminescent outputs from the luminometer are in relative light units (RLU). The ATP standards used in all assays were supplied with the ATP reagents for the tube assay (ATP Bioluminescence Assay Kit HSII, Roche Diagnostics Ltd., Burgess Hill, UK). Standards were reconstituted in Tris EDTA buffer.

Two types of ATP assay reagents were used: pre-packaged Merck HY-LiTE single-use devices (VWR International Ltd., Lutterworth, UK) and ATP Bioluminescence Assay Kit HSII (Roche Diagnostics Ltd., Burgess Hill, UK) in tube assays. The single use devices were used as per manufacturer's guidelines and worked in two stages. The first stage transfers ~30 µl of liquid sample into the device where it is exposed to a chemical lysis agent. The second stage allows contact of this mixture with the freeze dried bioluminescent reagents. Swabs were used in November 2007 for collection of sediment samples prior to ATP measurement with the single-use devices, and were tested once a visible sediment load could be seen on the swab. The swab was the submerged into liquid provided in the cap of the single-use device. The single-use device was then dipped into the cap and sample transferred to sample recessed and the device processed as for the liquid samples. In April 2006 standards of 0.165 and 1.65 nM ATP were tested in the field. During the November 2007 field campaign calibration curves between 0.1 and 100 nM ATP were completed on the first and final day and 10 nM ATP standard was assayed daily.

The ATP test kit used in the tube assay was implemented with minor modifications to manufacturer's guidelines. These modifications were the reduction of total assay volume from 200 µl to 100 µl, *i.e.* 50 µl of reagent and 50 µl of sample, and ΔRLU was used to measure the bioluminescence output, *i.e.* the difference between the bioluminescence value of the ATP reagents before and after the addition of the sample. Calibration curves were completed daily from 0.01 nM to 10 nM ATP along with appropriate blanks.

### *3.2.4. ATP extraction of water samples*

Prior to the second field campaign, the effectiveness of different extraction solutions and methods were tested on previously collected glacial samples, where the addition of boiling Tris EDTA extraction solution gave the most consistent results when compared with other techniques tested, including boiling in sodium bicarbonate and sodium triphosphate buffers and the chemical lysis cocktail supplied with the ATP bioluminescence assay kit (data not shown). Two 250 ml plastic sample bottles were rinsed three times in sample before collection. 490 ml of sample was vacuum filtered through sterile 47 mm diameter 0.22 µm pore size mixed cellulose ester membrane using a vacuum hand pump. The membrane was then placed into a 1.5 ml reaction tube and physically disaggregated using sterile tweezers. 1 ml of boiling Tris EDTA extraction solution was added to the reaction tube and then the tube was incubated in a boiling water bath for one minute. The extracts were cooled to +3˚C before testing with the ATP bioluminescence assay after ~1 hour. All extracts were tested in triplicate.

### *3.2.5. Shipping*

The ATP bioluminescence chemicals were hand-carried to the field site in cool boxes and refrigerated overnight. On arrival the single-use devices were stored at +3ºC (the ambient temperature of the bedrock tunnels immediately outside the field laboratory) and the tube assay reagents stored at -15ºC until reconstituted. Reagents were tested upon arrival at the field site with ATP standards to test viability. All consumables were shipped to the field.

Samples returned to institutional laboratories for cell counts from April 2006 field campaign were preserved in the field to a final concentration of 4% formaldehyde, shipped and then stored at +4˚C until laboratory analysis. Samples returned for cell counts from the November 2007 field campaign were frozen in the field, shipped frozen and then stored at -30˚C. Samples were defrosted and then preserved to a final concentration of 2% formaldehyde prior to counting.

### *3.2.6. Cell counts*

After preservation, an appropriate volume of the sample was filtered onto a 25 mm diameter, 0.2 µm pore size membrane (Whatman, FDP-495-010J, Fisher Scientific). The membrane was then stained with 400-fold dilution of a commercially supplied stock of SYBR® Gold nucleic acid gel stain (Invitrogen Ltd., S-11494). Between 20 and 250 fields of view were studied

with an Olympus epifluorescence microscope at maximum magnification to a total final count of 200 cells, unless 250 fields of view were reached during counting.

## 3.3. Results

The data and findings reported here were obtained from two field campaigns and additional institutional laboratory work conducted before, in-between and after the field campaigns. In summary, a preliminary field campaign in April 2006 used only commercial single-use ATP bioluminescent devices to detect ATP in glacial, bedrock tunnel and periglacial samples at field sampling sites. Whilst ATP was detected in some glacial, bedrock tunnel and periglacial samples, other samples – primarily glacial meltwater – did not show any detectable ATP, *i.e.* if any ATP was present, it was at a concentration below the lower limit of detection of the ATP bioluminescence reaction. Leading from this finding, a simple sample processing protocol was implemented in a second field campaign together with an alterative commercial ATP bioluminescence reagent to concentrate any microbial cells present and hence ATP in water samples to allow detection. The alterative ATP bioluminescence reagent test kit enabled for a greater flexibility in sample type acceptance and allowed for correction of interference from high-clay content sample which sequestered ATP. Commercial single-use ATP bioluminescent devices where only used for analysis of sediment samples in the second campaign. Fluorescent cell counts, as an established method to quantify microorganisms, were also conducted on the samples from November 2007 to enable comparison with the ATP measurements.

### *3.3.1. Initial application of single-use ATP assay devices - April 2006 field campaign*

To test the suitability of commercial single-use devices for ATP detection in the Engabreen system, samples of subglacial meltwater, basal ice (after melting) and water containing various levels of sediment from the bedrock tunnel system were assayed (Figure 3-1). In three of the four subglacial meltwater samples tested the bioluminescence values (in RLU) were equivalent to control samples comprising sterile water – *i.e.* no detection of ATP was seen. The value from the remaining subglacial meltwater sample, MW 4 was approximately double the value from the control samples. The bioluminescence values from the basal ice samples were nearly an order of magnitude higher than the meltwater samples. As the basal ice samples had visible sediment particles present, and with prior knowledge of the likely association of microorganisms with sediment particles, this lead to the initial assumption that the majority of microorganisms in subglacial samples were associated with sediment particles (Sharp *et al.* 1999). Similar findings arose in testing water samples within the ambient

bedrock tunnel system where two stagnant pools were sampled before and after disturbing the sediment that accumulated at their base (Pool 1 and Pool 2). The bioluminescence values in both pools increased by a factor of two to three with a higher sediment load in the samples achieved by disturbing the sediment. This supported the initial interpretation that ATP and hence microorganisms were associated with sediment particles. Therefore one approach that may allow for the detection of ATP in similar water samples is to increase the concentration of sediment and hence microorganisms.

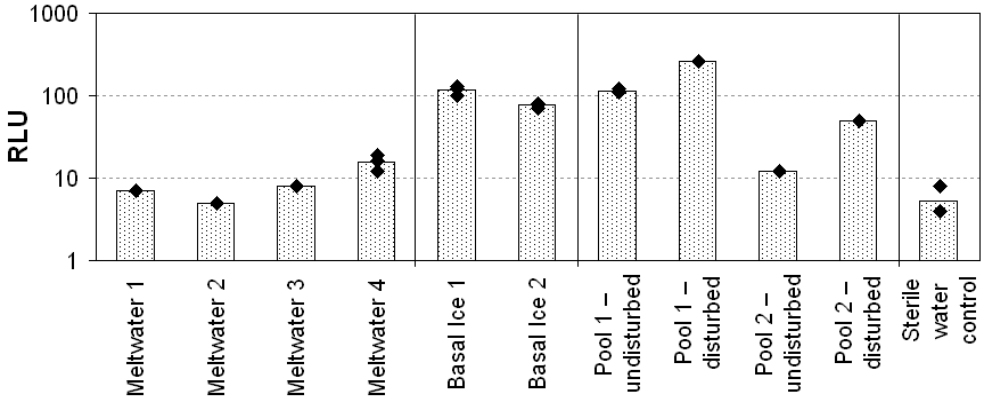


*Figure 3-1. Output from ATP bioluminescence single-use devices from April 2006 field campaign. Samples are from subglacial meltwater, basal ice and ambient bedrock tunnel system locations with up to three replicate assays per sample. Assay values are in units of relative light units (RLU) and which is proportional to ATP concentration Bars show mean values, and points show individual bioluminescence values.*

### *3.3.2. Application of single-use ATP assay devices to sediment samples in November 2007 field campaign*

In the November 2007 field campaign the single-use devices were used on sediment samples only. This was due to the association of ATP and sediment measured in samples during April 2006 (Figure 3-1), and hence it was assumed that the single-use devices would be sensitive enough to detect ATP in the sediment samples but not the water samples. As the sediment samples analysed in November 2007 were not necessarily suspended in water, samples were collected using sterile swabs. A total of nine sediments, from three different environments were collected with swabs prior to assessment with single-use devices. Figure 3-2 shows that nearly three orders of magnitude difference in bioluminescence values were observed in the nine sediment samples. The highest bioluminescence values and assumed ATP concentration were found in the periglacial sediments, intermediate levels were associated within the bedrock tunnel environment and the lowest levels were in the basal ice samples.

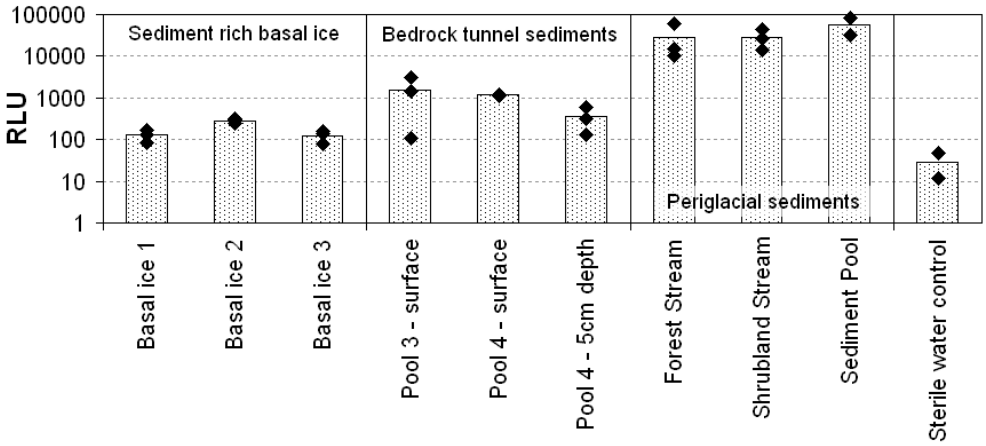


*Figure 3-2. ATP bioluminescence assay values from sediments collected from basal ice, ambient bedrock tunnel system and periglacial samples associated with water sources in November 2007 field campaign. Sediments were collected using swabs and analysed with single-use ATP assay devices. A bioluminescence signal of 1000 RLU approximates to an ATP concentration of 3.8 nM (~6 ng ATP.g$^{-1}$ sediment) from analysis of ATP standards. Bars show mean values from triplicates (except Sediment Pool and Sterile Water Control were from duplicates), and points show individual bioluminescence values.*

### *3.3.3. Application of ATP extraction protocol and alternative ATP assay reagent test-kit to water samples in November 2007 field campaign*

The in-field ATP extraction protocol was conducted within the field laboratory on water samples, three glacial and three periglacial, along with an extraction blank, which consisted of a filter paper processed in the same way as the samples (see method described in Section 3.2.4). The results in Figure 3-3 show the bioluminescence values for the periglacial samples are higher than the glacial water samples, with the Rock Pool having the highest RLU value. However the bioluminescence values of the two meltwater samples required the most attention as that they were nearly two orders of magnitude lower than bioluminescence value of the extraction blank.

To test the cause of unexpectedly low bioluminescence values in meltwater samples (Meltwater 5 and Meltwater 6 - Figure 3-3), all samples were autoclaved and an ATP spike of known concentration was added. Comparing the bioluminescence values from the spiked samples to a bioluminescence value from a spiked buffer blank, ATP spike recovery was calculated. The two meltwater samples had very low spike recovery values of 0.16 and 0.19%. The spike recovery values for the remaining four water samples were significantly higher and varied between 4.45 and 91.9% (Table 3-1). The variability of the ATP spike

recovery values lead to refinement of sample processing protocol to include a spike recovery step after initial ATP sample analysis. Along with the analysis of ATP standards, spike recovery values allowed for the conversion of bioluminescence values to ATP concentration (Table 3-1).

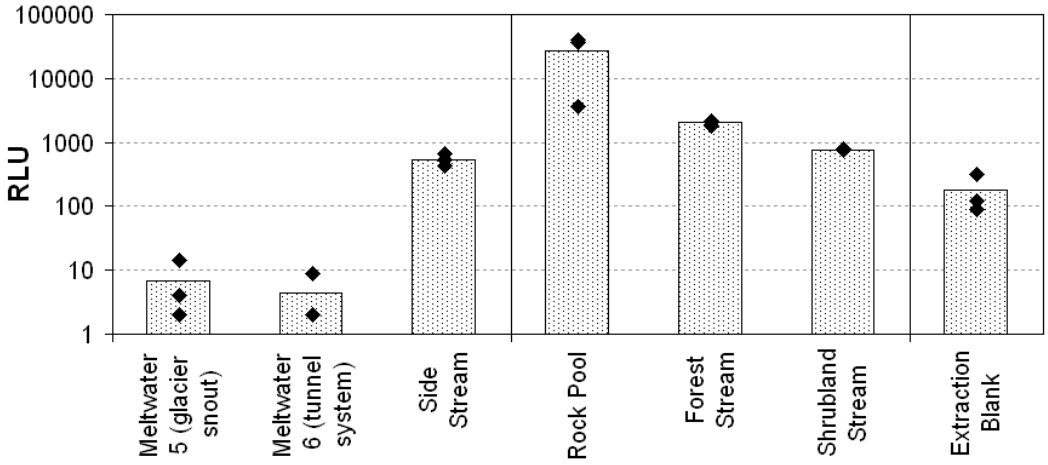


*Figure 3-3. Bioluminescence values from glacial and periglacial water samples and extraction blank obtained after use of an ATP extraction protocol and alternative ATP assay reagent test kit. Bars show mean values from analytical triplicates, and points show individual bioluminescence values.*

*Table 3-1. ATP concentrations for four water samples where the original bioluminescence values were greater than the extraction blank. All samples are corrected for ATP spike recovery and concentration during sample processing. Correlation is by linear fit to log-log plot of ATP standards co-assayed with samples.*

| Sample name | ATP spike recovery (%) | ATP concentration (pM) |
|---|---|---|
| Side Stream | 4.45 | 12.1 |
| Rock Pool | 13.5 | 225 |
| Forest Stream | 12.5 | 16.4 |
| Shrubland Stream | 91.9 | 0.730 |

### *3.3.4. Fluorescence cell counts and calculation of apparent ATP per cell*

Duplicate samples of the glacial and selected periglacial samples (in Figure 3-2 and Figure 3-3) were collected and returned to institutional laboratories to conduct fluorescent cell counts. Cell counts were used as an established measure of concentration of microorganisms and were considered to be an independent method of cell enumeration to the measurement of ATP concentration. To directly compare ATP concentrations and cells counts for the different

samples, an apparent ATP amount per cell was calculated by dividing ATP concentration by number of cells in each sample (molecular mass of 605.2 g.mol$^{-1}$ for ATP-$Na_2H_2$ • $(H_2O)_3$).

Figure 3-4 summarises the previous ATP measurement data together with the fluorescent cell count data. The glacial samples (Figure 3-4a) have a much narrower range of ATP concentration, cell counts and apparent ATP per cell, than the periglacial samples (Figure 3-4b). The results in Figure 3-4a show that only one of the glacial samples – sample "Side Stream" with apparent ATP per cell concentration of 0.08 ± 0.03 fg ATP cell$^{-1}$ – had an apparent ATP per cell concentration significantly different to previously published values of 0.16 to 2.25 fg ATP cell$^{-1}$ (Fairbanks *et al.* 1984). Figure 3-4b shows the periglacial samples exhibit a much greater spread, in terms of overall microbial concentration and the detected ATP concentration for equivalent cell counts, with only two of the six samples having concentration of ATP per cell equivalent to published values (to allow for visualisation of the non-glacial samples, the Forest Stream Sediment is not included in Figure 3-4). The Forest Stream and Shrubland Stream fell below the published values of ATP per cell and the Snow samples were above the published values.

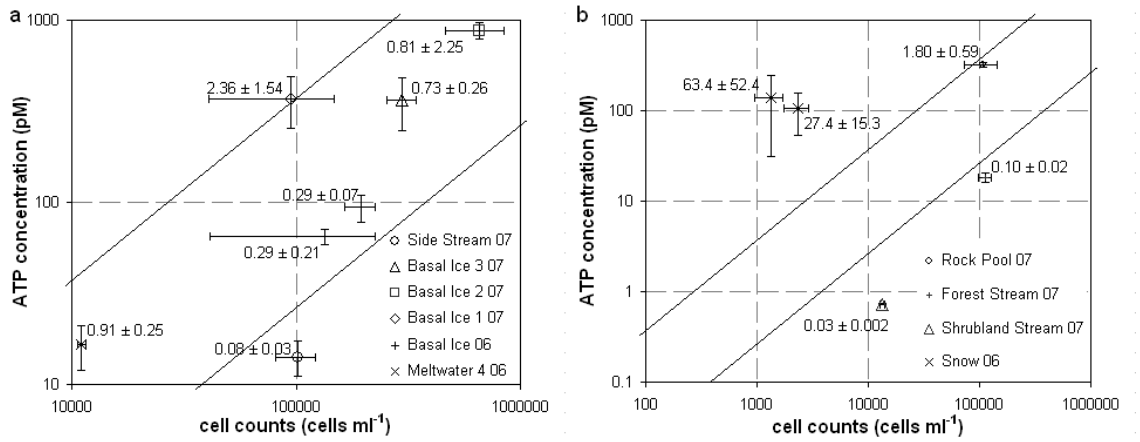


*Figure 3-4. Comparison of ATP concentration with cell counts for a range of samples from April 2006 and November 2007 field campaigns. Error bars show one standard deviation of ATP concentration from triplicates and cell counts from duplicates. The numbers and errors next to the data points are the apparent ATP per cell in fg ATP.cell$^{-1}$. (a) glacial samples, (b) periglacial samples. Forest stream sediment was not plotted in (b) to better visualise the spread in the other periglacial samples - 3.86 ± 0.12 x 10$^8$ cells ml$^{-1}$ and 120 ± 117 nM ATP giving 0.19 ± 0.18 fg ATP cell$^{-1}$. Lines represent standard curves, and are plotted using published values of ATP per cell: 0.16 and 2.25 fg ATP cell$^{-1}$ (Fairbanks* et al. *1984).*

## 3.4. Discussion

We have demonstrated, by applying an ATP bioluminescence assay, the in-field detection of ATP and therefore microorganisms in glacial and periglacial samples at low concentrations that are typical of subglacial environments (Figure 3-2, Figure 3-3 and Table 3-1).

### *3.4.1. ATP detection using the single-use devices*

The suitability of a commercially available ATP detection system consisting of ATP reagents packaged into single-use devices and portable luminometer was tested on a range of glacial and ambient bedrock tunnel samples from Engabreen in April 2006. Three of the four subglacial meltwater water samples were at or below the detection limit of the single-use devices, therefore for the subsequent field campaign in November 2007 a simple extraction protocol was implemented to analyse all water samples.

The main strengths of the single-use devices were the analytical stability and the packaging of the analytical reagents. The analytical stability meant that only one standard curve was required for each manufacturers' batch. This was emphasised by less than 8% change in RLU values for 1 and 0.1 nM ATP standards between standard curves produced on the first and final day during the November 2007 field campaign. The packaging of the analytical reagents meant that the majority of the measurements were made at the sampling site, therefore results were obtained within minutes of sample collection; problems were only encountered when the ambient temperature dropped below 5ºC as the instrument could not operate under these conditions. The main disadvantage of the single-use devices was the low flexibility in sample analysis. As the single-use devices were designed for monitoring bacterial contamination in the food and health industries the extraction chemicals used in the in-built sample processing are unlikely to be optimal for extraction of ATP from the glacial and periglacial samples tested in this study. Although extraction of ATP from samples was effective, as ATP was detected in the samples (Figure 3-2), the level of extraction efficiency and interference from sediment is unknown, hence the ATP levels measured directly with the ATP single use devices are difficult to interpret in the context of microbial enumeration.

### *3.4.2. Alternative ATP analysis using ATP reagent kit and bespoke sample extraction protocol*

The alternative assay analysis was a combination of separate assay reagent kit and extraction protocol, rather than having the two aspects combined in the form of the single-use devices. Application of the simple concentration and ATP extraction protocol (filtration of sample onto membranes, followed by subsequent extraction of ATP in boiling Tris EDTA) allowed

for lower concentrations of ATP to be detected in some of the water samples (Figure 3-3). The separate extraction and detection techniques are more flexible in terms of sample analysis, as parameters such as buffer ingredients and extraction time can be easily modified. Being able to control the contents of the extraction buffer, unlike the 'black box' system of the single-use devices, can aid in the interpretation of results and lead to improvements in ATP extraction in subsequent field campaigns. The disadvantages of the simple concentration and extraction protocol are that the technique takes longer, it requires additional, yet basic, equipment and some field 'laboratory' space. Also large samples may need to be transported to the in-field laboratory, although this can be overcome as samples can be filtered at the sampling site and then only the filter membranes need to be transported.

Using boiling Tris EDTA as an extraction buffer, ATP concentration was quantifiable in the water samples from the Side Stream, Rock Pool, Forest Stream and Shrubland Stream (Table 3-1), demonstrating that boiling Tris EDTA was a suitable extraction technique for the majority of glacial and periglacial water sources. No ice samples were processed with this method as only basal ice samples were tested during these field campaigns, and these were analysed with the single-use devices. It was not possible to detect ATP in either Meltwater 5 or 6 as they had RLU values significantly lower than the blank (Figure 3-3). The anomalously low concentrations of detectable ATP in these two subglacial water sources lead to the development of two hypotheses to the origin of these low concentrations. Either the concentration of microorganisms was below the limit of detection of the assay, or there was significant interference from the sample matrix with any ATP that was released during extraction. If the concentration of microorganisms was below the detection limit of the assay then levels equivalent to the blank would be expected, as the corresponding RLU values were significantly below this the second hypothesis was tested in the field. Samples were sterilised then spiked with a known concentration of ATP, the value of this spike was then compared to the value of a spike in Tris EDTA buffer without the sample. The spike recovery in both subglacial water samples was very low (0.16 and 0.19%), indicating that the cause for low ATP signals in the subglacial water sources is highly likely to be due to interference with the sediment. The presence of fine particulates is likely to cause significant adsorption of any released ATP during extraction. From the literature it is known that ATP recovery from soils is normally variable and incomplete and depends on soil type (Jenkinson & Oades 1979). The ATP bioluminescence reaction can be inhibited by the presence of ions (Wen *et al.* 2001) and ATP can be sequestered by the sediment matrix (Jenkinson & Oades 1979, Ciardi &

Nannipieri 1990, Contin *et al.* 2002, Wen *et al.* 2005). Wen *et al.* (2001) report interference with ATP bioluminescence reaction at ion concentrations >1 mM, however as typical ion concentrations in glacial meltwaters vary from 10s to 100s μM (Brown 2002), therefore they should not be sufficient to quench the ATP bioluminescence activity. ATP is strongly absorbed by sediment, especially by clay minerals and organic matter (Muljadi *et al.* 1966, Graf & Lagaly 1980). Due to the bedrock geology of the Svartisen Icecap (predominantly granite and schist), it is likely that the entrained sediment in the subglacial waters would be dominated by clay size particles; a visual inspection of the meltwater filter papers supports this. Therefore absorption of ATP is likely to be the dominant cause of ATP loss in these and other water samples tested here. Development of ATP extraction protocol to reduce this loss include the addition of chemical modifiers to block adsorption sites on the particulates is on-going in the authors' laboratory.

### *3.4.3. ATP concentration in Engabreen in terms of other icy environments*

The ATP concentrations found in the Engabreen glacial and periglacial system are similar to those found in other studies using ATP to detect life in the cryosphere. To enable easier comparison of the data in Figure 3-4 to other studies, some of the molar concentrations have been converted to $ATP.kg^{-1}$, assuming 1g water is equivalent to 1g sediment. The mass of ATP in the periglacial sediment (72 μg $ATP.kg^{-1}$) was within the range of concentrations of ATP measured in soils from Arctic Dry Valleys by Cowan *et al.* (2002) (~1.2 to 660 μg $ATP.kg^{-1}$), however the concentrations of ATP measured in the debris-rich ice were much lower (39 to 533 ng $ATP.kg^{-1}$). The ATP concentrations of 63.4 to 222 ng $l^{-1}$ (127 to 439 pM), found in glacial lake water by Simmons *et al*. (1983), and 120 ng $l^{-1}$ (237 pM) found in Arctic sea ice core by Dahlbäck *et al.* (1982) are equivalent to the 225 pM ATP in the highest of our water samples, Rock Pool (Table 3-1). Indicating the ATP concentrations detected at Engabreen and its surrounding environment are some of the lowest ATP concentrations detected using this technique in glacial and cryospheric environments.

### *3.4.4. Use of ATP concentration as a proxy for cell concentrations*

As ATP concentration is related to bacterial numbers it has been used as a proxy for bacterial concentration (Deininger & Lee 2001, Cowan *et al.* 2002, Seshadri *et al.* 2009). However, the mass of ATP per cell varies with cell size and activity (Bancroft *et al.* 1976, Fairbanks *et al.* 1984). Therefore cell counts were conducted on glacial and selected periglacial samples from Figure 3-2 and Figure 3-3, as a measure of cell numbers independent of ATP concentration. To compare ATP and cell count data between the different samples, the apparent ATP cell

was calculated. ATP concentration per cell is known to vary with bacterial size and activity (Fairbanks *et al.* 1984), but also additional factors including variations in preservation, processing techniques, environmental parameters and contributions from non-bacterial ATP could change apparent ATP per cell in these samples. The results in Figure 3-4 show that six of the seven glacial samples and two of the six periglacial samples had apparent ATP per cell values that were within published values of 0.16 and 2.25 fg ATP $cell^{-1}$. For the remaining samples several reasons are possible for the apparent ATP per cell values being outside of this published range, and are discussed concerning each sample. As the November 2007 samples were only preserved once returned to institutional laboratories, there could be a change in cell numbers between ATP analysis and conducting fluorescence cell counts, however this is unlikely in these samples as there are no clear differences between the apparent ATP per cell in the April 2006 and November 2007 samples. The high apparent ATP per cell in both Snow samples (27.4 ± 15.3 and 63.4 ± 52.4 fg ATP $cell^{-1}$) from April 2006 could have been artificially raised by sources of eukaryotic and extracellular ATP, hence raising apparent ATP per cell. Due to their significantly larger size, even a small number of eukaryotes and / or cyanobacteria in a sample could increase the average ATP per cell, however there is no evidence of larger organisms in the slides prepared for cell counts. Another source of non-bacterial ATP is from extracellular sources. To address the stability of extracellular ATP, studies in the Antarctic Dry Valleys indicate that ATP readily hydrolyses at low temperatures even in the Antarctic Dry Valleys where moisture content is low (Cowan *et al.* 2002, Cowan & Casanueva 2007). Engabreen has a much higher humidity therefore accelerating hydrolysis and making a significant contribution of free ATP even less likely. Three of the four filtered water samples from November 2007 (Side Stream, Shrubland Stream and Forest Stream) had lower than published apparent ATP per cell levels (0.084 ± 0.025, 0.033 ± 0.002 and 0.10 ± 0.018 fg ATP $cell^{-1}$ respectively). The low concentrations of ATP could be due to degradation in ATP, cell loss during processing, inefficient ATP extraction and / or insufficient spike recovery. The study by Cowan & Casanueva (2007) demonstrated that in natural Antarctic soil samples, free ATP had a half-life of approximately 3 hours, as the samples in this study where stored for less than an hour after ATP extraction and the boiling step in the extraction protocol should deactivate the ATPases that break down the majority of ATP, therefore ATP degradation in these samples should be limited. Inefficient extraction of ATP from samples and insufficient spike recovery values are inconsistent with the much higher apparent ATP per cell for the Rock Pool that was within published values of ATP per cell (1.8 ± 0.58 fg ATP $cell^{-1}$). None of the preceding explanations can singly satisfactorily explain the

discrepancy between published ATP per cell and some of the apparent ATP values per cell in Figure 3 however these discrepancies could be due to several of these factors. In addition it is not known if these published ATP values per cell are valid for the microorganisms that are present in these types of environments, as is has been demonstrated that psychrophiles react differently to mesophiles at low temperatures, including an increase in ATP concentration during cold shock whereas mesophiles lower their ATP concentration (Napolitano & Shain 2004). Further investigations into the effect of ATP concentration in bacteria in natural glacial and subglacial environments would be needed to confirm whether these apparent ATP per cell values are real or due to several of the aforementioned reasons. Despite the discrepancy between ATP concentration and cell count data ATP concentration is still a useful indicator of microbial concentration in samples, however if comparing data to work from other locations or between significantly different sample matricies, interpretation should be cautious, or independent confirmation of cell concentrations made.

## 3.5. Conclusions

Using ATP bioluminescence assays it was possible to detect ATP in subglacial samples in the field from Engabreen. Two commercially ATP bioluminescence assay formats were used (single-use devices and separate assay reagent and extraction protocol) and between them ATP was successfully analysed in ice, sediment and periglacial water samples. The single-use devices were convenient as could be used at the sampling site to obtain results within minutes of sampling, however these were only appropriate for samples with higher ATP concentration, such as in the sediments. The separate assay and sample extraction protocol provided greater flexibility in terms of sample type as it allowed for concentration of targets through filtration of sample onto membrane, ATP was then extracted in boiling buffer.

The distribution of ATP concentration in Engabreen was similar to the expected distribution of microorganisms; with higher concentrations of microorganisms being associated with the sediment. However limits should be placed on interpretation of what this data means in terms of microbiology. This study has highlighted two areas of further research which would improve confidence in data interpretation. Firstly, the development of a method for extraction of low levels of ATP from the subglacial meltwater samples, and other samples with similar matrix issues, *i.e.* high levels of clay minerals. Secondly, investigations to resolve the cause(s) of the discrepancies between ATP estimates and cell count data in the water samples. Understanding this would improve the robustness of ATP as a biomarker especially when comparing different sample types. Despite these caveats, the ATP concentration in most water

samples could be corrected for interference from the sediment, and periglacial rock pool had an apparent ATP per cell within published data and ATP concentration (225 pM) was comparable to water samples from Antarctic lakes (127 to 439 pM) and in Arctic sea ice core (237 pM) (Simmons *et al.* 1983, Dahlbäck *et al.* 1982).

The use of the ATP bioluminescence assay to detect ATP in samples in and around Engabreen allowed for the completion of multiple cycles of analysis. Generally these multiple cycles of analysis can lead to in-field development of hypotheses, sampling strategies and sample processing which can allow for the completion of unique studies and improve the efficiency of scientific return per field campaign. In this case, detecting ATP in the field has allowed for the in-field development and testing of the hypothesis that the low ATP concentration of ATP in subglacial waters was due to interference from the sediment, and the refinement of sample processing protocols (the addition of an ATP spike recovery step to correct for ATP absorbance).

## Acknowledgements

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# 4. In-field implementation of a recombinant Factor C assay for the detection of lipopolysaccharide as a biomarker of extant life within glacial environments

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## Abstract

The discovery over the past two decades of viable microbial communities within glaciers has promoted interest in the role of glaciers and ice sheets as contributors to subglacial erosion, global biodiversity, and the regulation of global biogeochemical cycles. *In situ* or in-field detection and characterisation of microbial communities is becoming recognised as an important approach to improve our understanding of such communities. Within this context we demonstrate, for the first time, the ability to detect Gram-negative bacteria in glacial field-environments (including subglacial environments) via the detection of lipopolysaccharide (LPS); an important component of Gram-negative bacterial cell walls. In-field measurements were performed using the recently commercialised PyroGene® recombinant Factor C (rFC) endotoxin detection system and used in conjunction with a handheld fluorometer to measure the fluorescent endpoint of the assay. Twenty-seven glacial samples were collected from the surface, bed and terminus of an Arctic valley glacier (Engabreen, Northern Norway), and were analysed in a field laboratory using the rFC assay. Sixteen of these samples returned positive LPS detection. This work demonstrates that LPS detection via rFC assay is a viable in-field method and is expected to be a useful proxy for microbial cell concentrations in low biomass environments.

Keywords
Lipopolysaccharide, endotoxin, pyrogen, portable life detection, recombinant Factor C, cryosphere, subglacial

## 4.1. Introduction

The ability to quickly quantify microbial populations at a sampling site has powerful applications across many areas of science and industry. The development of rapid in-field detection methods for generic microbial populations in both food and health industries has been made possible by the detection of biomarkers, *i.e.* chemical markers unique to extant life (Aycicek *et al.* 2006; Lipscomb *et al.* 2006; Rasmussen *et al.* 2008). The best known example of such an approach is the detection of the generic biomarker adenosine triphosphate (ATP) using off-the-shelf ATP bioluminescence kits (Davidson *et al*. 1999).

In the environmental sciences, sample analysis at the sampling site or in a field laboratory offers many advantages. These include: (i) reducing sources of error by removing the need to modify samples for transport and extended storage, (ii) collecting further samples to clarify unexpected results, and enabling the in-field development and refinement of (iii) protocols, (iv) sampling strategies and (v) hypotheses. Together these advantages offer reduced environmental impact and reduced resource usage, for a given scientific return, when compared to the well established approach of sample collection, transport and subsequent analysis in institutional laboratories. Finally, obtaining results in the field provides the opportunity for the completion of unique experiments not achievable by conventional approaches *e.g.* improved spatial and temporal resolution normally restricted by the logistics of sample transportation.

Icy ecosystems, in particular glaciers, are now known to be viable habitats for microbial life (Sharp *et al.* 1999, Skidmore *et al.* 2000, Foght *et al.* 2004, Gaidos *et al.* 2004, Mikucki *et al.* 2004, Skidmore *et al.* 2005), yet little is known about microbial abundances or their relevance to biogeochemical processes and therefore to global biogeochemical cycles. Due to the rapidly changing nature and the remoteness of many icy environments, they provide a prime example of where the development of in-field measurements is paramount (Siegert *et al.* 2007). One approach to address the difficulty of collecting microbiological data is to detect biomarkers (proxies for microbial abundance, activity and population diversity), as many of these biomarkers can be detected by simple assays. In order to apply these techniques to in-field biomarker detection and quantification in glacial samples they have to be compatible with environments that are remote, hostile, operationally difficult, and often have low

biomass (Hodson *et al*. 2008). Within the current work, we focus on the detection of lipopolysaccharide (LPS), as a biomarker, in samples from an Arctic glacier. LPS is an essential component of the cell wall of Gram-negative bacteria and, due to the abundance of Gram-negative bacteria, has been used as a proxy for extant microbial life (Watson *et al.* 1977). Gram-negative bacteria are widely found in icy environments having been identified in glacial samples from New Zealand (Foght *et al.* 2004) and China (Xiang *et al.* 2005); in Arctic and Antarctic pack ice (Brinkmeyer *et al.* 2003); in Antarctic lake ice (Mosier *et al.* 2007) and in snow from Svalbard (Amato *et al.* 2007). Thus they are likely to be present in most glacial environments.

Established techniques for the quantification of LPS, or endotoxins, exist in the pharmaceutical industry as these compounds are potentially toxic if they enter the blood stream (Bang, 1956, Levin *et al.* 1970). The terms LPS and endotoxin are often used interchangeably in the literature, but in this work we use the term LPS. Currently the standard method for the determination of LPS concentration is the *Limulus* amoebocyte lysate (LAL) assay. The LAL assay uses the hemolymph of horseshoe crabs (*Limulus*) which naturally clots in the presence of LPS. Commercial preparations of the hemolymph react to the presence of LPS producing various types of optically readable endpoints. The LAL assay has been used as a proxy for microbial concentration in various natural environments, particularly where biomass is low: hot springs in the High Arctic (Steele *et al.* 2004), marine sediments (Watson *et al.* 1977), marine water profiles (La Ferla *et al.* 2004) and in Subglacial Lake Vostok accretion ice (Karl *et al.* 1999). The standard LAL assay is relatively complex, requiring skilled operators and normally requiring multiple stages of analysis and therefore has been performed in established institutional laboratories on collected ice and water samples (Karl *et al.* 1999, La Ferla *et al.* 2004) and onboard a research vessel (Watson *et al.* 1977). In-field detection of LPS was conducted by Steele *et al.* (2004) where a commercially available microfluidic LAL-based LPS detection system was used (Endosafe®-PTS™, Charles River, MA, USA). Although this system is applicable to field use, being portable and rapid, the system has been developed for pharmaceutical use and is not flexible in terms of sample type and data analysis, thus making it difficult for development with environmental applications. The preceding work has shown the potential of LPS as a proxy for microbial abundance in low biomass environments, *e.g.* the detection of $<10^4$ cells per ml, (Karl *et al.* 1999). Here we build on this work by demonstrating the detection of LPS in the field in a glacial environment by the use of a recently released commercial product, the Cambrex PyroGene® recombinant

Factor C LPS detection system. Briefly, the LPS activated protein (Factor C) from the LAL clotting cascade, has been isolated (Nakamura *et al.* 1986) and a recombinant form produced. This recombinant Factor C (rFC) has subsequently been developed into a fluorescence-based assay using an artificial non-fluorogenic substrate for rFC. The rFC is activated in the presence of LPS and results in the cleavage of the artificial substrate and liberation of a fluorescent product. The rFC assay has sensitivity equivalent to the LAL test (Ding & Ho, 2001) and more significantly, is simpler to implement and therefore is more suited to LPS detection in the field than the traditional LAL assay.

Thus, the specific objectives of the current study are to (i) demonstrate that rFC assays can be performed in a field environment, (ii) demonstrate rFC assays can be used in the field to detect LPS in glacial samples and (iii) explore field-based sample processing protocols for the rFC assay. To achieve the study objectives the unique facilities at, Engabreen (Norwegian Arctic) were exploited, where the Svartisen Subglacial Laboratory allows direct access to pristine subglacial samples.

## 4.2. Materials and Methods

### *4.2.1. Field site*

Samples were collected and analysed at Engabreen in November 2007, November 2008 and March 2009. Development of an in-field assay protocol was conducted during November 2007, and during November 2008 and March 2009 samples were collected for systematic analysis. Engabreen is a temperate outlet glacier draining the Svartisen Ice Cap, Northern Norway (66˚41'N, 13˚46'E). Facilities provided by the Norwegian Water Resources and Energy Directorate (NVE) allowed direct access to pristine samples at the glacier bed via Svartisen Subglacial Laboratory (Lappegard *et al.* 2006). Access to the glacier bed was through a series of tunnels drilled into the bedrock. These lead to locations where fresh subglacial meltwater and basal ice (amongst others types of samples) were collected. Pristine basal ice samples were collected from a temporary ice cave bored into the glacier using hot water in the research shaft. All in-field laboratory analyses were conducted in a field-laboratory housed within the subglacial rock tunnels.

### *4.2.2. General materials, methods and shipping*

All consumable items were freighted or hand carried to the field-site. The assay reagents and fluorometer were hand carried to the field site where the rFC assay kit was transported in a cool box, with cool packs.

Pipette tips and Eppendorf tubes were sterilised by autoclave, both prior to shipment to the field-site and at the field-site. Glassware and aluminium foil were depyrogenated by heating to 250˚C for 4 hours prior to shipment and packaged in depyrogenated foil. The water used to dilute standards and blanks was certified endotoxin free (supplied with rFC assay kit) and to minimise shipping, local subglacial water (filtered through 0.22 µm pore cellulose membrane and autoclaved three times) was used to make buffer solutions needed in the additional sample processing protocols. All chemicals were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

*4.2.3. Sample collection and handling*

Sediment, ice and meltwater samples were collected from the glacier snout and proglacial plain (subaerial samples), and subglacial environments (via the Svartisen Subglacial Laboratory), and were collected on different days during three one-week-long field campaigns from the 19$^{th}$ to 27$^{th}$ November 2007, the 19$^{th}$ to 26$^{th}$ November 2008 and the 18$^{th}$ to 27$^{th}$ March 2009. In November 2008 and March 2009 a systematic sampling strategy was applied to collect equivalent samples, as far as practical, from both field campaigns. The November 2008 and March 2009 the samples clustered into three sample locations: subglacial meltwater collected from bedrock tunnels directly beneath the glacier; basal ice samples from the ice cave; and subaerial samples *i.e.* external to the Svartisen Subglacial Laboratory and tunnels (Figure 4-1). The subglacial meltwater samples (coded MW 1 to 5) were collected from different locations along a ~600 m tunnel (the Spiral Tunnel and Stream), sites MW 1 and 2 were under the centre of the glacier and site MW 5 was the closest to the glacier margin. The samples were collected from subglacial inlets in the tunnel roof (sites MW 1, 2 and 3) and from fast flowing portions of the meltwater stream (sites MW 4 and 5). Site MW 5 was at the confluence of an additional subglacial meltwater stream and associated tunnel close to the research shaft (RS in Figure 4-1). The Spiral Tunnel consists of purely subglacial meltwater and the additional stream mainly drains snowmelt and / or rainwater. Basal ice samples (coded BI 1 to 6) were collected along a transect, from ice in contact with bedrock into glacial ice, where the total perpendicular distance from the bedrock contact to the furthest bulk ice sample was ~5 m. BI 1 to 3 were debris rich ice samples, where BI 1 was in contact with the bedrock and BI 3 was near the contact with the glacial ice. BI 3 to 6 were glacial ice (or sediment poor ice), where BI 4 was near the contact with debris rich ice and BI 6 was furthest from the contact. The external samples consisted of snow, and water from englacial, proglacial, and forest streams (locations in Figure 4-1).

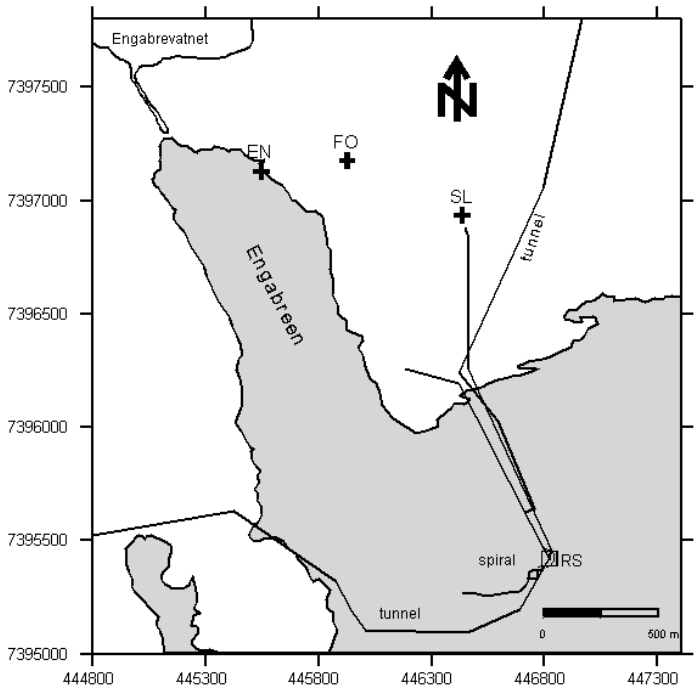


*Figure 4-1 Map of Engabreen area with locations of the subaerial stream sampling sites and water access tunnels (black lines). EN – englacial stream, FO – forest stream, SL – shrubland stream and Engabrevatnet is the proglacial lake. The proglacial stream sample in 2008 was in the position same as EN (2009), the snow samples were collected from near the shrubland stream site, and the basal ice samples were collected from the research shaft (RS). The outline of the glacier is from 1995, but similar to November 2008 and March 2009.*

All water samples were collected in autoclaved 250 ml Beckman bottles (Beckman, USA), or in sterile 1.5 ml Eppendorf tubes and returned to the field-laboratory for processing and analysis. All bottles were rinsed at least three times with sample water before being filled. Sediment samples were collected in 50 ml sterile centrifuge tubes. Snow samples were placed into sterile sample bags using gloves wiped in 96% ethanol. The basal ice samples were collected from the ice cave in the research shaft. In 2007 and 2008 this was conducted using an ice axe or chisel, previously wiped with 96% ethanol. In 2009 the samples were extracted using a chainsaw. Ice was wrapped in depyrogenated foil within the ice cave, moved to the field-laboratory and melted overnight in clean autoclave bags or sterile sample bags. The surface of the chainsaw collected samples was washed with ethanol and then with warm autoclaved water to remove surface contaminants. All samples were assayed with 24 hours of collection or melting.

*4.2.4. Recombinant Factor C assay*

The Cambrex PyroGene® recombinant Factor C (rFC) endotoxin detection system (product code 50-658U, Lonza, Slough, UK) was used for the study. Each assay comprised a 50 µl sample mixed with 50 µl of the rFC working reagent (proenzyme, fluorescent substrate and buffer), made up as per manufacturer guidelines. Assays were incubated at ambient field-laboratory temperature for 3 hours, where the temperature varied between +17˚C and +21˚C. Fluorescence measurements were made using a handheld *Pico*Fluor fluorometer (Turner BioSystems, CA, USA) at time 0, and then after 1, 2 and 3 hours incubation time in November 2007 and every 30 minutes for 3 hours in November 2008 and March 2009. Readout of the fluorometer was in Relative Fluorescence Units (RFU). To allow for the small reagent volumes indicated in the manufacturer guidelines, the *Pico*Fluor was used with a mini-cell adapter and 70-250 µl borosilicate glass cuvettes (product code WU-13095-56, Turner BioSystems, Cole Parmer, London, UK). All samples were assayed in triplicate.

The LPS standards supplied with the rFC kit are quoted in endotoxin units (EU), which is a measure of the biological activity of LPS. LPS standards of 10, 1, 0.1 and 0 $EU.ml^{-1}$, were tested for each batch of working reagent.

An alternative to the standard data analysis described in the rFC assay kit was implemented in the current study. This was implemented to allow for (i) non-standard and varying assay incubation temperatures and (ii) uncertainty in the time evolution of the assay under in-field conditions. The implemented analysis comprised of plotting the fluorescence intensity as a function of assay time. For the majority of samples analysed, when using an appropriate incubation time, this resulted in a linear relationship. Therefore for all samples and standards analysed, a linear function was fitted to the data (between time zero and the end of the incubation period) using the least squares method. $R^2$ values were calculated for each assay to assess the appropriateness of the linear model for each analysis. The gradient of the fitted function (termed assay response, in $RFU.min^{-1}$) was used for further data analysis. LPS concentrations in samples were quantified from calibration data sets produced from the LPS standards, where a linear function was fitted to a log-log plot of assay standards and assay responses. The lower limit of detection of LPS was defined as assay response of the appropriate blank plus three times the standard deviation of the assay response of the blank.

*4.2.5. Sample processing protocols*

A sample processing protocol was defined that reflected a desire to minimise field-based sample processing (minimal processing protocol). To assess whether more involved, but still

field deployable, sample processing would improve analytical performance (*i.e.* decreased lower limits of detection), two additional sample processing protocols were tested (additional protocol A and additional protocol B). Three sample types were considered: water, ice and sediment.

**Direct analysis (minimal processing protocol)**

The water samples were directly assayed. For sediment samples an equal volume of LPS free water was added to the sediment, sample was shaken vigorously for 10 seconds and immediately a liquid aliquot including suspended particulates was taken and assayed. Ice samples were melted at ambient laboratory temperature and moved to +3°C storage once melted. The melted ice samples were then agitated to suspend fine particulate and a representative liquid aliquot was assayed.

**Boiling in Tris EDTA buffer (additional protocol A)**

For water samples, 490 ml of sample was filtered through an autoclaved 47 mm diameter, 0.22 µm pore cellulose membrane (Millipore, FDR-293-050F, Fisher, Loughborough, UK) using a polysulfone filter unit and hand vacuum pump. The filter membrane was folded and placed into a 1.5 ml Eppendorf tube and physically broken, using flamed metal tweezers, to improve subsequent access to the extraction buffer. To this, 1 ml of boiling 100 mM Tris, 4 mM EDTA extraction buffer was added, followed by incubation in boiling water for one minute. Tubes were cooled and stored at +3˚C and representative liquid aliquots assayed within 24 hours. Due to the high volume of sample filtered, this extraction protocol was appropriate for water samples with low sediment concentrations. Thus, ice and sediment samples were not used for this method.

**Ultrasonic treated at ambient temperature in Tris saline buffer (additional protocol B)**

For water and melted ice 15 ml of sample and for sediment 2 g of sample was added to 15 ml of 25 mM Tris, 100 mM sodium chloride buffer in 50 ml centrifuge tubes. The tubes were subjected to three sets of 10 seconds duration in small ‘hobby’ sonicator (James Products Ltd, Ultra 6000WS, product code A49FW, Maplin Electronics, Rotherham, UK). Solutions were cooled in an ice bath between each sonication treatment. Samples were agitated and then a representative liquid aliquot was assayed. This processing protocol was most suited to sediment and sediment-rich ice samples, however water samples were also tested.

## 4.3. Results

### *4.3.1. Adaptation of rFC assay protocol for in-field analysis*

The manufacturer's protocol for performing the rFC assay state a requirement for incubation of the mixed sample and assay working reagent at +37˚C in a fluorescence microplate reader, and that fluorescent intensity measurements should be taken at time zero and after one hour incubation; the difference between the two values should be used to calculate LPS concentration. The logistics of establishing the rFC assay at a glacial field-site precluded the use of a standard laboratory specification fluorescent microplate reader with incubation temperature control. A non-temperature controlled handheld fluorometer compatible with reagent volumes needed for the rFC assay and easily transportable to the field site was identified and used. Incubation was conducted in a field laboratory with an internal ambient temperature in the range of +17˚C and +21˚C. This adaptation to the method necessitated some modifications to the manufacturer's assay instructions, and these were studied prior to the field campaigns.

Due to the expected lower and fluctuating incubation temperatures encountered in the field laboratory, a lower rate of fluorescence intensity development for the assay was anticipated, therefore requiring a longer incubation period to develop a suitable signal for a given LPS concentration. In order to test this hypothesis and to determine the required length of incubation time, the rFC assay was conducted with LPS standards (10, 1, 0.1, 0.01 and 0 $EU.ml^{-1}$) in an institutional laboratory with an ambient temperature of +20 to +22˚C over an incubation period of 6.5 hours. Figure 4-2 shows the fluorescent intensities recorded at half hourly intervals using the handheld fluorometer, and the corresponding calibration curves if the assay was stopped after 1, 3 and 6.5 hours. The 10 $EU.ml^{-1}$ LPS standard started to plateau after 4 hours (not shown in Figure 4-2a), but the 1 $EU.ml^{-1}$ LPS standard continued to increase in intensity linearly (by visual inspection) during this time. Figure 4-2b shows the assay response for the 0 and 0.1 $EU.ml^{-1}$ LPS standards is greatest when the incubation time is limited to one hour, which is an artefact of the low RFU values for each standard at time zero, as seen in Figure 4-2a. The lower time zero data points have a greater effect on the standards when the incubation time is short. Figure 4-2a is normalised to the blank, the standard deviations (SD) of triplicates of the standards including the blank are included to demonstrate the ability to discriminate between the standards.

The results in Figure 4-2a show that if the manufacturer's recommended one hour incubation time was to be followed with an incubation temperature similar to the expected field

laboratory conditions, then discrimination between the 10 and 1 EU.ml$^{-1}$ standards and between these and 0 EU.ml$^{-1}$ standard was possible however no significant discrimination between 0.1 and 0 EU.ml$^{-1}$ standards could be made. When the incubation time was extended to 3 hours, then discrimination among 10, 1 and 0.1 EU.ml$^{-1}$ standards and between these and 0 EU.ml$^{-1}$ standard was now possible. Extending the incubation time beyond 3 hours does improve the separation between the 0.01 and 0 EU.ml$^{-1}$ standards however it is only a minor increase. Also, extending incubation time to 3 hours is still practical for field analysis.

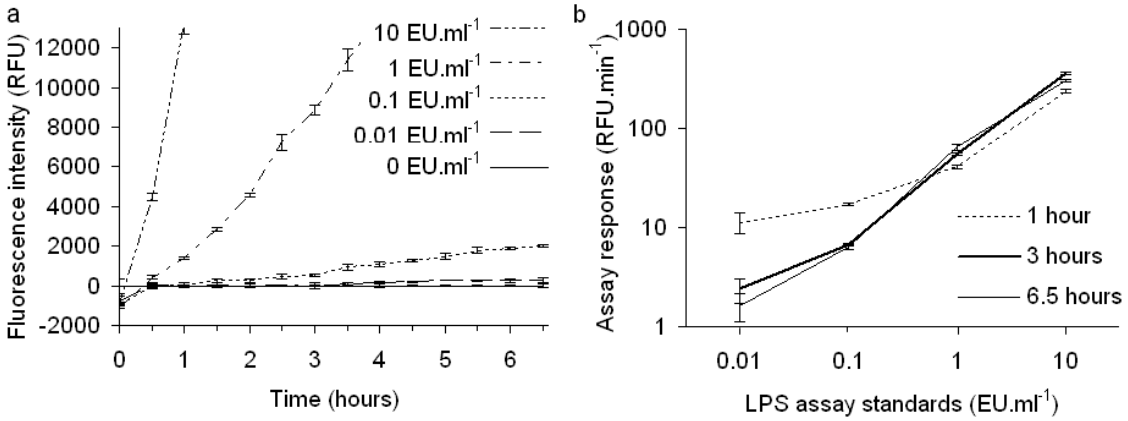


*Figure 4-2. Affect of non-standard incubation temperature on the rFC assay using LPS standards to determine modified rFC assay protocol for field deployment. The 10, 1, 0.1 and 0.01 EU.ml$^{-1}$ standards are normalised to the 0 EU.ml$^{-1}$ standard. a) Time evolution of the LPS standards, y-axis is truncated, the 10 EU.ml$^{-1}$ standard starts to plateau after 4 hours and 1 EU.ml$^{-1}$ standard continues to increase linearly (RFU = relative fluorescence units). b) Calibration curves at 1, 3 and 6.5 hours (i.e. assay response between t = 0 and time of interest). Error bars represent ±1 SD from triplicates.*

Since the in-field assay response of the rFC assay and its interaction with environmental samples was unknown prior to field deployment, it was considered prudent to collect fluorescence intensity data at a minimum of hourly intervals during the 3 hour incubation period in addition to the initial and final data points. This would allow confirmation of the expected time evolution of the assay response as determined in the laboratory (Figure 4-2a) and any significant deviation from this was identified by calculation of Coefficient of Determination ($R^2$) values.

### *4.3.2. Testing of additional sample processing protocols for glacial samples for use in rFC assays*

To assess the effect of minimal sample processing (*i.e.* minimum amount of processing required to obtain liquid samples, *e.g.* melting ice) compared to protocols with additional

sample processing for LPS detection, we conducted a range of tests on field samples during the November 2007 field campaign. The additional sample processing protocols comprised of filtration then boiling of the filter membrane in Tris EDTA buffer (additional protocol A) and ultrasonic treatment at ambient temperature in Tris saline buffer (additional protocol B). For each additional sample processing protocol, a set of six different samples was analysed and compared to the same sample set that underwent the minimal sample processing protocol (Table 4-1). Due to operational reasons, different sample sets for additional protocol A and B were used.

*Table 4-1 Assessment of two additional sample processing protocols for LPS determination in glacial samples when compared to the use of the minimal sample processing protocol. Note that all assay responses are derived from samples analysed in triplicate.*

| Sample description | Assay response ($RFU.min^{-1}$) | | $R^2$ | |
|---|---|---|---|---|
| | Default protocol | Additional protocol | Default protocol | Additional protocol |
| Boiling in Tris EDTA buffer (additional protocol A) | | | | |
| Blank | 0.74 | 0.74 | 0.240 | 0.215 |
| Pro-glacial stream (snout) | 14.31 | -0.98 | 0.946 | 0.312 |
| Pro-glacial stream (side) | 19.99 | -0.84 | 0.975 | 0.495 |
| Periglacial iron-rich pool | 1.83 | 1.22 | 0.626 | 0.658 |
| Subaerial forest stream | 14.82 | 12.49 | 0.994 | 0.969 |
| Subaerial scrubland stream | 4.37 | 0.23 | 0.962 | 0.165 |
| Subglacial meltwater 4 (day 4) | 5.57 | -0.72 | 0.856 | 0.322 |
| Ultrasonic treated at ambient temperature in Tris saline buffer (additional protocol B) | | | | |
| Blank | -0.66 | -0.92 | 0.914 | 0.592 |
| Subglacial meltwater 5 (day 6) | 1.22 | -0.53 | 0.657 | 0.962 |
| Bedrock tunnel sediment (site MW 5) | -0.32 | 10.47 | 0.916 | 0.981 |
| Bedrock tunnel water (stagnant pool) | 80.89 | 8.99 | 0.958 | 0.979 |
| Bedrock tunnel sediment (stagnant pool) | 26.25 | -3.71 | 0.998 | 0.972 |
| Glacial basal ice | -5.29 | -2.25 | 0.977 | 0.606 |
| Groundwater (borehole) | 16.62 | 0.21 | 0.950 | 0.057 |

The results in Table 4-1 show that three of the seven samples (including the blank) processed using additional protocol A had a similar assay response to the minimal processing protocol

(*blank*, *subaerial iron-rich pool* and *subaerial forest stream*). However, for the remaining four samples, the assay response of additional protocol A was significantly lower than the assay response of the minimal processing protocol, and was near or below zero (0.23 to -0.98 RFU.min$^{-1}$). Comparing the assay responses of the minimal processing protocol and additional protocol B, the assay responses were lower for additional protocol B than for the minimal protocol in all samples (including the blank) except for the *bedrock tunnel sediment* sample where it was higher. Table 4-1 shows neither the assay responses nor the $R^2$ values observed for both of the additional sample processing protocols showed a consistent improvement in comparison to the minimal sample processing protocol. Hence for the November 2008 and March 2009 field campaigns, the minimal sample processing protocol was used exclusively.

### *4.3.3. In-field use of rFC assay to detect LPS in glacial samples with minimal sample processing*

To confirm that the in-field rFC assay protocol was a repeatable technique when conducted in the field, LPS assay standards of 10, 1, 0.1 and 0 EU.ml$^{-1}$ were tested on each day the assay was completed during the November 2008 and March 2009 field campaigns, producing what is referred to as a LPS calibration set for each day. Figure 4-3 shows that within each LPS calibration set, there is a clear positive relationship between LPS concentration and rFC assay response, and that the individual LPS assay concentration standards are easily discriminated.

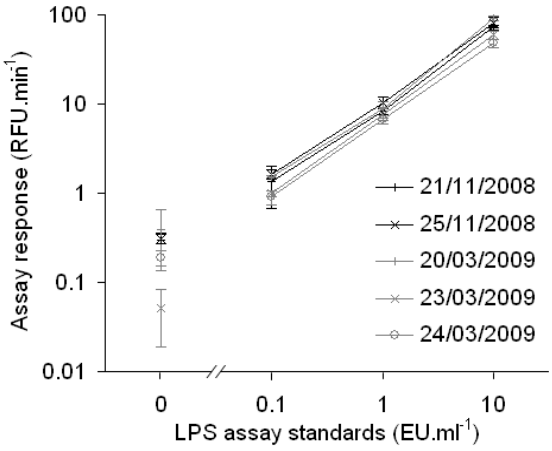


*Figure 4-3. Calibration curves produced on the five days during the 2008 and 2009 field campaigns* i.e. *rFC assay response for 10, 1, 0.1 and 0 EU.ml$^{-1}$ standards. Error bars indicate ±1 SD of the assay response from triplicates. Note log scale and broken x-axis to allow for visualisation of the 0 EU.ml$^{-1}$ standards.*

Furthermore, for a given LPS concentration standard, the spread of assay responses between the five different LPS calibration sets is acceptable.

To demonstrate that the rFC assay could detect LPS in a variety of glacial samples in the field, 27 samples from various subglacial and subaerial locations (locations described in Section 4.2.3) were tested for the presence of LPS. Figure 4-4 shows LPS concentrations measured in the samples assayed.

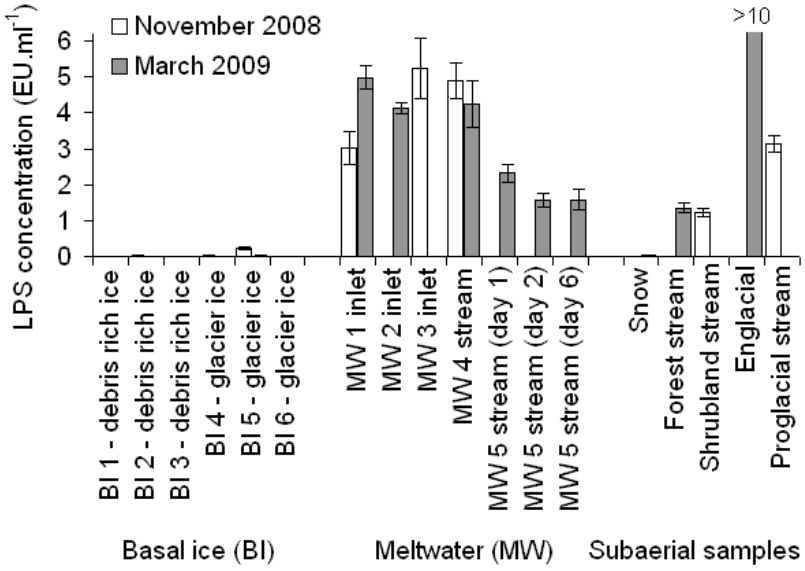


*Figure 4-4. Determination of LPS levels in 27 glacial samples using the rFC assay. Details of the sampling sites are described in Section 4.2.3. All assays were performed with no significant sample processing (minimal processing protocol). LPS concentration was quantified by using the daily LPS calibration sets, assuming a linear interpolation from a log-log plot of LPS standards (10, 1 and 0.1 EU.ml$^{-1}$) and the assay responses. The y-axis (LPS concentration) has been truncated (i.e. the value for 2009 englacial water is clipped) to enable better visualisation of the relationship of the LPS concentration values of the other samples. Error bars are ±1 SD of assay responses from triplicates.*

The results in Figure 4-4 show that LPS was detected in 16 of 27 samples (*i.e.* sample assay response was greater than the assay response of the blank, plus three times the standard deviation of the assay response of the blank). The presence of LPS was detected in all nine of the subglacial meltwater samples, in five of the six external samples (excluding sample *2008 snow*), and in only two of the twelve basal ice samples (samples from site BI 5 from both campaigns). Using the LPS calibration sets (10 to 0.1 EU.ml$^{-1}$ standards), LPS concentration could be quantified in 13 of the 16 samples with detectable LPS. Of the three samples where LPS was detectable but not quantifiable, two had an LPS concentration <0.1 EU.ml$^{-1}$ (samples

*2009 snow* and *2009 BI 5*) and one had an LPS concentration >10 EU.ml$^{-1}$ (sample *2009 englacial*). Figure 4-4 shows there is two to three fold variability in the nine subglacial meltwater samples (3.56 ± 1.46 EU.ml$^{-1}$). However the three samples collected from site MW 5 (1.83 ± 0.43 EU.ml$^{-1}$) are consistently lower than the six samples from sites MW 1 to 4 (4.43 ± 0.81 EU.ml$^{-1}$). The subaerial samples show the most variability, including samples below 0.1 EU.ml$^{-1}$ and above 10 EU.ml$^{-1}$. The $R^2$ values of the assay response to the RFU values for the 16 samples with detectable LPS ranged from 0.978 to 0.998, whereas the $R^2$ values of the remaining 11 samples ranged between 0.073 and 0.997 (data not shown).

## 4.4. Discussion

The results in Figure 4-2 show that, in order to use the rFC assay in the field under ambient conditions, modifications to the recommended manufacturer's protocol were necessary. Firstly, the manufacturer's protocol required an incubation temperature of +37°C. Since the laboratory at our glacier field site was between +17°C and +21°C we developed and demonstrated a modified assay protocol that involved a longer incubation period – increased from a manufacturer's stated time of 1 hour to 3 hours. This allowed sufficient assay signal to be developed to discriminate LPS concentrations over two orders of magnitude. The increased incubation period can be accounted for by the expected reduced reaction rate at lower temperatures. The assay of a LPS calibration standard data set in parallel with all sample analyses enabled for the correction of any variation in the assay reaction rate as a function of temperature and enzyme activity, which can be modified by non standard storage conditions such as those encountered during transportation. We note that under colder ambient conditions in many glacial environments, the incubation time may need to be increased above 3 hours. Further studies are required to define appropriate incubation periods for lower incubation temperatures. At very low temperatures, field deployable incubation equipment such as a small dry block heater may be required. A second modification was due to the manufacturer's stated assay protocol that required only fluorescent measurements at the beginning and end of the incubation period. Due to the uncertainty surrounding the effect of sample matrix on the time evolution of the assay response, the data set was increased by the collection of additional fluorescent measurements during the incubation period. For the November 2008 and March 2009 samples, unexpected, non-linear time evolution of the assay responses (meaning $R^2$ values < 0.95) was observed only in samples where LPS was not detectable.

As the manufacturer's application of the rFC assay is to test human and animal parenteral drugs, biological products and medical devices, the intended sample types for the commercial assay are considerably different to glacially relevant samples. Therefore two additional sample processing methods, additional to the minimal processing protocol needed to have samples as a liquid, were considered with the intention of decreasing the lower limit of detection, by filtration (concentration) and / or cell lysis (to liberate LPS into a more available form). The two additional processing methods were chosen as they were also being considered as sample processing methods for other analysis techniques being used in the same field campaign (November 2007). Additional protocol A consisted of filtration of a water sample to concentrate microbial cells on the filter membrane, followed by boiling the membrane in Tris EDTA buffer. Despite a 490-fold concentration step involved (see Section 4.2.5), no improved LPS detection was observed compared to the minimal protocol. The assumed reason for this was the strong adsorption of LPS to the cellulose filter paper used in the protocol (Douwes *et al.* 1995). Additional protocol B consisted of ultrasonic treatment at ambient temperature in Tris saline buffer, with no prior filtration. The ultrasonic treatment was intended to lyse microbial cells and thus liberate LPS into a more accessible form for the rFC assay reagents. No consistent improvement in LPS detection was observed, which is probably due to a number of factors including that the bacterial cells were likely to have been lysed by the rFC reagents, as observed with the LAL assay (Holzheimer, 1998), therefore the power of the 'hobby' sonicator used in this study was too low to disrupt significant additional Gram-negative cells. Hence during the November 2008 and March 2009 field campaigns only the minimal sample processing protocol was used. It is anticipated that further study will enable decreased lower limits of detection by sample processing protocols with appropriate consideration of material choices, *i.e.* to avoid LPS adsorption.

The use of LAL and rFC assays for the detection of LPS in pharmaceutical and other established applications is considered to have a very low limit of detection under laboratory conditions. Typical values reported under these conditions are 0.01 $EU.ml^{-1}$ which represents about ~$10^2$ $cells.ml^{-1}$, where 10 EU represents ~$10^5$ cells from Mueller *et al.* (2004) and La Ferla *et al.* (2004). The rFC assay's low limit of detection and the presence of environmental microorganisms make the rFC assay prone to contamination. Hence, the use of rFC assays in field environments may lead to levels of contamination that severely compromise the analytical utility of the technique. Within the current study, it was only practical to apply standard good microbiological practice (*e.g.* ethanol wiped gloves) due to the logistical issues

of working at a remote field site. To confirm whether contamination was a significant issue, the analysis of a blank sample (consisting of certified LPS free water) was included in each LPS calibration set. The assay responses of the blank samples were between 0.05 and 0.39 $RFU.min^{-1}$ which is below the range of the lowest standard used in the field (assay response of 0.1 $EU.ml^{-1}$ 0.90 to 1.65 $RFU.min^{-1}$). As all the sample blanks returned an assay response less than the 0.1 $EU.ml^{-1}$ standard the rFC assay could be conducted in the Engabreen field environment without significant contamination. In summary, in-field contamination does not represent a fundamental problem for field based implementation of the rFC assay, however to confirm this appropriate contamination controls should be completed with each analysis batch.

To demonstrate the in-field detection of LPS, a suite of contrasting samples was collected at or near Engabreen over two field campaigns (November 2008 and March 2009), and co-assayed with standards to allow for quantification (Figure 4-3 and Figure 4-4). The detection of LPS in 16 of the 27 subglacial and subaerial samples enabled the basic objective of demonstrating rFC assay as an in-field technique to be achieved.

For the initial interpretation of the preceding LPS data in the context of the microbiology of the Engabreen system, the 27 samples can be considered in three clusters; (i) subaerial samples, (ii) subglacial meltwater and (iii) basal ice. A systematic sampling strategy was applied to collect equivalent samples from both field campaigns. Unfortunately the practicalities of collecting equivalent samples in these two field campaigns and the range of samples that returned detectable LPS limit any inferences that can be made on the differences between the spring and autumn behaviour of Engabreen.

A range of subaerial sampling sites was chosen to contextualise the data from the subglacial environment. These comprised fresh snow, and waters from shrubland, forest, proglacial, and englacial streams. The fresh snow samples (both *2008 snow* and *2009 snow*) exhibited either no or very low levels of detectable LPS (<0.1 $EU.ml^{-1}$) as would be expected as these samples had very low particulate concentration (from visual inspection of the filter paper), which has been shown to correlate to bacterial concentration in snow (Segawa *et al.* 2005). Waters from shrubland and forest streams had similar levels of detectable LPS (0.99 and 1.00 $EU.ml^{-1}$). This is unexpectedly low in comparison to the subglacial samples, as it would be expected that the environment around the glacier would have a higher microbial concentration and hence LPS concentration (Gaidos *et al.* 2004, Kaštovská *et al.* 2007). To fully explain these results require further investigations into the proportion of the Gram-negative bacterial

populations in these systems and the hydrological controls *i.e.* the relative contributions of snowmelt and rainwater. The proglacial stream sample was expected to be predominately fed by subglacial meltwater and therefore would be expected to have had LPS values similar to the subglacial meltwater samples. For the single proglacial stream sample assayed, the detected level of LPS (3.13 $EU.ml^{-1}$) was within the spread of values obtained for the subglacial meltwaters. Lastly the englacial stream sample produced the highest LPS value (>10 $EU.ml^{-1}$) of all the samples during both field campaigns. Whilst unexpectedly high, englacial streams are ill-defined as they can be draining local features, including snowpacks, where bacterial numbers could be much higher than fresh snow, if sediment concentration increases (Segawa *et al.* 2005).

The sites for the subglacial meltwater samples fall into two groups. The LPS levels from all samples from sites MW 1 to MW 4 (the Spiral Tunnel) are similar (4.43 ± 0.81 $EU.ml^{-1}$ and n=6) and higher than all samples from site MW 5, which is at the confluence of two streams (1.83 ± 0.43 $EU.ml^{-1}$ and n=3). The interpretation of this is the LPS load in the additional stream contains significantly less LPS than in the Spiral Tunnel Stream and which accounts for a dilution effect at the confluence. This appears to be a reasonable suggestion as the two streams are distinct in terms of source, location and size of their catchment areas (described in Section 4.2.3). The high concentrations of LPS in these meltwaters, equivalent to approximately 2 to 4 x $10^4$ Gram-negative bacteria per ml, are consistent with other investigations that show significant microbial populations in subglacial environments (Sharp *et al.* 1999; Foght *et al.* 2004).

Six of the melted basal ice samples were considered to be glacier ice *i.e.* sediment poor ice away from the influence of bedrock erosion (samples from sites BI 4 to BI 6 defined visually) and six samples to be debris rich ice (samples from sites BI 1 to BI 3 defined visually). LPS was detected using the rFC assay in only two of the twelve samples (samples *2008 BI 5* and *2009 BI 5*), and LPS concentration was quantifiable in one of these (sample *2008 BI 5*). Both these samples were glacier ice. There are two potential explanations for non-detection of LPS in the majority of the basal ice samples; the concentration of LPS is below the detection limit of the rFC assay or there is interference with the rFC assay from the sample. The concentration of microorganisms in glacier ice is known to be low (from $10^2$ to $10^3$ $cells.ml^{-1}$ Midre Loveńbreen, Spitsbergen, Mader *et al.* 2006). The lack of detection of LPS in the majority of the glacial samples is consistent with this observation as it suggests that LPS concentration approaches the lower limit of detection that can be obtained with the in-field

rFC assay in this type of sample. It was observed that the sample with quantifiable LPS concentration (*2008 BI 5*) had some minor sediment inclusions, although substantially less than any of the debris rich ice samples, thus increasing potential concentration of microorganisms as the majority are associated with sediment load (Sharp *et al.* 1999, Junge *et al.* 2001, Tung *et al.* 2006). The association of microorganisms with sediment suggests that debris rich ice samples should contain a higher concentration of microorganisms than *2008 BI 5*, and hence an easily detectable concentration of LPS. Given that none of the debris rich samples gave a detectable LPS signal, we infer the non-determination of LPS in the debris rich ice (samples from sites BI 1 to BI 3) reflects interference of the sediment with the rFC assay. Detection of LPS in some of the glacier ice (but not sediment free) samples suggests that the effect of this interference scales with sediment concentration. The source of this interference could be due to one or more of the three following factors: adsorption of LPS by the sediment, the sediment acting as a fluorescence quencher or the sediment denatures rFC enzyme. Possible methods for reducing the interference involve (i) removal of the sediment (but this could also remove the cells), (ii) reduce the sediment concentration, or (iii) increase the availability of LPS. These methods could be achieved by one or more of the following; cell lysis or, dilution, filtration or centrifugation of sample prior to analysis.

## 4.5. Conclusion

We have demonstrated for the first time, in a glacial environment, the in-field detection of LPS, a proxy for Gram-negative bacteria, and thus the presence of extant life. This was conducted using a recently commercialised bioassay kit (rFC assay) together with a modified assay protocol and a portable fluorometer to enable use. The suitability for in-field detection of LPS by the rFC assay included the analysis of samples directly without the need for significant sample processing. A diverse range of glacial sample types was tested for LPS including basal ice, subglacial meltwater and samples external to the subglacial environment. LPS was detected successfully in each of these sample groups. The limitations of the current in-field rFC assay protocol were encountered only with very low biomass samples (~$10^3$ cells.ml$^{-1}$, where 10 EU is ~$10^5$ cells from Mueller *et al.* 2004 and La Ferla *et al.* 2004), and samples with a significant fraction of fine particulates. Hence further investigation into sample processing approaches will be required to optimise the rFC assay for these sample types. The modified rFC assay, as demonstrated by the detection of LPS in glacial samples, has potential applications in other environmental low biomass samples, as it offers (i) a new technique for the in-field detection of extant life using LPS as a proxy and (ii) the ability to

determine additional microbial community structure by the detection of LPS as a proxy of Gram-negative bacteria when employed in conjunction with other less specific biomarker techniques, *e.g.* ATP bioluminescence or fluorescent cell counts.

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# 5. In-Field Assessment of Subglacial Microbial Populations: A Multi-Technique Approach

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## Abstract

The suitability of four techniques for the detection of microbial communities using biomarkers in subglacial environments was assessed. The techniques were applied to samples from Engabreen in Northern Norway during two field campaigns (November 2008 and March 2009). The four techniques were real-time PCR, fluorescence cell counts, ATP bioluminescence assay and recombinant Factor C (rFC) assay (to detect lipopolysaccharide). Real-time PCR was used with three taxonomic (two bacterial and one archaeal) and five functional pathway primers (for dsrA, nirK, pmoA, mxaF and nifH genes). Each technique was applied to twelve subglacial ice samples, twelve meltwater samples and two snow samples, and additional four water samples from the external environment (two glacial and two non-glacial). Advantages of the individual techniques included: rapid analysis (ATP bioluminescence and rFC assay), quantification of all samples analysed (cell counts) and versatility (real-time PCR). All the biomarker concentrations were highest in the Debris-rich Ice and non-glacial water sources, moderate in glacial meltwaters and low in Glacier Ice (debris-poor) and Snow samples. PCA analysis of the data revealed clustering of the different sample types ($P$<0.001 for Factor 1), where significant differences are seen between

Debris-rich Ice and all other sample types except one of the glacial meltwater sample sites, and between this meltwater sample site and the Glacier Ice. Analysing samples with multiple techniques, as used in this study, has the potential for greater insight to be made on the microbiological influence on biogeochemistry of subglacial systems.

Keywords

Engabreen, real-time PCR, fluorescence cell counts, ATP bioluminescence, LPS endotoxin detection

## 5.1. Introduction

The discovery of active microbial communities at glacier beds (Sharp *et al.* 1999, Skidmore *et al.* 2000) lead to an increased interest in the potential influence that microbial communities may have on the chemistry of subglacial environments. Recent efforts have gone into characterising the microbial communities, estimating rates of metabolism and identifying potential metabolic pathways underneath different glaciers and in different types of subglacial environments (Christner *et al.* 2001, Skidmore *et al.* 2005, Tung *et al.* 2006, Cheng & Foght 2007). Together these studies have revealed distinct subglacial microbial communities that may influence their local chemical environment (Gaidos *et al.* 2004, Bhatia *et al.* 2006, Kaštovská *et al.* 2007) by influencing geochemical cycles including carbon, nitrogen, sulphur and iron (Christner *et al.* 2008 and Hodson *et al.* 2008).

Various microbial-based analytical techniques have been employed to study subglacial ice and related environments. These techniques are dominated by the analysis of microbial biomarkers *i.e.* methods to visualise microbial cells and the detection of cellular biomolecules including nucleic acids and lipids (Bhatia *et al.* 2006, Kaštovská *et al.* 2007, Liu *et al.* 2009, Mikucki *et al.* 2009). However these techniques also include detection of microbial activity by measuring the uptake of labelled substrates (Foght *et al.* 2004) or through inferences from chemical composition (Sharp *et al.* 1999, Wadham *et al.* 2004), and can be used to determine the presence, abundance, diversity and activity of microorganisms.

Due to the variety of information that microbial techniques provide, previous studies have applied two or more biomarker techniques on the same samples. For example Kaštovská *et al.* (2007) compared subglacial and supraglacial assemblages using fatty acid analysis as a qualitative measure of microbial diversity and fluorescent microscopy to quantify the bacterial, cyanobacterial and algal abundances. Foght *et al.* (2004) applied sequencing of 16S

DNA and viable cell counts to subglacial sediments. Multiple techniques are applied as biomarkers or biomolecules can be selected that represent generic microbial life (*e.g.* ATP, total DNA) or be specific to a taxonomic group or species (16S rDNA sequences or distribution of lipid biomarkers) hence in combination can be very powerful.

The use of multiple life detection techniques is likely to reveal a more complete understanding of sub-glacial microbial communities. Real-time PCR, fluorescence cell counts, ATP bioluminescence and LPS detection (using recombinant Factor C assay) were selected as a representative selection of biomarker detection techniques and that were compatible with in-field analysis. All four techniques were applied in a combination of analyses conducted in the field and in institutional laboratory to analyse subglacial ice, subglacial meltwater and non-glacial environmental water samples from Engabreen (the Enga Glacier) in Northern Norway. Real-time PCR primers were selected encoding for gene sequences for taxonomic classification of bacteria (two primer pairs) and archaea, and functional pathways involving sulphur, nitrogen and carbon cycles (specifically dsrA, nirK, pmoA, mxaF and nifH genes). With the following objectives: (i) To conduct multiple life detection techniques on multiple glacial samples from Engabreen in the Norwegian Arctic. (ii) To assess the suitability of the multiple techniques (individually and in combination) to analyse the microbial populations in subglacial environment in and around Engabreen. (iii) To apply multi-variant data processing techniques to the dataset acquired by the multiple life detection techniques. (iv) To interpret the data in terms of microbiological populations in the sample types and identify relationships among them.

## 5.2. Materials and Methods

### *5.2.1. Field site*

All samples were from Engabreen, an outlet glacier of the Svartisen Ice Cap that straddles the Arctic Circle in the Norwegian Arctic (glacier snout at 66˚41’N, 13˚46’E). A unique aspect of Engabreen is the Svartisen Subglacial Laboratory, which is a facility run by the Norwegian Water and Energy Resources Directorate (NVE) providing access to pristine subglacial samples. Access to the laboratory was gained via tunnels drilled into the bedrock underneath and around the glacier. The pristine ice samples were accessed through a research shaft from the bedrock tunnels to the glacier bed. An ice cave was excavated in the base of the glacier by hot water drilling. Other sampling sites included meltwater not exposed to the external environment (Spiral Tunnel and Alex’s Stream) and water from the environment external to the tunnel system (Subaerial samples).

*5.2.2. Sample collection and processing*

The samples were collected from the environment in and around Engabreen from 19th to 27th November 2008 and 18th to 27th March 2009. A total of six sample types were analysed: debris-rich and debris-poor glacial ice (from the ice cave, where a visible boundary separated the two types of ice), water samples from the Spiral Tunnel (ST), Alex's Stream (AS), snow (Snow) and environment external to the tunnel system (Subaerial). The sampling point for AS Water was just beyond the confluence of water from the stream in the Spiral Tunnel and another stream which drained a much larger area of Engabreen and hence a larger catchment area. In addition, there were four samples collected from outside the bedrock tunnel system (subaerial samples). During each field season two subaerial water samples were collected, one glacial and one non-glacial. In November 2008 the non-glacial water sample was collected from a stream flowing through snow covered shrubland (Shrubland Stream - SS), and the glacial sample was collected from stream flowing underneath the glacier bed (Glacier Bed - GB). In March 2009, it was not possible to access the shrubland stream due to deeper snowcover, therefore the non-glacial sample was collected from a stream flowing through a forest (Forest Stream - FS). The glacial sample was collected from the same location from the glacier as in November 2009, however the water was flowing from a crevasse in the glacier (Englacial - EN) rather than a stream beneath it.

Ice and snow samples were melted at ambient laboratory temperature (~18ºC) and then, if necessary, moved to 3ºC storage prior to processing. Between 1 ml (Debris-rich Ice) and 50 ml (all water sources) of each sample was preserved with 2% glutaraldehyde for cell counts. Between 10 ml (Debris-rich Ice) and 3000 ml (Glacier Ice) of sample was filter onto 0.22 µm pore size filter membranes. The filter membranes where then split in two; one half was processed for ATP bioluminescence and the other half for real-time PCR. 1 ml of each sample was aliquoted for LPS quantification. All samples (with the exception of AS Water in March 2009) were assayed, preserved or extracted with 24 hours of collection or upon completion of melting. The AS Water samples were collected and processed daily (filtered or preserved) and were extracted and assayed on the sixth and final day. All AS Water samples were stored, prior to analysis, either at -15ºC for ATP bioluminescence, LPS determination and real-time PCR or at +3°C for cell counts.

All ATP and LPS measurements and DNA extractions were conducted in the field laboratory. In November 2008 preserved samples for cell counts and the DNA extracts were returned from the field (between +2 and +8°C) and analysis was conducted in institutional laboratory.

In March 2009 the majority of cell counts and real-time PCR analyses were conducted in the field. Due to time constraints some samples preserved for cell count and some of the DNA extracts from 2009 were returned and the analysis was conducted in the institutional laboratory. For cell counts, AS Water, Debris-rich Ice and Glacier Ice were all conducted in institutional laboratory, and for real-time PCR analysis all primers for AS Water samples and eubacteria and mxaF primers for Debris-rich Ice, Glacier Ice and Subaerial Water samples were conducted in institutional laboratory.

### *5.2.3. DNA extraction and real-time PCR conditions*

DNA was extracted directly off disaggregated filter membranes using UltraClean™ DNA Isolation Kit (CamBio Ltd, UK product code UC-12800 Mo Bio Laboratories Inc.). The manufacturer's protocol was followed as closely as possible, the two exceptions were that all centrifuge steps were performed at 7200 x g instead of 10000 x g and samples were shaken manually instead of using a vortex mixer. Extraction blanks consisting of a moistened filter membrane were conducted with each extraction batch (three in March 2008 and two in November 2009). Real-time PCR was conducted using the CFX96 for the samples from November 2008 and in MiniOpticon™ Monitor in March 2009 (both instruments from Bio-Rad Laboratories Ltd., UK). The total PCR reaction volume was 10 µl and included 1 µl of DNA extract, 500 nM of forward and reverse primer and iQ SYBR green supermix (Bio-Rad Laboratories Ltd.). The real-time PCR conditions used in both instruments were 3 minutes at 95ºC then 40 cycles of denaturing at 95ºC, annealing at temperature stated in Table 5-1 and extension at 72ºC, each for 30s. Two primers code for bacteria however to distinguish between then they are referred as bacteria and eubacteria, nomenclature from original papers, see Table 5-1. PCR amplification was followed by a melt curve, where the samples were held for 1 second every 1ºC between 65 and 95ºC. Selection of cycle threshold (Ct) line and calculations of Ct values was conducted using CFX Manager™ Software v 1.0 (Bio-Rad Laboratories Ltd.). The full primer sequences and optimisation of real-time PCR conditions are in Appendix B.

### *5.2.4. Cell counts*

Glutaraldehyde preserved samples were filtered onto 0.2 µm pore size filter membrane (Millipore, FDR-312-020W, Fisher Scientific). The filters were then stained with 400 x dilution of SYBR® gold (Invitrogen Ltd., UK) for 15 minutes in the dark. Cells were then counted manually using CyScope (Partec UK, UK) with RB (455 nm) excitation filter and

520 nm emission filter. Duplicate slides were prepared per sample and between 20 and 50 fields of view were counted per slide.

*Table 5-1. Genes targeted with real-time PCR and the annealing temperatures used in this study.*

| Gene | Forward / reverse primer | Annealing T. (ºC) | Reference |
|---|---|---|---|
| 16S rDNA (bacteria) | E8F / E533R | 54 | Baker *et al.* 2003, Manerkar *et al.* 2008 |
| 16S rDNA (eubacteria) | PLK1 / PLK2 | 60 | Klaschik *et al.* 2002 |
| 16S rDNA (archaea) | ARC787F / ARC1059R | 54 | Yu *et al.* 2005 |
| dsrA for (bi) sulphite reductase for sulphate reduction | DSR1-F+ / DSR-R | 51 | Leloup *et al.* 2007, Kondo *et al.* 2004 |
| nirK for nitrite reductase (copper) for denitrification | nirK 1F / nirK 5R | 54 | Braker *et al.* 1998, Geets *et al.* 2007 |
| pmoA for particulate methane monooxygenase for methanotrophy | A189 F / Mb661 R | 54 | Costello & Lidstrom 1999, Kolb *et al.* 2003 |
| mxaF for methanol dehydrogenase for methane and methanol consumption | mxa1003f / mxa1561r | 51 | McDonald & Murrell 1997, Inagaki *et al.* 2004 |
| nifH for nitrogenase reductase for nitrogen fixation | nifH F / nifH R | 60 | Rosch *et al.* 2002, Ogilvie *et al.* 2008 |

### *5.2.5. ATP extraction and determination*

ATP Bioluminescence Assay Kit HSII (Roche Diagnostics Ltd, UK) was used to analyse sample extracts. The extraction procedure was based on a standard procedure recommended by the manufacture. Briefly 1 ml of boiling 100 mM Tris, 4 mM EDTA buffer was added to the disaggregated filter membrane. Samples were then incubated in boiling water bath for 2 minutes. Once cool (at +3ºC), samples were shaken and 50 µl of sample was added to 50 µl of ATP bioluminescence reagents. Luminescence was measured using a HY-LiTE 2 luminometer (Merck, VWR International Ltd., Lutterworth, UK). Samples were quantified using standards from $10^{-8}$ to $10^{-11}$ M ATP. After analysis samples were autoclaved and 100 µl of $10^{-8}$ M ATP spike was added all samples and the blank to correct for interference from the sediment (details in Chapter 3). The bioluminescence value of this spike was compared to the bioluminescence value of the blank to calculate spike recovery.

### *5.2.6. LPS determination*

The Cambrex PyroGene® recombinant Factor C (rFC) endotoxin detection system (product code 50-658U, Lonza,, UK) was used to quantify LPS concentration. Due to the non-temperature controlled conditions in which the assay was conducted, the manufacturer's protocol was modified (details in Chapter 4). Briefly, 50 µl of sample was added to 50 µl of working reagent (made up as per manufacturer's protocol). Fluorescence measurements

(*Pico*Fluor handheld fluorometer-Turner BioSystems, CA, USA), were made after the addition of sample (0 h) then every 0.5 h for 3 hrs. Fluorescence was quantified using standards between 0.1 and 10 $EU.ml^{-1}$. For further calculations the assay response in $RFU.min^{-1}$ of the rFC assay for each sample and standard was used (by fitting linear least squares fit of fluorescence values to time). $R^2$ values were calculated to measure the goodness of fit.

### *5.2.7. Statistical analysis*

The ATP, LPS and cell count data was $\log_2$ transformed to achieve a normal distribution. To analyse the real-time PCR, Ct values were corrected for volume filtered and, as lower Ct values represent higher DNA concentration in the sample, the negative of the corrected Ct value was used. Outliers were identified and removed from the real-time PCR data by visual inspection of melting curves and by p-plot of residuals data. Missing data for ATP, cell counts and real-time PCR was replaced by the nearest equivalent sample giving an interpolated data set (Tabachnick & Fidell 2007). Conducting Principal Component Analysis (PCA) of the original data set and the interpolated data gave similar relative results in terms of their positions in the 1 x 2 factor plans (data not shown), and therefore interpolated data was not considered a significant source of bias when analysing the samples. All statistical analyses were conducted using Statistica 9.0 (StatSoft Inc. OK, USA).

## 5.3. Results

Of the 30 samples (total), only 27 samples were analysed by the rFC assay due to the loss of three of the AS Water samples. An additional six Debris-rich Ice samples were analysed with real-time PCR and ATP bioluminescence as duplicate samples were collected and processed to assess extraction efficiency. To describe the results the techniques are divided into real-time PCR, where the results are relative, and the other techniques (cell counts, ATP bioluminescence and rFC assay), where the results are quantifiable. The results from both field campaigns are clustered into six different sample types, however the subaerial samples are reported individually (summarised in Figure 5-1 and Table 5-2).

### *5.3.1. Individual techniques*

The ‘successfully detected’ data in Figure 5-1 caption and column in Table 5-2 indicates the number of samples where the biomarker concentration of each target was distinguishable from the blank. For the ATP and rFC assays these samples were also above the lowest concentration standard, and for real-time PCR, amplification occurred within 40 cycles and

these samples had an appropriate melt curves for that primer pair (the melt curves were assessed visually).

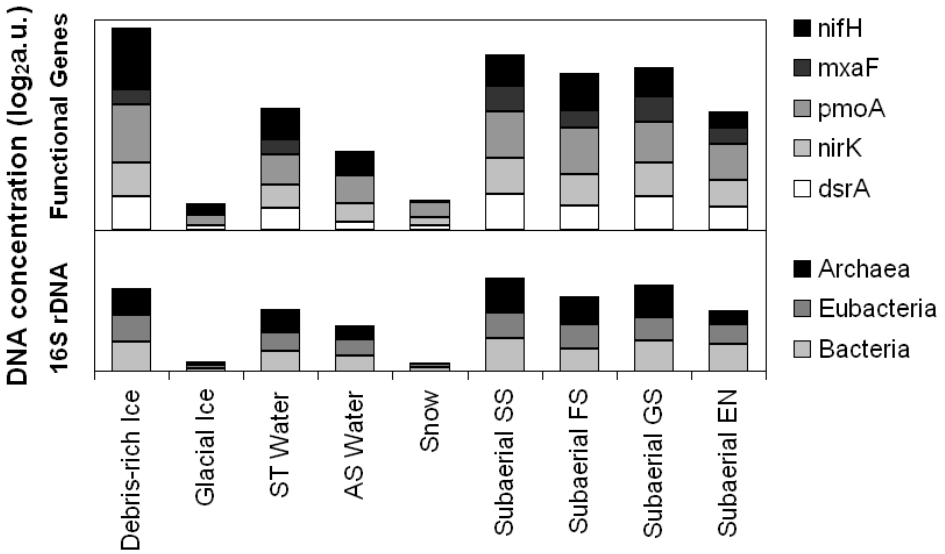


*Figure 5-1 Real-time PCR results for the different sample types. The y-axis values are mean Ct values for blank minus mean Ct values for sample or sample type and then corrected for volume of sample filtered prior to extraction. As the y-axis is related to Ct values it has a $log_2$ scale. A total of 36 samples were analysed with each real-time PCR primer pair. The DNA sequence was 'successfully detected' in for the different primers (mean coefficient of variance (CV) in parentheses): 34 for bacteria (23.2%), 35 for eubacteria (29.5%), 34 for archaea (31.4%), 32 for dsrA (35.5%), 26 for nirK (28.3%), 29 for pmoA (39.7%), 30 for mxaF (27.6%) and 30 for nifH (30.5%). Total of 250 from 288 samples and mean CV of 30.7%.*

All the sample types could be analysed with the real-time PCR primer pairs for bacteria, eubacteria, archaea, dsrA and mxaF. However, no nirK was detected in the Glacier Ice samples, no nifH was detected in the Snow samples and no mxaF was detected in the AS Water samples (Figure 5-1). The mean CV for the technique was 30.7%, however the pmoA gene had the highest individual CV of the primers and techniques at 39.7%.

The results in Figure 5-1 show that the highest concentrations of DNA were found in Debris-rich Ice, with Glacier Ice and Snow samples having the lowest. The DNA target sequences were highest for the bacterial and eubacterial primers, and the most abundant functional primer was pmoA in all sample types except for Debris-rich Ice where it was nifH. The lowest concentration of the DNA target sequences was generally mxaF, this excluded the sample types where there was no detection of particular genes.

*Table 5-2. The results for the techniques with biomarkers, including how many of each sample type returned quantifiable values above the blank compared to the number analysed by that technique 'successfully detected', range of values and the mean CV for each technique. Sample location abbreviations ST – Spiral Tunnel, AS – Alex's Stream, SS – Shrubland Stream, GB – Glacier Bed, FS – Forest Stream and EN – Englacial.*

| Technique / Sample Type | Cell Counts (cells.ml$^{-1}$) | | ATP bioluminescence (pM) | | rFC assay (EU.ml$^{-1}$) | |
|---|---|---|---|---|---|---|
| | Successfully detected | Range of sample means | Successfully detected | Range of sample means | Successfully detected | Range of sample means |
| Debris-rich Ice | 6 of 6 | 34300 – 1150000 | 3 of 12 | 0.196 – 157 | 0 of 6 | |
| Glacier Ice | 6 of 6 | 177 – 1470 | 6 of 6 | 0.00958 – 1.15 | 1 of 6 | 0.244 |
| ST Water | 6 of 6 | 774 – 3150 | 6 of 6 | 0.0205 – 0.900 | 5 of 6 | 3.03 – 5.25 |
| AS Water | 6 of 6 | 274 – 714 | 6 of 6 | 0.195 – 2.11 | 3 of 3 | 1.57 – 2.32 |
| Snow | 2 of 2 | 96 – 247 | 2 of 2 | 0.222 – 1.62 | 0 of 2 | |
| Subaerial - SS | 1 of 1 | 73900 | 1 of 1 | 1.41 | 1 of 1 | 1.23 |
| Subaerial - GB | 1 of 1 | 4050 | 1 of 1 | 9.11 | 1 of 1 | 3.14 |
| Subaerial – FS | 1 of 1 | 10000 | 1 of 1 | 3.43 | 1 of 1 | 1.35 |
| Subaerial – EN | 1 of 1 | 9060 | 1 of 1 | 0.843 | 1 of 1 | >10* |
| Total / mean CV | 30 of 30 / 37.2% | | 27 of 36 / 30.4% | | 13 of 27 / 11.9% | |

*fluorescence value greater than the highest standard of 10 EU.ml$^{-1}$

Using the 'successfully detected' column in Table 5-2 to compare the analytical techniques shows that all samples could be assessed by cell counts, and some of each sample type could be analysed with ATP bioluminescence. However, ATP was quantifiable in only three of twelve the Debris-rich Ice samples. None of the Debris-rice Ice, Glacier Ice or Snow samples could be assayed for LPS using the rFC assay.

Overall, the results in Figure 5-1 and Table 5-2 show that there were significantly higher concentrations of biomarkers in the Debris-rich Ice compared to the Glacial Ice with up to three orders of magnitude more cell counts ($P$<0.001 for cell counts). The concentrations of biomarkers in the water samples were intermediate to these extremes. The range of values for the water source samples was narrower when compared to both types of ice sample and generally less than an order of magnitude.

Looking at the details of the different biomarkers in the different sample types, summarising the distribution becomes more complex. Therefore to simplify the variation in the dataset and to aid in the interpretation of these differences, multivariate statistical analysis was applied to the data.

*5.3.2. Multivariate analysis*

Principal Component Analysis (PCA) was applied to the multi-factorial dataset. The rFC assay data was not included in the PCA analysis (Figure 5-2) as rFC assay data was incomplete and highly biased (only one LPS measurement in the twelve ice samples).

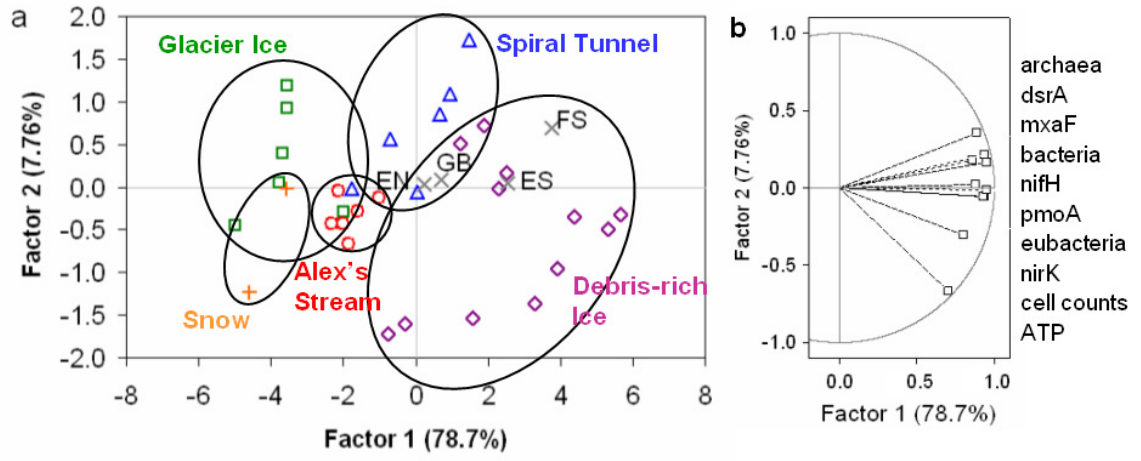


*Figure 5-2. Principal Component Analysis (PCA) on the real-time PCR, cell counts and ATP. a) Projection of the samples (cases):* x *are the Subaerial samples (SS - Shrubland Stream, GB - Glacier Bed, FS- Forest Stream and EN-Englacial). The ovals are placed arbitrarily to distinguish among the different sample types. b) Projection of the biomarkers (variables) on the factor plane. One-way ANOVA was conducted to test the significance of the sample type (using five of the sample types* i.e. *excluding subaerial data), for Factor 1* P*<0.001 and for Factor 2* P*=0.007.*

In terms of the spread of the samples within the sample types in Figure 5-2a, the AS Water samples have the narrowest range and the Debris-rich Ice samples have the widest range. Factor 1 explains a high proportion of the variation in the dataset (78.7% total). One-way ANOVA (unequal N HSD post-hoc analysis) was applied to PCA Factor 1 and showed that the Debris-rich Ice samples were not statistically related to the other sample types except ST Water and that the Glacier Ice was statistically different from ST Water ($P$<0.05), however Factor 2 can distinguish between Debris-rich Ice and ST Water samples Water ($P$<0.05).

Comparing the results in Figure 5-2a and Figure 5-2b PCA demonstrates that: (i) from the position of the biomarkers on the factor plane that all the biomarkers provide a significant contribution to describe the overall variance, all of the biomarkers positively correlate with Factor 1, the bacterial real-time PCR primer data contributed the most to the overall variance, and to Factor 1 and that ATP bioluminescence data contributed to the most of the variance to Factor 2. (ii) The PCR primers cluster into three groups with similar statistical behaviour as

they plot in similar locations on 1 x 2 Factor plane: archaea; dsrA, mxaF and bacteria; and nirK, pmoA, nifH and eubacteria. (iii) Differences in samples can be related to differences in the biomarkers as they will plot in similar locations in Figure 5-2a and Figure 5-2b, *e.g.* generally the most significant biomarker to describe the variation between the Debris-rich Ice and Glacial Ice samples, considering all the sample types, is ATP concentration; the sample types plot diagonally opposite each other and parallel to the projection of the ATP bioluminescence biomarker data.

## 5.4. Discussion

### *5.4.1. Suitability of individual and combination of techniques for biomarker detection*

Multiple life detection techniques could be conducted on glacially relevant samples from Engabreen in the Norwegian Arctic. These techniques relied on the detection of biomarkers: DNA sequences, stained microbial cells, ATP and LPS. Each biomarker provides distinct information on microbiological life. Considering all of the biomarkers in Figure 5-1 and Table 5-2, there were some common issues where particular sample types could not be assayed by more than one technique. The cause of this non-detection can be divided into situations where either the concentration of biomarker was below the detection limit of the assay (Glacier Ice and Snow samples by selected primers for PCR and LPS) or there was interference with the assay reagents by sample matrix (Debris-rich Ice samples by ATP and LPS).

Real-time PCR values are proportional to the copy number of the sequence amplified by the particular primer pair, this copy number varies for the different genes in an organism and the copy number of the same gene can vary among different species (Farrelly *et al.* 1995, Klappenbach *et al.* 2000). The choice of primer pairs for real-time PCR was used to target 16S rDNA to identify taxonomic groups (bacteria, eubacteria and archaea) and genes encoding for proteins involved in a variety of functional pathways (dsrA, nirK, pmoA, mxaF and nifH). The main strength of real-time PCR analysis is the diversity of information that can be collected by a single technique on the same extract and can be used to characterise microbial populations by the selection of appropriate primer pairs. However, the experimental set-up and the extraction protocol was the most elaborate of the analyses tested here and required instrumentation for sample extraction (a microcentrifuge) hence analysis had the greatest mass requirement. However conducting real-time PCR analyses with the instrumentation used here requires less equipment and time than other laboratory nucleic acid detection techniques with separate amplification and detection steps.

The SYBR Gold® fluorescent dye used in this study for fluorescence cell counts stains both live and dead bacteria; hence cell numbers reported include non-viable bacteria. The main strengths of fluorescence cell counts are the ability to easily compare among studies conducted on different sample types and in different laboratories and, within this study, the ability to apply it to every sample (as the technique does not rely on biochemical reagents which might be influenced by differences in the sample matrix). Also, preservation can be conducted quickly in the field or at the sampling site and extraction of targets is not required, therefore removes any additional complications that these processes may introduce. However, manual fluorescence cell counts, as conducted here, are operator-dependent and time consuming, taking significantly longer than any of the other techniques used here as samples have to be processed individually. These disadvantages can be addressed by the use of flow cytometry as portable instruments are now commercially available (*e.g.* CyFlow® SL Partec, Germany). However, prior to field deployment methods to distinguish between cells and sediment would be required.

As ATP hydrolyses quickly in natural environment, it is a measure of extant life. The ATP bioluminescence assay used here was simple to perform, with the measurement of bioluminescence values requiring less than a minute. The addition of the concentration, extraction and incubation steps and analysis of standards applied here increased this time to between three and five hours due to required incubation steps (Section 5.2.5) and depended on the time required to filter the samples. The addition of ATP spike recovery step and subsequent calculations allowed for the quantification of interference with the ATP bioluminescence assay caused by sequestering of some of the ATP by the sediment. However when the sediment concentration of the sample was high, seen in the majority of the Debris-rich Ice samples, so much ATP was sequestered by the sediment to be able to quantify this adsorption (see Chapter 3 for detailed discussions). Development of a suitable extraction protocol is on-going in the authors' laboratory to address this issue.

LPS is present in the cell wall of Gram-negative bacteria and hence measures only a portion of the total microbial community. To measure LPS using rFC assay minimal processing was used removing the need for additional laboratory equipment (like real-time PCR assay) or addition additional stages to analysis with sample extraction (real-time PCR and ATP assays). Although successful with the water samples, LPS could only be detected in one Glacial Ice sample and in none of the Snow or Debris-rich Ice samples, reasons discussed at the beginning of this section and in detail in Chapter 4.

All of the techniques used here measure microbial biomass, real-time PCR combines biomass and gene copy number, ATP concentration combines biomass and activity, cell counts enumerate live and dead cells and LPS measures Gram-negative bacteria. Conducting multiple techniques on the same samples can lead to more robust results as they provide independent measurements of microbial concentrations, and the differences among the techniques focus on different aspects of microbiology. Combining the data from the four different techniques on samples from Engabreen lead to the acquisition of a large dataset. As the techniques measure different targets, summarising the overall microbial distribution can be complex. For example, all techniques show the lowest concentration of biomarkers in Glacial Ice and the real-time PCR and cell count levels were equally low in the Snow samples, however the ATP concentrations in the Snow samples were higher or comparable to those in AS and ST Water samples (Figure 5-1 and Table 5-2). To simplify the variation in the dataset and to aid in the interpretation of these differences, PCA was applied to the dataset (Figure 5-2). The different sample for each sample type cluster together, with some statistically significant differences among some of the sample types. These differences are discussed with respect to the microbiology in the following section.

### *5.4.2. Microbiology of Engabreen system*

Both the data from the different techniques and statistical analyses allowed for the interpretation of the data collected on the microbiology of the Engabreen subglacial system (Figure 5-1, Table 5-2 and Figure 5-2).

The data shown in Figure 5-1 and Table 5-2 agree with general distribution of microorganisms from published data, where microbial biomarkers and hence assumed microorganisms are higher in the Debris-rich Ice compared to the Glacier Ice, (Foght *et al.* 2004, Mader *et al.* 2006). Foght *et al.* (2004) found viable cell counts between 2 and $7x10^6$ cells.ml$^{-1}$ and were typically 3-4 orders of magnitude higher in the debris rich ice than glacial ice, which is equivalent to the difference we found here of $3x10^4$ to $10^6$ cells.ml$^{-1}$ in Debris-rich Ice and $2x10^2$ to $10^3$ cells.ml$^{-1}$ of Glacier Ice. This general trend is reflected in all the techniques, and distribution of sample types along Factor 1, although smaller differences can be seen with some the techniques and sample types.

Previous studies into the diversity of glacial ice or meltwaters have shown a dominance of bacterial over archaeal species (Sjöling & Cowan 2003, Simon *et al.* 2009). Within this study this is reflected in the higher concentration of bacterial DNA markers over archaeal DNA markers. Apart from the absence of some biomarkers in certain samples (Figure 5-1) all DNA

sequences could be detected in all of the sample types, suggesting a diverse range of microbial functions including different pathways and both oxidation and reduction in the Engabreen system. However as DNA was extracted and amplified for this study, the detection of the functional pathway sequences demonstrates the presence of microorganisms with the potential for these metabolic pathways, and does not necessarily mean they are active.

With the exception of mxaF all the functional genes used here have been previously studied using PCR techniques in samples from icy environments: polar soils (Pacheco-Oliver *et al.* 2002, Martineau *et al.* 2010), alpine glacier foreland samples (Kandeler 2006, Duc *et al.* 2009b), on Antarctic microbial mats (Jungblut & Neilan 2010) and ice cover of lakes in McMurdo Dry Valleys, Antarctica (Olson *et al.* 1998, Karr *et al.* 2005). However these studies have generally concentrated on the analysis of the diversity of a particular gene or in the case of Pacheco-Oliver *et al.* (2006) two functional genes for methanotrophs and with Kandeler (2006) a set of genes related to denitrifying bacteria, thus none have targeted more than one ecological function as in this study. Analysis of a functional gene has been conducted on subglacial brine samples from Blood Falls, Antarctica however this targeted phosphate reduction (Mikucki *et al.* 2009). As an alternative to PCR identification of nucleic acids, functional microarrays have been applied to whole samples from icy environments as they analyse a high number of targets in a single sample. Yergeau *et al.* (2007) used array targeting >24000 oligonucleotides involved in carbon and nitrogen cycles and applied to Antarctic soil samples as a tool to study controls on nutrient cycling. Duc *et al.* (2009a) applied a microarray specifically for the identification of species based on nifH gene on Alpine glacial foreland soils.

As all of the biomarkers positively correlate with each other along Factor 1 (Figure 5-2b), it is likely that the variance described by Factor 1 reflects microbiological abundance. Therefore, indicating that the primary differences in sample types are related to cell concentration. The real-time PCR results have a stronger influence on Factor 1 than the ATP and cell count data. This could be because the real-time PCR results are due to cell concentration and gene copy number; thus can have several ‘hits’ per one for cell counts. The clustering of the real-time PCR primers suggests that the greatest differences in these results can be described with three dominant groups. Further investigations would be required to elucidate whether these either reflect differences in community structure among the samples, or are an artefact of sample processing.

The ability to remove the effect of biomass would allow for inferences on the relationships among the sample types to be made on community structure alone, rather than the combined effect of structure and biomass. One method to explore relationships involved normalising the real-time PCR data to bacterial Ct (although this assumes the copy number for each of the targets genes is the same as for 16S rDNA). Conducting unequal N HDS (post-hoc analysis) on Factor 1 from PCA on this normalised dataset revealed two distinct clusters of sample types; Debris-rich Ice and ST Water as one cluster and Glacial Ice, AS Water and Snow as the other, $P>0.6$ between the sample types within the clusters and $P<0.01$ between the sample types from the two clusters. If these relationships are dominated by changes in microbial community then it suggests that the ST Water originates from melting of the Debris-rich Ice and that the AS Water from melting of Glacier Ice.

## 5.5. Conclusions

Multiple microbiological techniques can be applied to ice, meltwater, snow and stream water samples from Engabreen subglacial environment. The four techniques used in this study individually show advantages of: rapid analysis (ATP bioluminescence and rFC assay), applicability to a greater variety of sample types (cell counts) and versatility (real-time PCR). Together the techniques provide a large dataset requiring analysis.

Analysis of functional genes reveals the potential for a diverse range of microbial function in Engabreen and surrounding environment; including carbon (pmoA and mxaF), sulphur (dsrA) and nitrogen (nirK and nifH) cycles, and potential for reduction (nirK and dsrA) and oxidation (pmoA, mxaF and nifH).

PCA can be applied to the dataset of the multiple biomarkers, where samples of the same sample type cluster together (Figure 5-2). Some of the sample types show significant differences, most noticeably the Debris-rich and Glacier Ice ($P<0.05$). Using the projection of the biomarkers on the 1 x 2 Factor plane, all of the biomarkers contribute significantly to the variance among the samples. Most of variance in the dataset is likely to be related to population as all of the biomarkers are correlated with Factor 1 and it explains 78.7% of the variation in the dataset. However some variation remains and by removing the effect of population, differences and relationships in community structure among sample types could be more clearly understood.

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# 6. A Simple ATP Extraction Method for Low Biomass, High Clay Content Glacial Samples

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## Abstract

Adenosine triphosphate (ATP) extraction followed by quantification via ATP bioluminescence assay has previously been used to estimate microbial life in a variety of environmental samples. Prior to detection ATP needs to be extracted from cells. This causes problems in sediment-rich glacial samples as clay particles and minerals readily adsorb ATP, and chemicals that are frequently used to prevent adsorption can interfere with ATP bioluminescence assay reagents. These chemicals cannot be diluted out as expected ATP concentration in glacial samples is too low. Thus, the effect of thirteen chemical modifiers on the adsorption of ATP was investigated using a model system where bentonite clay, ATP and modifier were incubated in a standard buffer (Tris EDTA) prior to ATP assay. The modifiers included phosphates, polyanions, cationic and non-ionic surfactants, EDTA, magnesium sulphate and proteins. In the model system, most modifiers were unsuccessful as they reduced output of the ATP assay (cationic surfactants and magnesium sulphate), had lower or equivalent ATP recovery to the standard buffer (phosphates), or both (EDTA and polyanions). Only 1% w/v Tween 20, 1% w/v bovine serum albumin and 0.2, 1 and 5% w/v casein had higher ATP recovery than the standard buffer (mean recovery 11.2 ± 1.6%); 5% w/v casein was the highest at 103.6% ± 3.0. The addition of 5% w/v casein was developed into an extraction protocol, compatible with quantification by an ATP bioluminescence assay. Testing the protocol on sediment from a glacial ice sample spiked with bacterial culture showed a >200-fold increase in ATP recovery compared to extraction in the standard buffer alone, detecting $3.4x10^4$ CFU in 100 mg fine grained sediment.

Keywords
Adenosine triphosphate, ATP bioluminescence, clay adsorption, glacial, casein, life detection.

## 6.1. Introduction

There is significant current interest in understanding life in extreme environments and therefore there is a requirement for tools to study such life. A key question is how much life, typically microbial life, is in a given environment. As adenosine triphosphate (ATP) is found in all known organisms its quantification in samples can be used as a proxy for microbial cell numbers. Also ATP hydrolyses easily in natural environments, therefore measuring ATP concentration is viewed as a proxy for extant life. In many environmental microbiology and industrial applications the determination of ATP concentrations is well established (Jørgensen *et al.* 1992, Deininger & Lee 2001, Costa *et al.* 2006). The commercial products used for the determination of ATP concentration are based upon the detection of ATP-induced bioluminescence using an enzyme and substrate (luciferase and luciferin respectively) derived from the tails of fireflies and responsible for the ATP activated bioluminescence that fireflies are renowned for. ATP bioluminescence assays are used as they allow for the rapid and sensitive detection of ATP with low lower detection limits often in the picomolar concentration range (Chapter 3).

In extreme environment applications in the Earth's cryosphere, the interest of the authors, ATP concentration measurements are rare. A few studies have investigated ATP concentration in polar sea water (Dahlbäck *et al.* 1982, Vosjan *et al.* 1987, Fabiano & Danovaro 1998, Naganuma *et al.* 2006), Antarctic lake water and sediments (Simmons *et al.* 1983), Antarctic soils (Cowan *et al.* 2002) and sea ice and associated sediment (Dahlbäck *et al.* 1982). The ATP concentrations detected in these samples range from 127 to 439 pmol.$l^{-1}$ for lake water, maximum of 237 pmol.$l^{-1}$ for sea ice and between 2.3 nmol $kg^{-1}$ and 1.3 μ mol $kg^{-1}$ for Arctic mineral soils. Using published values of ATP per cell from 0.16 to 2.25 fg ATP $cell^{-1}$ (Fairbanks *et al.* 1984) these ATP concentrations equate to $2x10^4$ to $1x10^6$ cells $ml^{-1}$ for lake water, $3x10^4$ to $4x10^5$ cells $ml^{-1}$ for sea ice and between $5x10^5$ to $4x10^9$ cells $g^{-1}$ for mineral soils. These conversions from ATP concentrations are consistent with cryospheric environments supporting microbial life in a diverse range of environments, however they are frequently at low concentrations (several orders of magnitude lower than soils) (Karl *et al.* 1999, Sharp *et al.* 1999, Laybourn-Parry 2009). Therefore to detect this low biomass, techniques are required that need to be more sensitive than for detection in soil samples.

The authors have determined ATP concentrations in low biomass, glacial and subglacial samples to levels that equate to $2.4x10^5$ to $3.5x10^6$ cells $ml^{-1}$ of water or melted ice (unpublished data). In this latter work, although successful with some sample types, problems were encountered regarding samples with high clay content, *e.g.* subglacial meltwater and basal ice, where measured ATP concentrations were significantly lower than blank control samples (unpublished data). When an ATP standard was added to these high clay content samples, less than 5% of the added ATP could be detected by the ATP bioluminescence assay.

To address the preceding problem, the application of suitable sample processing methodology was required. A fundamental consideration when detecting ATP in samples is the need to liberate ATP from microbial cells and for it to remain in the liquid portion of the sample so it can be accessed by ATP bioluminescence reagents. For processing rarely studied low biomass, clay-rich samples the best foundation for methodology development is from those applied to soil and sediment samples. Several studies have been conducted to compare and optimise ATP extraction methods on soils (Ciardi & Nannipieri 1990, Inubishi *et al.* 1989, Contin *et al.* 1995), and particular methods have been developed and tested on specific types of samples, including high latitude soils (Sparrow *et al.* 1988), marine sediments (Bancroft *et al.* 1976) and whey waste water (Lee *et al.* 2006). Of these different methods only Bancroft *et al.* (1976) specifically target what they describe as clay sediments, although no further information as to the clay concentration was given. Through the other studies that compared and developed ATP extraction techniques, the highest clay content was a soil with ~60% clay (Ciardi & Nannipieri 1990). Ciardi & Nannipieri (1990) used three different extraction buffers: trichloroacetic acid, paraquat and phosphate (the TCAPP method, from Jenkinson & Oades 1979), sodium bicarbonate, chloroform, phosphate and adenosine (the BCPA method, from Martens 1985) and phosphoric acid, urea, dimethylsulphoxide, adenosine and EDTA (the PA method, modified from Webster *et al.* 1984). Bancroft *et al.* (1976) developed and used one extraction buffer; sodium bicarbonate (the BC method). The clay rich soil used by Ciardi & Nannipieri (1990) had ATP recovery rates of 65.5% for BCPA and 93% for TCAPP method, the ATP recovery for the PA method was not specifically mentioned but ranged from 55 to 65%. ATP recovery using the final protocol for the BC method in Bancroft *et al.* (1976) using BC method gave ATP recoveries between 20 and 133% for seven different sediments. Considering the details for these ATP extraction methods, they were unsuitable for applications for in-field detection of low biomass samples as: they contained toxic

components therefore making transport to and disposal in the field logistically difficult (TCAPP and BCPA methods); involve several stages of centrifugation, incubation, shaking and / or filtration hence requiring multiple additional equipment to be transported to the field (TCAPP, PA and BC methods); or have highly variable ATP recovery (BC method). Therefore an alternative approach to the development of an ATP extraction method for low biomass, clay-rich samples compatible with in-field deployment was required.

Within other disciplines the adsorption of nucleotides, including ATP, by clay minerals has been investigated due to their potential role in the prebiotic synthesis of oligonucleotides and hence the beginning of life on Earth (Ponnamperuma *et al.* 1982, Porter *et al.* 2000, Perezgasga *et al.* 2005, Lambert 2008). The majority of clay minerals have three types of adsorption sites: face sites which are present on the face and in the interlamellar spacing of the crystals, aluminol and silanol sites which are both at the crystal edges (Weaver & Pollard 1973). Generally a net negative charge on the face sites attracts cations, and a net positive charge at clay edges attracts anions (*e.g.* ATP) however these charges depend on pH of solution hence edge sites can become negatively charged at low pH (Graf & Lagaly 1980, Lagaly & Zieseman 2003). Of the two edge sites the aluminol sites are favoured as the site of ATP adsorption. This conclusion has been made from the observation that clay minerals more strongly adsorb ATP than silica and silica only contains silanol sites. To investigate the prebiotic synthesis of oligonucleotides the face and edge sites have been modified with different chemicals to prevent adsorption. Phosphates have been shown to modify edge sites of clay minerals, as after adsorption they make the total charge density at the edges more negative (Lyons 1964, Ponnamperuma *et al.* 1982). The cationic surfactants HDTMA and DDTMA have been used to block the interlamellar spacing of clays (Ertem & Ferris 1998, Perezgasga *et al*. 2005, Li & Gallus 2007) as they make the interlamellar absorption sites hydrophobic when adsorbed (Boyd & Mortland, 1986). Perezgasga *et al.* (2005) used sodium triphosphate and HDTMA to block adsorption sites of clays and reported significant absorption of ATP into interlamellar spacing of clay minerals (66% of total ATP absorbed at pH 2 and in the absence of a buffer). The information on adsorption sites, behaviour under different conditions and the chemicals and methods used in these studies can be applied to develop new methods for preventing adsorption of ATP by clay minerals after ATP extraction.

Given the preceding considerations, the work presented here focuses on the development of a simple ATP extraction procedure for use with low biomass, clay-rich samples by assessing

the ability of different chemicals to modify (block) the adsorption sites in clay minerals. This protocol needs to be compatible with detection with standard commercial ATP bioluminescence assay reagents. The requirement for a simple procedure arises from a desire for the procedure to be compatible with implementation in a field deployable assay.

The objectives of the current work, to enable the development of a simple ATP extraction method for low biomass, high clay content glacial samples, were as follows. (i) To create a model system using clay minerals that behaves as a natural system regarding ATP adsorption *i.e.* adsorbs a high fraction of ATP. (ii) To assess the stability of ATP under the conditions experienced during the extraction protocol to ensure that there is limited degradation of ATP during sample processing. (iii) To use the model clay system to test the efficiency of different types of chemical modifiers (phosphates, polyanions, surfactants, divalent cations and proteins) in reducing ATP adsorption by clay and to test the compatibility of the chemical modifiers with ATP bioluminescence reagents. (iv) To propose a protocol, using an appropriate chemical modifier, for a simple ATP extraction method for low biomass, high clay content glacial samples and compatible with an ATP bioluminescent assay. (v) To demonstrate the extraction protocol using glacial samples spiked with a representative psychrophilic bacteria culture.

## 6.2. Materials and Methods

### *6.2.1. Chemicals and abbreviations*

All chemicals were from Sigma-Aldrich (Poole, UK) unless otherwise stated, product codes follow in parentheses: ATP (adenosine triphosphate) (supplied as part of the ATP Bioluminescence Assay Kit HSII, Roche Diagnostics, Ltd., UK), BSA (bovine serum albumin) (A4503), casein (C5890), DDTMA (dodecyltrimethylammonium bromide) (D8638), dextran sulphate sodium salt (D4911), EDTA (ethylenediaminetetraacetic acid) (E5134), HDTMA (hexadecyltrimethylammonium chloride) (Fisher Scientific, Acros Organics-0050), heparin sodium salt (Fisher Scientific, 41121-0010), magnesium sulphate (20809-4), ovalbumin (A5503), sodium diphosphate (P8010), sodium phosphate (S0876), sodium triphosphate pentabasic (72061), Tris (Trizma® Base) (T6791) and TWEEN 20 (P7949).

The standard buffer used in all reactions was 100 mM Tris, 4 mM EDTA (TE). All modifiers were tested in TE buffer except testing the effect of EDTA when 0 mM and 40 mM EDTA were added to 100 mM Tris. All buffers including chemical modifiers were adjusted to pH

7.75 (using HCl) as the optimum pH for the ATP bioluminescence assay stated by the manufacturer.

### *6.2.2. Other materials*

**Sediments**

For the model system, bentonite clay (Sigma-Aldrich, UK B3378), was used. The bentonite clay was wrapped in foil and sterilised by autoclave at 121ºC for 20 minutes prior to use. The modal grain size for bentonite clay was 5.7 μm.

**Bacterial culture**

A bacterial culture was bought from the National Collection of Industrial and Marine Bacteria (NCIMB). *Flavobacterium xanthum* (NCIMB ID 2069) was grown at 4°C, on nutrient agar and harvested after one week. Dilutions of culture were made in 4‰ saline. *F. xanthum* was chosen as a model organism as it was a psychrotolerant bacterium; isolated from mud pool, Showa Station, Antarctica (Inoue, 1976).

**Environmental samples**

Two glacial samples were used to test the extraction protocol. Engabreen Glacial Ice (EBI) contained fine grained sediment and Greenland Surface Sediment (GSS) contained coarse grained sediment. EBI was melted, and to concentrate the sediment prior to use the sample was filtered with a 0.22 μm pore size mixed cellulose ester membrane (Millipore, FDR-293-050F, Fisher Scientific). GSS was used directly. EBI was predominantly fine grained with high visible-clay content and was collected from Engabreen, Norwegian Arctic in November 2007. GSS was courser grained dominated by quartz particles and was collected in May 2008, from Russell's Glacier, Greenland. GSS was kindly provided by Dr. Jemma Wadham, Bristol Glaciology Centre, University of Bristol. Sediments were dried at 37ºC overnight and autoclaved in foil at 121ºC for 20 minutes prior to use to degrade indigenous ATP.

### *6.2.3. ATP detection protocol and recovery calculations*

ATP Bioluminescence Assay Kit HSII (product code: 11699709001, Roche Diagnostics, Ltd., UK) was used for ATP bioluminescence assay reagents and ATP standards (manufacturer's stated detection limit 0.1 pM ATP). For the assay 50 μl of sample or ATP standard was added to 50 μl of bioluminescent reagent in glass test tubes (212-0021, VWR International Ltd. UK). Luminescence was measured immediately after mixing at ambient laboratory temperature

(18°C) in Merck HY-LiTE 2 portable luminometer (VWR International Ltd. UK). All readouts are stated in Relative Light Units (RLU), which includes compensation of the temperature dependency of the ATP bioluminescence reaction between 5 and 35ºC. For each sample the bioluminescence assay was conducted in triplicate.

To standardise the effect of the chemical modifiers and of bentonite clay across the different experiments, ATP recovery was calculated. ATP recovery was defined as the RLU value from the sample of interest as a percentage of the RLU value from the ATP standard in TE buffer. The errors presented are the standard deviation of triplicate assays per sample.

### *6.2.4. Protocol for the addition of chemical modifiers to prevent ATP adsorption onto clay*

The effects of thirteen chemical modifiers on clay adsorption sites were investigated. To mix the sediments, ATP standard and buffer, each sample was vortexed for 5 seconds between each step of the following protocol, *i.e.* after the addition of bentonite clay and ATP standard, and after both incubation periods. 1 ml of modifier without bentonite clay and 1 ml of modifier with 50 ± 0.5 mg bentonite clay were placed in a shaking incubator for 18 to 20 hours (the blocking step) under the following conditions: ambient temperature at 200 revolutions per minute (from Lyons 1964). 100 µl of 100 nM ATP standard was added to all samples. The samples were then placed in the shaking incubator for a further 4 hours (the equilibration step) and then centrifuged for 5 minutes at 7200 x g. The supernatant was then tested for ATP concentration.

### *6.2.5. Protocol for ATP extraction*

To release intercellular ATP, an extraction protocol based on one recommended by the manufacturer of the ATP test kit was used (Roche Diagnostics, Ltd., UK). In the development of the final ATP extraction protocol, prior to ATP extraction, glacial sediment samples were spiked with bacterial culture. 100 µl of a bacterial spike or blank was added to 100 ± 0.5 mg of each sediment. After 1 hour incubation of bacteria and sediment, 1 ml 5% casein in TE buffer was incubated at ambient laboratory temperature with the sample in locking 1.5 ml vials (to prevent lid opening during subsequent incubation in boiling water). After an hour, samples were placed in a boiling water bath for 5 minutes (the extraction step), cooled at 4ºC and then were left to equilibrate for 90 minutes (the equilibration step at 4°C). Extracted samples were centrifuged for 5 minutes at 7200 x g and then the supernatant was tested at room temperature using the ATP bioluminescence assay protocol in Section 6.2.3.

To quantify remaining interference from the glacial sediments with ATP concentration, an ATP spike recovery experiment was conducted. After the supernatant of each sample was assayed for ATP concentration, 100 µl of 100 nM ATP was added to remaining samples and buffer blank. These samples were incubated for 90 minutes, centrifuged for 5 minutes at 7200 x g and ATP concentration was measured in the supernatant as described in Section 6.2.3. The spike value was the difference between the mean RLU value of the sample before and after spike addition. To calculate spike recovery for each sample, the spike value for that sample was compared to the spike value from an extraction blank (casein in TE only).

## 6.3. Results and Discussion

### *6.3.1. Establishing a model system*

Bentonite was used as the model clay as it is commonly occurring in natural environments. To test that bentonite clay was a suitable model sediment to simulate clay-rich natural samples 1 ml of 10 nM ATP was added to 5, 10 and 50 mg clay. 10 nM ATP is equivalent to 2 x $10^6$ to 3 x $10^7$ to cells.ml$^{-1}$, using conversions of ATP cell$^{-1}$ from Fairbanks *et al.* (1984).

The model system used here behaves as published data on real samples and other model systems. Specifically a significant fraction of clay minerals caused loss of ATP and that the loss was greater with increasing clay mineral concentration (ATP recovery of 18.7% ± 6.1% for 5 mg, 13.2% ± 3.1% for 10 mg and 8.1% ± 2.0% with 50 mg).

### *6.3.2. Confirmation of ATP stability under temperature incubation conditions*

ATP hydrolyses easily in the natural environment with a half life on the order of a few hours at 2-4ºC (Cowan & Casanueva 2007). Most of the hydrolysis is attributed to activity of ATPases released by microorganisms although natural hydrolysis of pure ATP solutions at room temperature still occurs but with a half life on the order of days (Schubert *et al.* 2003). As the intended protocol for the assessment of modifiers and ATP extraction involved incubation of samples for prolonged periods or elevated temperatures, the effect of ATP hydrolysis on ATP concentration under these conditions was investigated. The specific conditions of interest were incubation at room temperature for 4 hours (simulating the equilibration step of the ATP assay protocol) and incubation in boiling water for 5 minutes (simulating the extraction step of the ATP assay protocol).

Experimentally, after 24 hour incubation at room temperature (an over test for the 4 hour equilibrium incubation step), a 10 nM ATP solution exhibited a reduction to 80.0% ± 6.1% of

the original concentration. An identical solution placed in boiling water for 5 minutes exhibited a reduction of the ATP concentration to 90.0% ± 8.0% of original value.

The slight reduction in ATP concentration during the conditions of the equilibration (4 hours only) and the extraction steps of the ATP assay protocol does not pose a problem for this study. As the ATP standards used in the recovery calculations underwent the same incubation conditions as the samples, any hydrolysis effect should be compensated for. However the observed reductions in ATP concentration demonstrate that the time for the incubation periods should be kept at a minimum.

### *6.3.3. Effect of chemical modifiers on ATP adsorption*

To prevent ATP being sequestered by the bentonite clay, thirteen chemicals were individually added to the model system in an attempt to modify the ability of bentonite clay to adsorb ATP prior to the addition of ATP. The chemical modifiers were separated into five classes comprising: phosphates, polyanions, surfactants, divalent cations and proteins. The effect of the chemical modifiers on: (i) ATP standard solutions without clay - to observe compatibility of the modifiers with the ATP assay and (ii) ATP standard solutions after exposure to clay samples - to observe the effect of the modifier on the ATP adsorption, were conducted in parallel, *i.e.* ATP recovery was calculated for each modifier with and without bentonite clay (Figure 6-1).

Each chemical modifier was tested at a minimum of two concentrations, which depended on class of chemical modifier. The modifiers and concentrations used are indicated in Figure 6-1. To select the concentrations used, two different approaches were taken.

Approach 1 was to use low concentrations of modifiers to limit interference with the subsequent ATP bioluminescent assay. This approach was applied to phosphates only. Due to the interference of (mono) phosphate with ATP bioluminescent reaction at >100 nM concentrations (Wen *et al.* 2001), the concentrations of the phosphates were selected to be 0.1 and 10 nM and nominally equivalent to 1 and 100 times the final ATP concentration.

For approach 2 the concentration of the chemical modifiers was selected to saturate potential adsorption sites, this approach was applied to all other classes of modifier. For the surfactants, 1 and 5% (w/v) HDTMA and DDTMA were chosen to approximate the maximum and minimum concentrations of HDTMA used by Perezgasga *et al.* (2005) to block a clay mineral. Perezgasga *et al.* (2005) quote the HDTMA concentration in term of percentage of the ion exchange capacity of their clay (50 to 250%), therefore the ion exchange capacity of a

typical bentonite clay from Meier & Kahr (1999) was used to calculate the concentration required. For polyanions, dextran sulphate and heparin, 0.05% and 0.5% w/v was used, based on the experiments in Lagaly & Ziesmer (2003). For the divalent cations, 4, 40 and 400 mM magnesium sulphate was added as 4 mM EDTA was already present in the standard buffer. As EDTA chelates divalent cations, EDTA concentrations were also modified and set at 0 and 40 mM (400 mM EDTA was not tested as it would not dissolve in 100 mM Tris). For proteins the concentrations used were typical of those used for blocking in immunoassays of 1% to 10% (Gosling 2000).

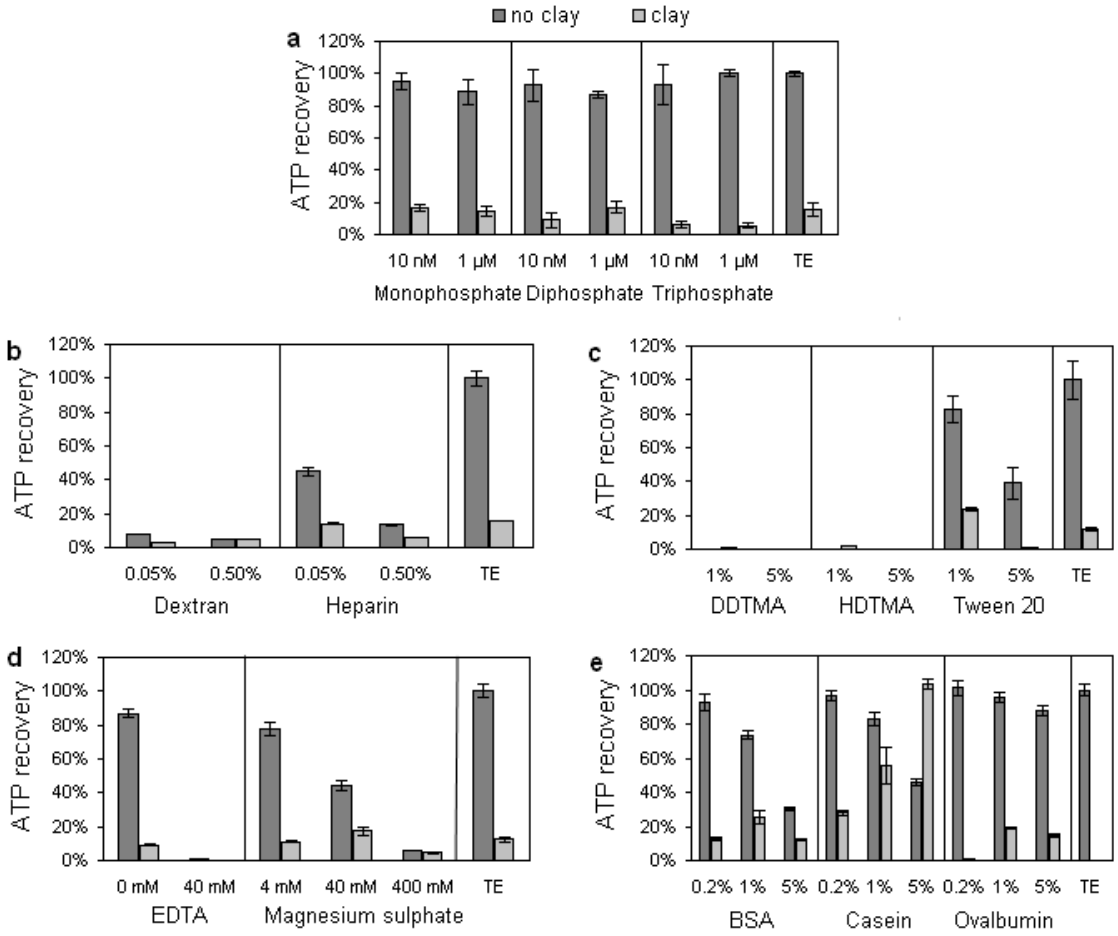


*Figure 6-1. ATP recovery for no clay (dark shading) and clay (light shading) with different chemical modifiers and their comparison to recovery with TE buffer control. ATP recovery is bioluminescent output of a sample in relative light units (RLU) as a percentage of the RLU value from TE buffer control (no clay or chemical modifier) of the same experiment. Solution ATP concentration is proportional to the RLU value. Chemical modifier classes are: a) phosphates, b) polyanions, c) surfactants, d) divalent cation, e) proteins. All concentration percentages are w/v. Error bars show ± 1 SD from triplicates.*

**Phosphates**

Monophosphate and triphosphate interact with the edges of clay minerals and increase the charge density (Berg & Joern 2006), and sodium triphosphate has previously been used to block edge sites of clay minerals including montmorillonite (Lyons 1964, Perezgasga *et al.* 2005); the predominant mineral in bentonite. As ATP is a polyphosphate, it is expected that if additional phosphate is added it will compete for the clay adsorption sites reducing the adsorption of ATP.

Results in Figure 6-1a show that there was no significant interference with the ATP bioluminescence reaction from any of the phosphates at the concentrations tested (ATP recovery ranged from 86.9% ± 1.9% to 100.0% ± 2.2%). When bentonite clay was added, ATP recovery was similar to ATP recovery using TE buffer only, from 5.7% ± 1.5% to 16.8% ± 3.5% (TE buffer 15.6% ± 4.4%). Therefore, the addition of phosphates at these concentrations has little effect on ATP recovery from bentonite clay.

The concentrations of triphosphate previously used to block adsorption sites of clays (0.1 and 0.05 M) (Lyons 1964, Perezgasga *et al.* 2005), were significantly higher than the concentrations used in this study. Also, higher concentrations of phosphates have been reported to increase the edge charge density of montmorillonite causing coagulation of the clay minerals (35 mM for 0.5% dispersion clay in triphosphate from Lagaly & Ziesmer 2003). This increased charge density should lead to the blocking of a significant number of edge adsorption sites. However if these higher concentrations were used as modifiers, they are likely to interfere with ATP bioluminescence reaction (Lundin & Thore 1975, Wen *et al.* 2001).

**Polyanions**

Polyanions such as tannate and polyphosphate have been reported to impart the same effect on clay minerals as phosphates; increasing charge density at the edge of minerals (Lagaly & Ziesmer 2003). Therefore the added polyanions should reduce the number of adsorptions sites available for ATP adsorption, are less likely to have the same direct inhibitory effect on ATP bioluminescence assay as phosphates, and therefore may be able to be used at higher concentrations. Dextran sulphate and heparin were chosen as representative polyanions as they are readily available.

The results in Figure 6-1b show that, without bentonite clay, at the concentrations of heparin and dextran sulphate tested, the polyanions interfered with the ATP bioluminescent reaction.

Dextran sulphate interferes more with ATP bioluminescent reaction than heparin – ATP recovery of 7.9% ± 0.3% compared to 44.8% ± 2.5% for 0.05% dextran sulphate and heparin respectively. The higher concentration of polyanions reduced ATP recovery further - down to 4.7% ± 0.2% for 0.5% dextran sulphate. Of the four samples, two modifiers each at two concentrations, only for the 0.5% dextran sulphate sample was the ATP recovery similar with and without the addition of clay however ATP recovery remained below 5%.

The reduction in ATP recovery without the addition of bentonite clay is likely to be due to an inhibitory effect of the polyanions on the ATP bioluminescence assay. Polyanions have been shown to form complexes with a multitude of different types of proteins (Jones *et al.* 2004, Joshi *et al.* 2008, Sedlák *et al.* 2009). Hence if the polyanions complex with the luciferase then they are likely to inhibit its activity, reducing the effectiveness of ATP bioluminescent reaction. For the 0.5% dextran sulphate sample, the similar ATP recovery with and without clay could indicate effective blocking of ATP adsorption sites; however the overall reduction in ATP signal by over 90% precludes further use of this modifier. It is noted that reducing the dextran sulphate concentration by a factor of ten (to 0.05% concentration) does not significantly increase the ATP recovery without clay, *i.e.* does not reduce the inhibition, although the blocking effect of the polyanion is reduced as indicated by the reduced ATP recovery in the presence of clay. Therefore further dilution of the dextran sulphate is not considered appropriate.

**Surfactants**

Surfactants, such as HDTMA, are adsorbed into the interlamellar spacing of clay minerals (Xu & Boyd 1995). The cationic surfactants DDTMA and HDTMA were chosen as they have been used previously to modify clay adsorption sites (Perezgasga *et al.* 2005, Ertem & Ferris 1998). Tween 20 was chosen as an additional surfactant as it is routinely used to generically block non-specific binding sites on surfaces in biotechnology applications.

The addition of DDTMA and HDTMA at the concentrations tested interfered with the ATP bioluminescence reaction. Figure 6-1c shows that 5% and 1% (w/v) DDTMA and HDTMA produced <1% of ATP recovery. When clay was added to both cationic surfactants, ATP recovery increased up to 9 times (1% w/v DDTMA), however ATP recovery remained below <2%.

Tween 20 had a much higher ATP recovery than both cationic surfactants, up to 82.6 ± 7.9% without clay. With 1% Tween 20 the ATP recovery in the presence of bentonite clay was

approximately twice that for TE (24.1 ± 1.0% and 12.0 ± 1.0% respectively), showing some potential to improve recovery of ATP in clay-rich samples.

Cationic surfactants including DDTMA have been shown to enhance the bioluminescence effect of firefly luciferase at low concentrations (Tani *et al.* 2008). After testing the cationic surfactants it was discovered that when concentrations of cationic surfactant increased the same cationic surfactants inhibit luciferase activity, this inhibition being complete at 10 mM (~0.3% (w/v)) for DDTMA (Tani *et al.* 2008). The increase in ATP recovery with the addition of clay to samples with HDTMA and DDTMA could indicate blocking of ATP adsorption sites, as the clay could bind to and hence remove some of the cationic surfactants, therefore reducing the concentration of the surfactant in the supernatant and its inhibitory effect. Perezgasga *et al.* (2005) found treating clays with HDTMA significantly reduced the adsorption of ATP onto sodium montmorillonite. As any excess reagent was removed prior to the addition of ATP and detection of ATP was via UV spectrometry, the interference from surfactants with ATP bioluminescence reaction was not a factor for ATP recovery in that study, meaning blocking these sites with surfactants is not suitable for the application in this study. Non-ionic surfactants such as Tween 20 are likely to have a lesser effect on the inhibition of luciferase because the interactions between protein and cationic surfactants have been shown to be due to ionic interactions (Yan *et al.* 2009).

**Divalent cation concentration**

Divalent cation concentrations have been observed to affect the ATP bioluminescence reaction, enhancing it at low concentrations and quenching it as the concentration of divalent cation increases (Wen *et al.* 2001). Also it has been demonstrated that divalent cations can enhance the adsorption of ATP in model clay systems (Rishpon *et al.* 1982). These effects were tested by the addition of a divalent cation and a chelating agent. Magnesium ions were chosen for the divalent cation as they are already present in the ATP bioluminescence reagent. EDTA was used as it is expected to chelate any magnesium ions (possibly in the ATP bioluminescence assay reagents) and therefore modify the concentration of magnesium ions.

The results in Figure 6-1d show that the addition of divalent magnesium ions, in the form of magnesium sulphate, without clay, reduces the ATP recovery. As the concentration of magnesium sulphate increases, the ATP recovery reduces, at 400 mM magnesium sulphate ATP recovery was 5.9% ± 0.2%. When clay was added the highest ATP recovery was

observed with 40 mM magnesium sulphate in TE (17.2% ± 2.1%). The effect of EDTA concentration of ATP recovery is discussed later in this section.

After the experiment was performed it was discovered that divalent cations have been observed to quench the ATP bioluminescence reaction at increasing concentrations (Wen *et al.* 2001). Although Wen *et al.* (2001) did not observe significant quenching with magnesium ions, the highest concentration tested in their study was 1 mM. However quenching was observed with sulphate ions at concentrations >1 mM (and up to 60% of original bioluminescence values at 6.4 mM). Therefore the quenching seen here may be an effect of the sulphate ion, all of the anions tested a measurable quenching effect was observed at 6.4 mM concentration. The highest ATP recovery with bentonite clay at 40 mM magnesium sulphate is likely to be due to increased blocking of the edge sites by the sulphate ions (Alves & Lavorenti 2004) at 40 mM. At 400 mM the quenching effect of magnesium and sulphate ions overcomes any benefit gained by blocking edge sites and at 4 mM the blocking of the sites was insufficient to increase ATP recovery.

As 4 mM EDTA was part of the standard buffer used with all chemical modifiers, the effect of 0 and 40 mM EDTA, without additional magnesium sulphate, was tested. Without clay, the removal of EDTA from the buffer slightly decreased ATP recovery to 87.0 ± 2.9%. At the higher 40 mM EDTA concentration ATP recovery dropped drastically to 0.6 ± 0.1%. The ratio between ATP recovery with and without clay for each of the EDTA concentrations remained similar. This indicates that EDTA had little or no interaction with the bentonite clay. It is expected that the increased levels of EDTA effectively reduced the available concentration of divalent cations (magnesium) by chelation and which are vital for the ATP bioluminescence reaction and therefore resulted in the drastic reduction in ATP recovery with 40 mM EDTA.

**Proteins**

Proteins are used to block adsorption sites surfaces in various bioanalytical techniques such as immunoassays. BSA, casein and ovalbumin are three readily available proteins commonly used in bioanalytical techniques. The three proteins were tested to assess their suitability for blocking bentonite clay adsorption sites to reduce ATP adsorption to clay.

The lowest concentration of all three proteins tested (0.2% w/v) showed little interference with the ATP bioluminescence assay, ATP recovery varied from 92.8% ± 4.6% for BSA to 101.2% ± 4.2% for ovalbumin (Figure 6-1e). As the concentration of the proteins increased

the inhibition of the ATP bioluminescence signal also increased as reflected in a decrease in calculated ATP recovery. This decrease was greatest with BSA where ATP recovery dropped to 30.4% ± 0.9% with 5% BSA. The highest ATP recovery with BSA and ovalbumin was with 1% protein giving 25.3% ± 4.2% and 18.7% ± 0.4% recovery, respectively. However a different pattern was observed with casein, as the concentration of casein increased, so did ATP recovery from 28.1% ± 1.3% for 0.2% casein to 103.6% ± 3.0% for 5% casein.

Evidence suggests that interactions between ATP and casein can prevent the luciferin and luciferase from accessing the ATP (Richardson *et al.* 1980, Simm *et al.* 2008), hence decreasing the availability of these biochemicals for the ATP bioluminescent reaction. This interference can explain the observed decrease in bioluminescence with increasing protein concentration. When clay is introduced to the system it is likely that the proteins are adsorbed to the surfaces and faces of mineral, as have been observed with casein on mica (Chowdhury & Luckham 1995), and whey proteins on faces and edges of kaolinite (Barral *et al.* 2008). The protein that is bound to the clay is unable to inhibit the ATP bioluminescence reaction as it is no longer present in the supernatant when tested. This view is supported by the higher ATP recovery with 5% casein and clay, in contrast to 5% casein without clay. The highest ATP recovery for samples with BSA and clay and ovalbumin and clay are observed at 1% (w/v) BSA and ovalbumin and appears to result from a balance to two competing processes. This is interpreted as follows, at 0.2% protein the concentration of protein is not adequate to effectively block the clay and ATP is lost be adsorption to clay, and at 5% protein, the concentration of protein not bound to the clay is sufficient to inhibit the ATP bioluminescence reaction and therefore overcome any benefit from the blocking of the bentonite clay surfaces.

**Modifiers summary**

The results shown in Figure 6-1 demonstrate the importance of testing the effect of the chemical modifier on the ATP bioluminescence reaction, as most of chemical modifiers tested interfered with this reaction. Only the phosphates at all concentrations tested and the proteins at the lowest concentration tested showed no significant interference with the ATP bioluminescence reaction (Figure 6-1a and Figure 6-1e). However, the phosphates showed no improvement in ATP recovery when clay was added, therefore only the proteins were considered further. Of the proteins, 5% (w/v) casein had the highest ATP recovery with clay at 103.6 ± 3.0%. Further investigations with different concentrations of the other modifiers tested here could improve ATP recovery. However the success, simplicity and non-hazardous

nature of the addition of casein to the buffer to minimise ATP adsorption meant that this approach was pursued further for the use in ATP analysis of low biomass, clay rich sediment samples.

### *6.3.4. Development of extraction protocol*

The prevention of ATP adsorption by sediment is only one aspect of the ATP extraction process. An extraction protocol needed to be developed to allow for processing of low biomass, high clay content samples compatible with measurement by the ATP bioluminescent assay. In addition, considerations were made so the protocol was compatible with field deployment, for example the elimination of shaking incubator steps.

As ATP is an intercellular biochemical, it has to be released from cells to be made available to the assay reagents. Also ATP is quickly degraded by cellular ATPases, therefore these need to be deactivated during extraction. To release intercellular ATP, an extraction protocol based on boiling in TE buffer as recommended by the manufacturer of the ATP test kit (Roche Diagnostics, Ltd., UK) was used. The new protocol is described in Section 6.2.5. The short incubation of the sample in a boiling water bath deactivates ATPases and, along with EDTA in the buffer and aids in cell lysis. To reduce adsorption of ATP by the sediment, 5% casein was added to the TE extraction buffer, and incubated with the sample prior to ATP extraction. This blocking time allowed for interaction between casein and sample. The manufacturer's extraction protocol heated the extraction buffer before addition to sample. Due to preincubation of buffer with sample this was not possible for this protocol. Therefore the incubation time in the boiling water bath was increased from 2 to 5 minutes.

**Sterilisation of casein chemical modifier**

Testing 5% (w/v) casein in TE buffer for background levels of ATP returned readings in the order of 1000 to 3000 RLU, equivalent to ~1 nM ATP (calibration using ATP standards), which was deemed to be too high for practical use. The source of the high background is likely to be from indigenous ATP in the casein used. Therefore to assess a standard method to reduce ATP background signal from casein, the casein was autoclaved 5% w/v in TE buffer. Autoclaving casein solutions reduced background RLU values about 100-fold to ~0.01 nM ATP, thus improving the lower limit of detection by about two orders of magnitude. However the ATP recovery was reduced from 103.6 ± 3.0% to 55.6 ± 0.6% of ATP in TE only. Despite this reduction in blocking efficiency of 5% casein after autoclaving, as indicated by the lower ATP recovery, it was still the most effective chemical modifier tested. The details behind the

change in efficiency are unknown but are likely to be related to minor modifications to the structure of casein during heat treatment (Le Ray *et al.* 1998, Raikos 2010).

**Shortening protocol time**

The protocol used to test the chemical modifiers took approximately 24 hours to complete. Reducing this time would limit hydrolysis of ATP and make the extraction protocol more practical especially when used in the field. Equilibrium concentration was chosen to test the ATP concentration as time is required for the samples to cool, due to the temperature dependence of the ATP bioluminescence reaction, and it allows for samples to be extracted in batches. Otherwise, if samples were tested immediately there would be a difference in ATP concentration between the first and last sample tested exclusively due to adsorption of ATP by the sediment. Both the length of the blocking step (*i.e.* the time between the addition of casein to the sediment and the ATP extraction) and the equilibration step (*i.e.* the time between the extraction of ATP from the blocked sediment and testing ATP concentration), were investigated.

To reduce the time of the blocking step five aliquots of 50 ± 0.5 mg bentonite clay with 1 ml 5% casein in TE buffer were prepared. ATP was added after 30, 60, 120 minutes and 18 hours. After ATP was added samples were left to equilibrate with ATP for 90 minutes (the equilibration step time was established before investigations into blocking step time), centrifuged for 5 minutes at 7200 x g and the supernatant tested. All samples were vortexed for 5 seconds after the addition of new liquids and after each incubation period. The results in Figure 6-2a show a decrease in ATP concentration to 56.0% of original value in the first hour of equilibration at room temperature. The change in ATP concentration then reduces much more slowly, *i.e.* it takes nearly 17 hours for an equivalent percentage decrease in the RLU value. If allowed to incubate for one hour then equilibrium is reached and allows time for analysis of samples without significant change in ATP concentration.

To investigate the equilibration time eight 1 ml aliquots of 5% casein were incubated with 50 ± 0.5 mg clay for 18 hours. To simulate ATP extraction of environmental samples 100 µl of 100 nM ATP solution was added to each aliquot. Aliquots were centrifuged at 7200 x g for 5 minutes before testing at 5, 20, 40, 60, 90, 120, 180 and 240 minutes after the addition of ATP standard. Figure 6-2b shows there is little change in ATP concentration during equilibration over the four hours tested, but the error between triplicates reduces after approximately 90 minutes. Therefore, the post ATP extraction equilibration time was reduced from 4 hours to a

minimum of 90 minutes. The new incubation times reduced the total protocol time from approximately 24 hours to approximately 4 hours.

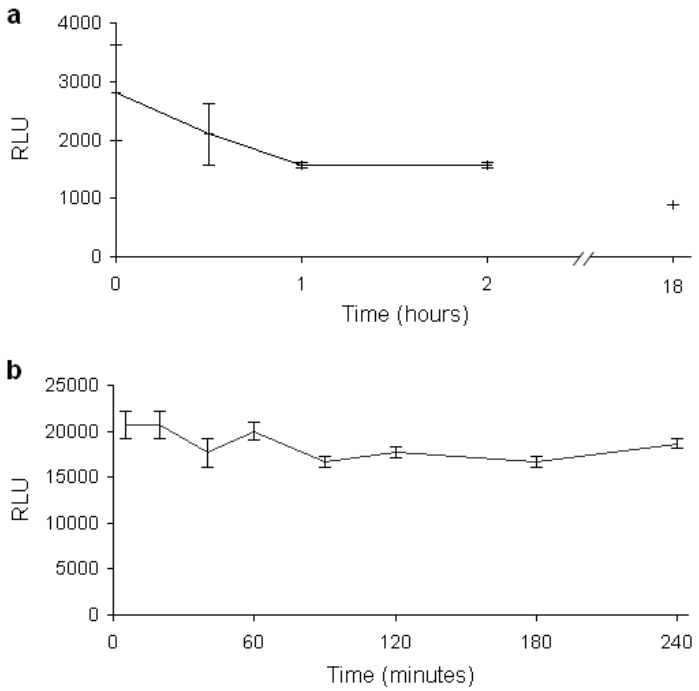


*Figure 6-2. Reduction in protocol time by monitoring the effect of ATP concentration by varying the a) time of the blocking step (*i.e. *the time after the addition of casein in TE to sediment), b) the time of the equilibration step (*i.e. *time after extraction). Readings are in RLU, and errors ± 1 SD from triplicates.*

### *6.3.5. Testing of new extraction protocol with glacial sediments*

The suitability of the field-deployable ATP extraction technique was established by testing with two glacial sediment samples and bentonite clay spiked with bacterial culture and the bacterial culture with no sediment. As the study was aimed at processing glacial samples, a psychrotolerant organism *F. xanthum* isolated from a low temperature environment was chosen. 100 µl of a blank, *F. xanthum* (cultured psychrotolerant bacteria), and 10- and 100-fold dilution of this primary culture were added to bentonite clay, fine glacial sediment, coarse glacial sediment and no sediment (*i.e.* buffer only). Two ATP extraction buffers were tested; TE buffer only and TE with 5% casein. After 1 hour incubation of bacteria or blank and sample, ATP was extracted and assayed.

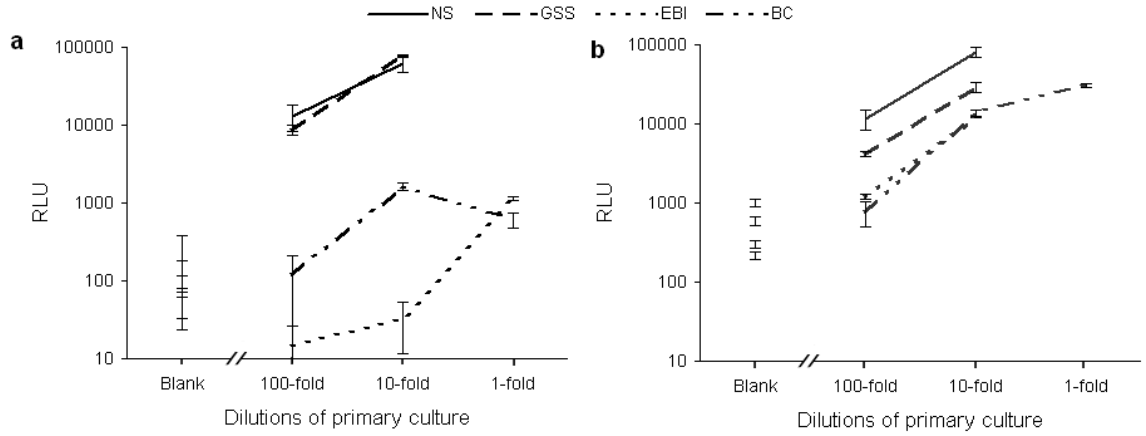


*Figure 6-3. The effect of 5% casein on ATP extracted from different samples with psychrotolerant bacteria. a) TE buffer only, b) 5% casein. There is no data for the highest concentration of bacteria spike in some samples as there was too much light to be detected by the instrumentation used. NS - no sediment, EBI - fine glacial sediment, GSS - course glacial sediment, BC - bentonite clay. Bacteria concentrations are relative to each other as it was not possible to quantify absolute bacterial concentration. Errors are SD from triplicates.*

By comparing the RLU values for the same sediment type extracted by the two buffers in Figure 6-3, it can be seen that the effect of casein in the extraction buffer strongly depended on the sample matrix. There was no significant change in RLU values when ATP was extracted from pure bacterial cultures, once sediment was added differences in the RLU values between the two extraction buffers were observed. With the coarse glacial sediment sample the RLU values halved with casein in the extraction buffer, but ~20 and ~200-fold increases in the RLU values were observed with the casein buffer for the bentonite clay and the fine glacial sediment, respectively.

To quantify interference from the glacial sediments with ATP concentration, an ATP spike was added to the sample after ATP concentration was assayed. This was performed in an experiment additional to that in Figure 6-3. 50 µl of a blank, a bacterial spike and 10- and 100-fold dilution of this primary culture (approximately $7x10^7$ $CFU.ml^{-1}$, CFU – colony forming units) were added to 100 ± 0.5 mg of no sediment, fine glacial sediment and coarse glacial sediment. After the sample was assayed 100 µl of 100 nM ATP was added to the remainder of the sample and buffer blank, incubated for 90 minutes and then ATP measured and spike recovery calculated as described in Section 6.2.5. The original RLU values were then corrected for spike recovery (Figure 6-4).

Correcting for ATP adsorption using spike recovery gives linear fit to log-log with $R^2$ >0.998 and gradients of 0.95, 0.82 and 0.96 for no sediment, fine glacial sediment and course glacial sediment respectively (using the data in Figure 6-4). The corrected bioluminescence values still show some variation between the different types of sediment for the same concentration of bacteria; the bioluminescence values are the highest with coarse glacial sediment, then no sediment with and lowest bioluminescence values from fine glacial sediment.

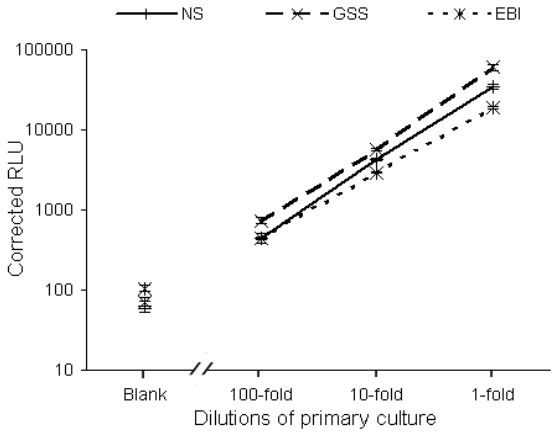


*Figure 6-4. ATP extracted from glacial sediments with bacteria, corrected for incomplete ATP recovery by using an ATP spike. Primary culture concentration was ~7x10$^7$ CFU.ml$^{-1}$, CFU – colony forming units. Errors are SD of analytical triplicates.*

The new protocol shows a significant improvement in ATP quantification in samples with high clay content, and can still be applied to low clay content samples. The new protocol is suitable for our purposes as small changes in ATP concentration are of most interest when they are within a specific sample type. However there is room for some further improvements, as after applying a spike recovery step, Figure 6-4 demonstrates that there is still some effect from the sediment on ATP concentration that has not been corrected for (bioluminescence values vary from 55 to 173% of the equivalent bacterial spike concentration with no sediment). This is can be seen because the same concentration of bacterial spike gives different bioluminescence values depending on the sediment type. This inconsistency could be due to incorrect spike value, differences in equilibrium concentration or changes in the extraction efficiency of this protocol with the different sediments. Therefore, if accurate (*i.e.* within a factor of 2), comparisons of ATP concentration between different sample types are needed then clarification of the sources of these inconsistencies is required. In summary, this technique is a simple, field deployable technique and particularly robust if comparing samples with similar sediment matrix. As this method can be applied in the field, amount of casein can

be optimised to balance the effects of adsorption by the sediment and interference with the bioluminescence assay prior to conducting analyses on a particular sample type, or range of sample types.

## 6.4. Conclusions

Most of the chemical modifiers tested in this study, reported to reduce ATP adsorption onto clays by blocking adsorption sites, interfered with the ATP bioluminescence reaction (Figure 6-1). Therefore, a balance between the blocking ability of the chemical modifier and any interference with ATP bioluminescence reaction was required. Of the five classes of modifiers tested, proteins were the most successful, with addition of 5% w/v casein having an ATP recovery of 103.6% ± 3.0 (Figure 6-1e).

Development of casein in TE buffer into a protocol for ATP extraction allowed the detection of ATP in clay-rich glacial samples with a total assay time of 4 hours. Implementing a spike recovery step after assaying, for the two glacial sediments tested, a linear relationship between cell concentration and RLU values was observed. However these bioluminescence values showed some variance depending on the sediment (Figure 6-4), meaning care should be taken when comparing different sediment types. The addition of spike recovery step increased the total assay time to 6 hours.

Using sterilised 5% casein in TE buffer as the extraction buffer, a two-fold decrease in sensitivity of the ATP bioluminescence assay was observed over TE extraction buffer when using the coarse glacial sediment, this decrease was significantly outweighed by >20 and >200-fold improvement in ATP recovery when using clay rich samples (bentonite clay and clay-rich glacial sediment respectively). This demonstrates that the casein in TE extraction buffer shows the most benefit to ATP extraction from clay-rich sediments but can be applied to other sediment types, hence wider applications of this technique include the analysis of ATP in clay-rich samples, especially if expected biomass is low ($3.4x10^4$ CFU in 100 mg sediment demonstrated in this study).

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# 7. In-Field, High-Temporal Resolution Analysis of Microbial Biomarkers in Subglacial Runoff from the Greenland Ice Sheet

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## Abstract

A field laboratory was established 1 km from the snout of a South West Greenland glacier, this enabled the collection of the first high-temporal resolution dataset on microbial biomarker concentrations in subglacial runoff. Four analytical techniques were performed in the field laboratory: real-time PCR; ATP bioluminescence; recombinant Factor C (rFC) assay to detect lipopolysaccharide (LPS); and fluorescent cell counts. Seven primer pairs were used with real-time PCR, with two targeting bacterial and archaeal 16S rDNA genes, and the remaining five targeting functional genes, specifically for sulphate reduction (dsrA), denitrification (nirK), methane oxidation (pmoA), methanogenesis (mcrA) and nitrogen fixation (nifH). These techniques were applied to 33 subglacial runoff samples (collected daily or twice daily) and 12 supraglacial samples (stream and cryoconite holes) between 16th July and 8th August 2009. ANOVA analysis could distinguish among the samples of three origins ($P<0.001$) with each of the analytical techniques applied. However there was also a correlation between some of the biomarkers and extraction batch or storage time ($P<0.05$). Therefore, further statistical analysis (PCA) was conducted on the remaining biomarker data. Studying the subglacial runoff samples an increase was observed in Factor 2 (related to ATP concentration – loading of 0.8303) especially after 27th July, indicating potential increase in microbial activity. By conducting these analyses in the field the sampling strategy used to collect the dataset and sample processing protocols specific to Leverett Glacier were refined. Results support the use of in-field biomarker analysis as an alternative approach to performing glacial

biogeochemical research, promising immediate results that can be used to inform future sampling, and enabling high rates of data acquisition.

Keywords

Leverett Glacier, South West Greenland, real-time PCR, ATP bioluminescence, rFC LPS detection

## 7.1. Introduction

The existence of active microbial populations at glacier beds has been known for a decade, and was initially detected from chemical changes in subglacial runoff and through culturing microorganisms isolated from debris-rich basal ice under *in situ* temperatures (Sharp *et al.* 1999, Skidmore *et al.* 2000). Since the initial findings, numerous microbiological techniques and chemical analyses have been applied to various subglacial environments to understand the extent of subglacial microbial activity and hence estimate its effects, and include detection of nucleic acid biomarkers, direct microbial enumeration and measurements of isotopic signatures (Skidmore *et al.* 2000, Mikucki *et al.* 2004, Wadham *et al.* 2004, Lanoil *et al.* 2009). These studies have provided insights into the taxonomic and metabolic diversity and activity levels of the subglacial communities from high latitude and high altitude glaciers, subglacial lakes and ice streams (recently reviewed by Christner *et al.* 2008 and Hodson *et al.* 2008), and demonstrate the ubiquitous nature of microbial communities in the subglacial environment. Microbiological activity at the glacier bed may have implications for the behaviour of glaciers, in particular the ability to draw down $CO_2$ during times of glacial retreat or to release methane produced during glaciation (Sharp *et al*. 1995, Wadham *et al.* 2008), making it important to understand microorganisms role in this biogeochemical cycling to feed into models of past and future climate. These subglacial microbes have already been found to modify their local chemical environment, including influencing the cycling of carbon, sulphur, nitrogen and iron (Sharp *et al.* 1999, Skidmore *et al.* 2000, Bottrell & Tranter 2002, Mikucki *et al.* 2004, Wadham *et al.* 2004, Wynn *et al.* 2007).

Microbial communities have been found in subglacial environments that are distinct from surrounding supraglacial and proglacial environment (Gaidos *et al.* 2004, Bhatia *et al.* 2006, Kaštovská *et al.* 2007). Hence, subglacial runoff is studied as it provides insight into an environment that is otherwise logistically difficult to access (Hodson *et al*. 2005, Mikucki *et al.* 2009). High-temporal resolution chemical analysis of subglacial runoff has shown changes over diurnal cycles within a melt season (Brown 2002). In one particular example, a periodic

drop in the nitrate concentrations in subglacial runoff of Haut Glacier d'Arolla, Switzerland, has been interpreted to be of potential microbiological origin (Bottrell & Tranter 2002). However, no studies have specifically targeted high-temporal resolution changes in microbiology in subglacial runoff. This is required to understand how subglacial activity may change throughout a melt season in order to more accurately estimate glacial contribution to global microbial reserves and activity and therefore reactions to future changes in climate (Priscu *et al.* 2008, Wadham *et al.* 2008).

Much of cryosphere is geographically remote. Analysis conducted on easily accessible glacial environments such as many of high altitude glaciers are not necessarily representative of the entire cryosphere, especially when considering the relative size of the polar ice sheets. However collecting and returning samples from remote locations increases logistical issues regarding sample preparation and transportation. We propose in-field analysis will vastly improve the efficiency and productivity of field campaigns as it will enable in-field refinement of sample processing and analytical protocols relevant to a given field site; enable in-field hypothesis development and testing; enable an "intelligent" approach to sampling strategies; and reduce the burden and detrimental effects of sample preservation and transport to institutional laboratories.

The aim of the present study was to collect high temporal resolution samples of subglacial runoff through multiple rounds of in-field analysis of microbiological biomarkers using multiple analytical techniques. This was applied to runoff and supraglacial samples at Leverett Glacier on the south western margin of the Greenland Ice Sheet. Four analytical independent techniques were selected that complimented each other, and each fulfilled the following criteria: microbiologically relevant targets with relative or quantifiable results, commercially available portable instrumentation for analysis and sample preparation and low lower limits of detection. ATP bioluminescence detects ATP, which is a biomarker for all known extant microbial life and represent both cell numbers and activity. Real-time PCR provides the opportunity to detect several specific markers with one technique through the choice of primers. Recombinant Factor C (rFC) assay detects lipopolysaccharide (LPS) which is a proxy for Gram-negative bacteria. Finally, fluorescence cell counts were selected as a standard technique and was found to be applicable to all sample types (Chapter 5).

## 7.2. Materials and Methods

### *7.2.1. Field site*

The Leverett Glacier is a land terminating glacier draining part of the western margin of the Greenland Ice Sheet just north of the Arctic Circle (snout at 67°03'N 50°11'W). A field camp was established on land, 1 km west of the glacier snout and between the drainage waters of Leverett Glacier and Russells Glacier (Figure 7-1a). Leverett Glacier was selected for sampling as the subglacial runoff could be seen to exit the portal (Figure 7-1b). All microbiological analyses were conducted in the field laboratory, however some sample processing was conducted on the glacier surface with hand vacuum pump (Figure 7-1c).

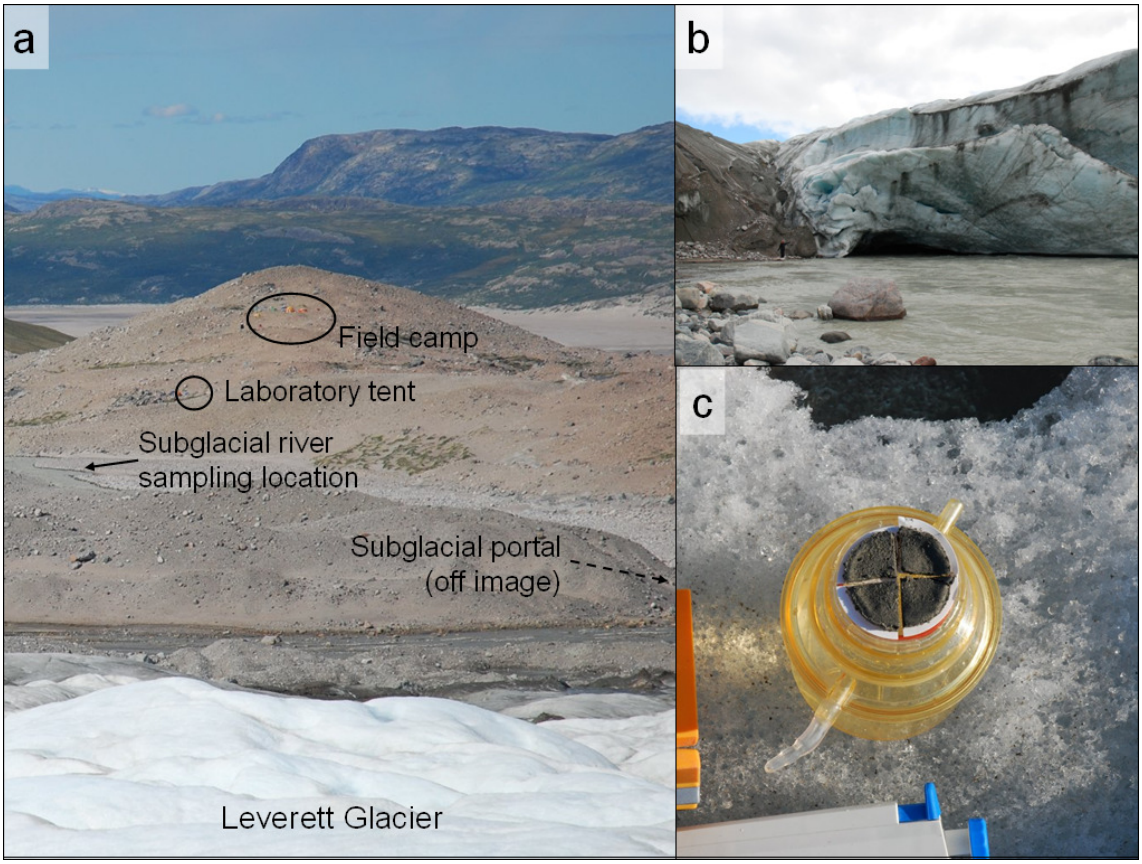


*Figure 7-1. Photos to give an indication of conducting in-field life detection at Leverett Glacier. a) overview of field camp taken 1km from the snout of Leverett Glacier (away from the subglacial portal) looking north east, b) Leverett Glaciers' subglacial portal on 27th June 2009, and c) filter membrane of cryoconite hole sample after filtering on the glacier (filter membrane 47 mm diameter).*

*7.2.2. Sample collection and processing*

Subglacial runoff samples were collected daily from 16th July 2009 (Day 197) to 7th August 2009 (Day 219) at 9 am and every other day at 6 pm (all local times), the only exception was for the morning of 3rd August (Day 215) as personnel were absent for logistical reasons. Subsequently all days are referred to in Julian day and sample labels include 0.38 or 0.75 depending on whether collected at 9am or 6pm, respectively. The daily samples were collected during the final phase of the melt season (Bartholomew *et al.* 2010). All subglacial runoff samples were collected from the proglacial stream ~400 m from the major subglacial portal of the glacier (Figure 7-1b). Supraglacial samples were collected from sites between 1 and 2 km from the glacier snout on Days 197, 204, 214 and 220. An additional four cryoconite hole samples were collected on Day 214 at 4, 6, 8 and 10 km from the glacier snout. Cryoconite samples were pooled from three separate holes at the same sampling distance and included a representative mix of water and sediment. All sample containers were rinsed at minimum of five times in sample water prior to collection of final sample.

For ATP bioluminescence and real-time PCR analyses, 100 or 150 ml of subglacial runoff, 125 ml of cryoconite hole sample or 2 litres of supraglacial stream was filtered onto 0.22 µm pore size membranes. The filter membranes were split; half to extract DNA for real-time PCR and the other half for the ATP bioluminescence assay (Figure 7-1c). The membranes were stored separately at -13°C in a generator powered freezer until processed within 4 or 8 days for the extraction of DNA or ATP respectively. For cell counts, 1 ml of subglacial runoff or cryoconite hole samples and 20 ml of supraglacial stream sample were preserved with formaldehyde to give final formaldehyde concentration of 2.5% (v/v). For LPS determination, 1 ml aliquots of each sample were taken. The samples for cell counts and LPS determination were stored at 2-8°C until processed, up to 2 months for cell counts or 8 days for LPS determination.

*7.2.3. DNA extraction and real-time PCR conditions*

DNA was extracted directly from filter membranes that were disaggregated using flame steriled tweezers. UltraClean™ DNA Isolation Kit (Mo Bio Laboratories Inc product code UC-12800, CamBio Ltd, Cambridge, UK) was used. The manufacturer's protocol was followed as closely as possible, the two exceptions were that all centrifuge steps were performed at 7200 x g and samples were shaken manually instead of using a vortex mixer. Extraction blanks consisting of a disaggregated filter membrane were conducted with each extraction batch.

Real-time PCR was conducted in a MiniOpticon™ (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). A total PCR volume of 10 µl was used with iQ SYBR Green Supermix (Bio-Rad Laboratories Ltd.). 1 µl sample and 500 nM for forward and reverse primers were used for all analyses except for cyanobacteria where 150 nM forward and 450 nM reverse primer was used. The real-time PCR conditions were 3 minutes at 95ºC then 40 cycles of denaturing at 95ºC for 10s, annealing at 51°C, 54°C or 60°C for 20s and extension at 72ºC for 27s (Table 7-1). This was followed by a melt curve held at 1ºC increments each for 1 second between 65ºC and 95ºC. Selection of the cycle threshold (Ct) line and calculation of Ct values was conducted using CFX Manager™ Software v 1.0 (Bio-Rad Laboratories Ltd.).

*Table 7-1. Genes targeted using real-time PCR and the associated annealing temperatures, see Appendix B for primer sequences and optimisation conditions.*

| Gene | Forward / reverse primers | Annealing T. (ºC) | Reference |
|---|---|---|---|
| 16S rDNA (bacteria) | E8F / E533R | 54 | Baker *et al.* 2003, Manerkar *et al.* 2008 |
| 16S rDNA (eubacteria) | PLK1 / PLK2 | 60 | Klaschik *et al.* 2002 |
| 16S rDNA (archaea) | ARC787F / ARC1059R | 54 | Yu *et al.* 2005 |
| 16S rDNA (geobacteraceae) | CYA359F / CYA781R | 51 | Nübel *et al.* 1997, Yergeau *et al.* 2008, |
| 16S rDNA (cyanobacteria) | Geo564F / Geo840R | 51 | Cummings *et al.* 2003 |
| 18S rDNA (fungi) | NS5 / NS6 | 51 | White *et al.* 1990, Wu et al. 2002 |
| dsrA for (bi) sulphite reductase | DSR1-F+ / DSR-R | 51 | Leloup *et al.* 2007, Kondo *et al.* 2004 |
| nirK for nitrite reductase (copper) | nirK 1F / nirK 5R | 54 | Braker et al. 1998, Geets et al. 2007 |
| pmoA for particulate methane monooxygenase | A189 F / Mb661 R | 54 | Costello & Lidstrom 1999, Kolb *et al.* 2003 |
| mcrA for methyl coenzyme-M reductase | ME1f / ME2r | 51 | Hales *et al.* 1996, Inagaki *et al.* 2004 |
| nifH for nitrogenase reductase | nifH F / nifH R | 60 | Rosch *et al.* 2002, Ogilvie *et al.* 2008 |
| mxaF for methanol dehydrogenase | mxa1003f / mxa1561r | 51 | McDonald & Murrell 1997, Inagaki *et al.* 2004 |

### *7.2.4. ATP extraction and determination*

An ATP extraction procedure for high-clay content, low biomass samples, developed in Chapter 6, was used. 1 ml of 3.3% w/v casein in 100 mM Tris, 4 mM EDTA buffer was added to disaggregated filter membrane and left to block for 60 minutes. Samples were then incubated in a boiling water bath for 5 minutes, cooled and stored at +6ºC for 90 minutes to equilibrate. Samples were shaken and then centrifuged at 7200 x g for 5 minutes. ATP Bioluminescence Assay Kit HSII (Roche Diagnostics Ltd, Burgess Hill, UK) was used to

analyse environmental extracts and ATP standards (provided in the assay kit). 50 µl of the supernatant was then added to 50 µl of ATP bioluminescence reagents. Luminescence was measured in a HY-LiTE 2 luminometer (Merck, VWR International Ltd., Lutterworth, UK) with output in Relative Light Units (RLU). Standards between 10 and 0.01 pM ATP and appropriate blanks were assayed with each analysis batch and used to quantify ATP concentration. To correct for interference from the sediment, a spike recovery step was conducted. Post analysis, 100 µl of 100 pM ATP standard was added to each sample, the sample was shaken vigorously and after 90 minutes, centrifuged again and the supernatant re-tested. The difference in the bioluminescence value before and after spike addition was compared for the bioluminescence value of a spiked blank to calculate spike recovery.

All extractions were conducted in the field, however due to a problem with contamination of some of the stock of analysis tubes transported to the field site, the ATP samples from Sample 203.38 onwards were assayed for ATP concentration in an institutional laboratory after the field campaign. To preserve the ATP concentration during storage and shipping, a 210 µl aliquot of the supernatant was taken after the first centrifuge step, this aliquot and the remaining liquid and filter membrane were frozen in the field and transported to the institutional laboratory. The supernatant aliquot was used to quantify ATP concentration and the remaining liquid and filter membrane was used for spike recovery calculations. ATP standards and extraction blanks were frozen in the field and used along with fresh ATP dilutions in institutional laboratory to quantify ATP concentration.

### *7.2.5. Lipopolysaccharide (LPS) determination*

The Cambrex PyroGene® recombinant Factor C (rFC) endotoxin detection system (product code 50-658U, Lonza, Slough, UK) was used to quantify LPS concentration. Prior to analysis the samples were centrifuged for 5 minutes at 7200 x g and the supernatant was tested. Due to the non-temperature controlled conditions that the in-field assay was conducted, the manufacturer's protocol required modification (Chapter 4). Briefly, 50 µl of sample was added to 50 µl of working reagent (made up as per manufacturer's protocol). Measurements were made at time zero, then every hour for 3 hours in a *Pico*Fluor handheld fluorometer (Turner BioSystems, CA, USA). Output from the fluorometer was in Relative Fluorescence Units (RFU). Standards between 0.1 and 10 $EU.ml^{-1}$ were co-analysed with every assay batch. The assay response of the rFC assay to each sample and standard was calculated by performing a linear least squares fit of fluorescence intensity verses time, giving a rate of

fluorescence intensity change in units of RFU.min$^{-1}$. The assay response of the samples and standards was then used to quantify LPS concentration.

### *7.2.6. Microbial enumeration*

The formaldehyde preserved samples (see Section 7.2.2) were filtered onto a 0.2 µm pore size filter membrane (Millipore, FDR-312-020W, Fisher Scientific). Samples from subglacial stream and cryoconite holes were diluted prior to filtration to disperse samples. The water used to dilute the samples was prepared locally and immediately prior to use by filtering water using sterile, disposable 0.22 µm pore size in-line filters and syringes. The filter membranes with sample were stained with 400 x dilution of SYBR® Gold nucleic acid stain (Molecular Probes™, Invitrogen Ltd., OR, USA) for 15 minutes in the dark, and the slide was assembled using SlowFade® Antifade kit (Molecular Probes™, Invitrogen Ltd, OR, USA). Counts were performed with a CyScope fluorescence field microscope (Partec UK, Canterbury, UK) with 455 nm excitation and 520 nm emission filters. Due to limited time during field work 25 fields of view for each sample were counted. The fluorescence cell counts were completed in the field only when the ambient field tent laboratory temperature was below 22°C, due to concerns of fading of the fluorescent dye. Due to time constraints some samples were stored cold and transported to an institutional laboratory and for cell count analysis.

### *7.2.7. Sediment concentration calculations*

To calculate the sediment concentration in the subglacial runoff samples, 1 ml aliquots of each sample were collected and sealed in the field, and a larger volume (250 ml) of sample collected on Day 220 was used as a calibration sample. Following the field campaign, the optical density of 50% dilution of each sample, plus a Milli-Q water blank and calibration set of 25, 35, 40, 45, 50, 75 and 100% of original concentration of the calibration sample was measured at a wavelength of 300 nm in a benchtop microtitre plate reader (Varioskan Flash, Thermo Scientific, MA, USA). Each measurement was performed in duplicate. Additionally, three sets of 40 ml of calibration sample was filtered onto separate 0.22 µm pore size membranes and dried at 37°C until the mass stabilised. The resultant sediment mass per unit volume used to convert to optical density readings.

For cryoconite samples, 40-50 ml aliquot of each sample was collected and sealed in the field. Upon return to institutional laboratory these were then filtered, dried and the dry mass measured.

### *7.2.8. Data treatment for statistical analysis*

To obtain a normal distribution, a $\log_2$ transformation was applied to the ATP, LPS, cell count and sediment concentration data. Test for normality was performed by visual inspection of p-plot of residual data. For the real-time PCR data, the Ct values were corrected for sample volume, and as higher Ct values indicate lower DNA concentration, the negative of the corrected Ct value was used to give positive correlation between input data and abundance. Outliers were identified and removed from the real-time PCR data by visual inspection of the melt curves and by p-plot of residuals data. Missing data were treated by substitution to give an interpolated data set (Tabachnick & Fidell 2007). The missing values were replaced by the minimum Ct value for nifH for real-time PCR (Sample 203.38 to 205.38) as there was no amplification of this primer within 40 cycles. The means of the nearest samples were used for ATP (204.38 and 214.38) as these samples were missing and the mean of the sample type were used to complete the cell count data. All statistical analyses were conducted using Statistica 9.0 (StatSoft Inc. OK, USA).

## 7.3. Results

### *7.3.1. Preliminary in-field experiments to finalise protocols for sample collection and processing*

Before subsequent analysis could be performed, a suitable source of water for sample processing and negative controls was required. These sources differed among the techniques. For real-time PCR and ATP bioluminescence, supraglacial water was filtered through 0.22 µm pore size filter membranes and then autoclaved at 121°C for 15 minutes. This was sufficient to reduce blanks below the lowest standard, and the blanks remained low if the water was used within two days of preparation. To provide water to dilute the samples for cell counts the water was used immediately after filtration without autoclaving. LPS free water was supplied with the rFC LPS detection assay kit and was used when required in all LPS analyses.

To refine sampling strategies and processing protocols preliminary analyses were conducted on two subglacial runoff samples, two cryoconite samples and one surface stream sample, all collected on Day 187. Analysis of these samples demonstrated that positive results could be obtained for all the techniques used, confirming equipment, reagents and consumables survived transportation and could be used to detect biomarkers above blank values (data not shown). These initial analyses were also used to select primer pairs for real-time PCR. As the concentration of cells and the community structure in Leverett runoff was unknown prior to

commencement of the field campaign more primer pairs were transported to the field than would be used routinely on the samples. Seven primer pairs were selected and applied to subsequent subglacial and supraglacial samples (bacteria, archaea, dsrA, nirK, pmoA, mcrA and nifH). These provided representatives of the expected kingdom level phylogeny and a range of metabolic pathways. In the five remaining pairs, DNA could not be detected in either of the subglacial runoff samples, using a cut off of 3.3 Ct cycles (one order of magnitude) of the negative control.

These initial analyses also lead to modifications of sample processing protocols, specifically a reduction of concentration of casein in ATP extraction buffer from 5% to 3.3% (w/v), and the addition of a centrifuge step prior to the testing of the LPS samples.

Further samples were collected to refine sampling strategy, specifically chose sample frequency, on Day 190, 192 and 194 when the height of the river approximated the minimum and maximum of the diurnal cycle, in this case at 9 am and 6 pm. These samples were analysed using real-time PCR with bacteria, archaea, dsrA, nifH and mcrA primer pairs and with the rFC assay. Over the six samples, the real-time PCR varied up to 6 Ct values (mcrA) (*i.e.* a factor of $2^6$) and LPS by a factor of 2. Differences could be seen between the morning and afternoon samples, the afternoon samples having lower DNA concentrations than the morning samples in the first two samples, however the DNA markers were higher in the afternoon samples from the final day. Therefore it was chosen to collect samples daily in the morning and on alternate days in the afternoon.

### *7.3.2. The temporal experiment*

All 33 subglacial runoff samples were analysed by real-time PCR with seven primer pairs, ATP bioluminescence assay and rFC assay. Due to time constraints, only selected samples analysed for fluorescent cell counts. Additionally, parallel samples were collected to assess sediment concentration after the field campaign. A near complete record of analysis was conducted on samples collected in the temporal experiment, only a few exceptions are observed in Figure 7-2. For the real-time PCR analysis this is due to the target concentration being below the detection limit of the assay, for the ATP bioluminescence assay taken on day 203.75 and 214.38 the samples were lost, and for LPS on 207.75 the level was above quantifiable LPS concentration (*i.e.* >10 $EU.ml^{-1}$).

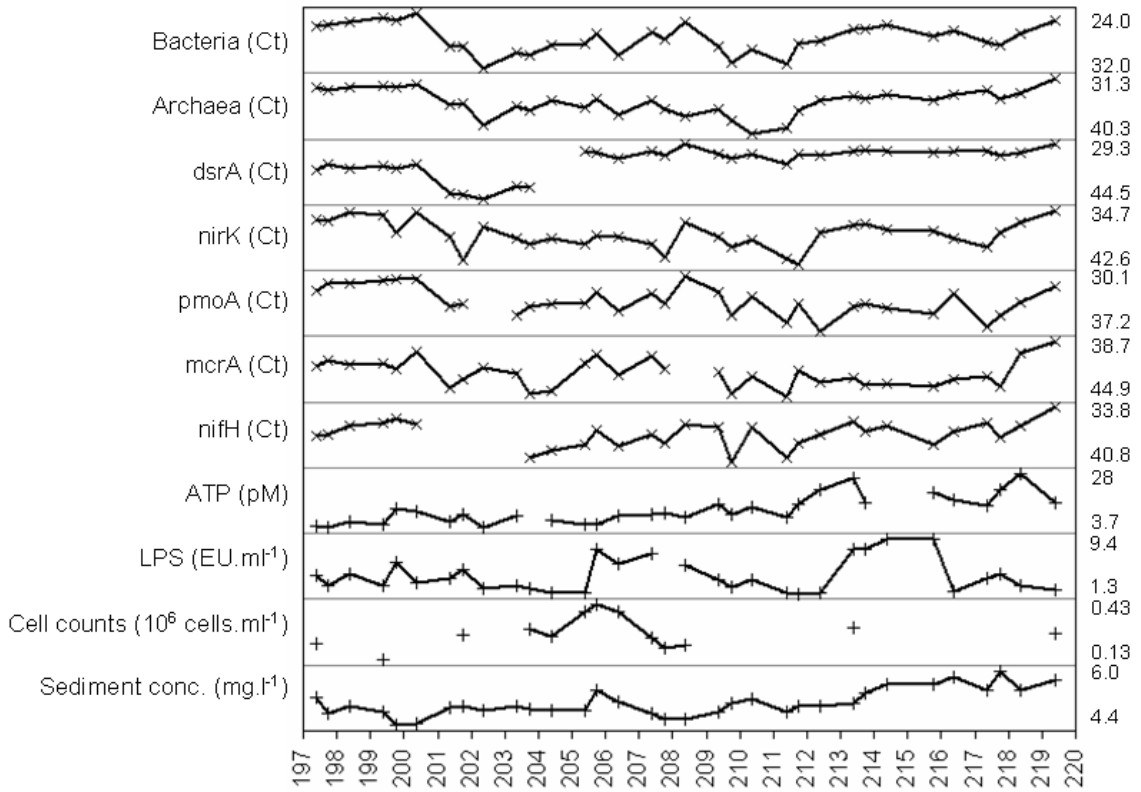


*Figure 7-2. High-temporal resolution biomarker concentration in the subglacial meltwaters of Leverett Glacier. The biomarkers were seven primer pairs analysed by real-time PCR, and data for ATP, LPS, cell and sediment concentration. The real-time PCR data are the Ct values corrected for filter volume and positively correlate with DNA concentration (i.e. $log_2$ scale and Ct values are reversed). Shaded areas indicate the afternoon samples.*

There are no significant differences ($P$>0.05) between morning and afternoon subglacial runoff samples nor obvious trends that appear in all the biomarker concentrations. From the real-time PCR data, there are periods of relatively minimal change (*i.e.* from Sample 201.38 to 203.38 and Sample 213.38 to 217.75 in bacteria, archaea and mcrA), periods of general increase (from Sample 197.38 to 200.38 and from Sample 217.75 to 219.38 in all primers) and periods of more rapid change (from Sample 208.38 to 213.38 in all primers except archaea). A general increase is observable in ATP and sediment concentration through the sampling period, starting at about Sample 205.38. The LPS concentration shows little variation, however large increases are observed after Sample 205.38 and Sample 212.38 and a decrease after Sample 215.75. Variations can be seen in the cell count data, although these are limited due to the number of samples assayed. At different times during the collection period some of the biomarkers trace each other; the pattern of the ATP and LPS concentration in the

first nine samples is very similar, as is the pattern in the majority of the primer pairs and ATP concentration between Sample 209.38 and 211.38.

Some of the changes observed in the dataset could be a result of the strategy used to analyse the samples, *i.e.* in-field storage of samples for up to 8 days, then extraction of a batch of samples. Of note is the high concentration of all of the PCR primers after the first six samples, which coincides with the first extraction batch and increase in LPS concentration after Sample 206.38 and 212.38, which coincides with the commencement of a new cycle of in-field storage.

To enable trends to be better visualised from the combined dataset of all the various biomarker values, additional data analysis was performed - specifically analysis of variance (ANOVA) comparing the results and sample processing *i.e.* extraction batch and sample storage time as categorical predictor and Principal Component Analysis (PCA) (see Section 7.3.4).

### *7.3.3. Supraglacial microbial input composition*

Supraglacial samples from Leverett Glacier, consisting of supraglacial stream and cryoconite holes, were collected on four separate days to provide insight into potential microbiological inputs from the glacier surface to the subglacial runoff. These were analysed with the same techniques as for the temporal experiment with the results from ATP, LPS and cell counts shown in Table 7-2.

*Table 7-2. Quantifiable biomarkers and sediment concentrations in the different glacial sample types. Numbers are the minimum and maximum values from the individual samples.*

| Biomarker/variable | Cryoconite holes | Supraglacial meltwater | Subglacial meltwater |
|---|---|---|---|
| Number of samples | 8 (2)* | 4 (2)* | 33 (12)* |
| ATP (pM) | 240 – 2000 | 0.75 – 2.8 | 3.7 – 29 |
| LPS ($EU.ml^{-1}$) | 0.50 – 6.1 | 0.24 – 2.6 | 1.3 – 19.4$^{\dagger}$ |
| Total cell counts ($cells.ml^{-1}$) | $1.6 \times 10^6 – 4.1 \times 10^6$ | $4.3 \times 10^3 – 8.6 \times 10^3$ | $1.3 \times 10^5 – 4.3 \times 10^5$ |
| Sediment ($mg.ml^{-1}$ dry) | 3.3 – 6.4 | n.d. | 4.4 – 6.0 |

*Numbers in brackets indicate the number of samples that cell counts were conducted on.

$^{\dagger}$Upper limit may be artificially high, see discussion on correlation with storage time.

n.d. – not determined.

The results in Table 7-2 show significant ($P$>0.001) differences in biomarker concentrations between the subglacial and supraglacial samples, and between different types of supraglacial samples (*e.g.* surface streams versus cryoconite holes). The ATP and cell count concentration is highest in the cryoconite hole samples, about an order of magnitude higher than the subglacial runoff samples, despite similar sediment concentrations being measured. The

subglacial runoff samples are just over an order of magnitude higher than the supraglacial stream samples. This order of magnitude change is not seen in LPS concentration among the sample types. Within each sample type, the range of each of the biomarkers is just under an order of magnitude, however the relative range in the concentration of ATP and LPS is slightly higher than for cell counts.

### *7.3.4. Application of ANOVA and PCA to analyse the temporal experiment dataset*

To objectively assess the effects of sample type and sample processing (*i.e.* short term storage and extraction batch), one-way ANOVA was conducted on the subglacial and supraglacial dataset. The sample type has the most significant effect on results of each technique or primer pair ($P<0.001$ for all analyses) meaning all biomarkers vary among the sample types and can be used to differentiate some sample types. Looking into the effect of batch processing, LPS showed strong correlation with storage time ($P=0.003$) and the primer pair for dsrA showed a strong correlation with extraction batch ($P<0.001$) meaning there is a significant effect on the results of these aspects of sample processing. In addition there was correlation between the amount of bacterial and archaeal DNA and extraction batch (bacteria $P=0.013$ and archaea $P=0.029$) meaning there is a smaller, yet still statistically significant effect between these primers and extraction batch.

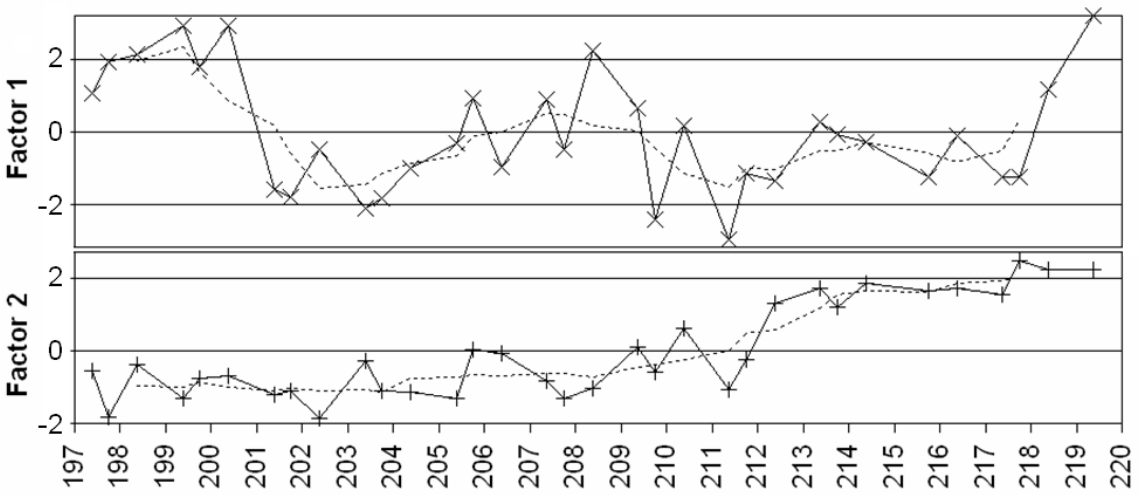


*Figure 7-3. Temporal evolution of Factor 1 and Factor 2 from PCA analysis on subglacial runoff samples with the following variables: real-time PCR primer pairs for nirK, pmoA, mcrA and nifH, ATP concentration, cell count data and sediment concentration. Dashed lines represent the running mean of five samples around (two either side) that data point. Factor 1 explains 38.6% of the variation in the dataset and Factor 2 explains a further 25.0% of the variation. x-axis is date in Julian days.*

To simplify the data from the temporal experiment dataset, multivariate statistical analyses were considered and PCA was chosen to emphasise the variation in the dataset. Due to the statistical correlation between the LPS data and storage time and the bacteria, archaea and dsrA primer pairs and extraction batch, PCA analysis was conducted without these variables, *i.e.* only included the real-time PCR data obtained using primers for nirK, pmoA, mcrA and nifH, ATP concentration, cell count data and sediment concentration (Figure 7-3).

Factor 1 is related to the functional primers (pmoA, mcrA, nirK and nifH) and Factor 2 to ATP and sediment concentrations (Table 7-3).

*Table 7-3. Loadings of Factor 1 and Factor 2 from variables included in Figure 7-3.*

| Biomarker | Factor 1 | Factor 2 | Total |
|---|---|---|---|
| nirK | 0.7451 | 0.2247 | 0.7782 |
| pmoA | 0.8661 | -0.2840 | 0.9115 |
| mcrA | 0.8014 | -0.0252 | 0.8018 |
| nifH | 0.7840 | 0.4887 | 0.9239 |
| ATP | -0.1161 | 0.8303 | 0.8384 |
| Cell counts | -0.3078 | 0.1604 | 0.3471 |
| Sediment | -0.1701 | 0.8150 | 0.8326 |

The temporal changes seen in Factor 1 and 2 in Figure 7-3 show that the first six samples have high Factor 1 before a decrease, and then become somewhat erratic. However an increase in Factor 2 is visible over the second half of the time collected. Using the 5-point running mean to remove short-term temporal, including diurnal, variations this increase lasts from Sample 208.38 to 215.38.

Further PCA was conducted on the temporal experiment dataset plus the supraglacial samples to confirm that the different sample types appeared to be significantly different. The results are shown in Figure 7-4, with projection of the samples and the biomarkers on the 1 x 2 Factor plane.

The results of the PCA clearly exhibited clustering of the samples according to sample type (Figure 7-4), where $P<0.001$ for Factor 1 and 2, however no significant difference was seen between the subglacial runoff morning and afternoon samples. The results in Figure 7-4 show that all variables used in this analysis are significant to explain the variance and contribute to Factor 1 in the same direction. Generally the real-time PCR data has the strongest influence (primers nirK, pmoA and mcrA). The strongest contributor to Factor 2 is nifH primer and then ATP concentration.

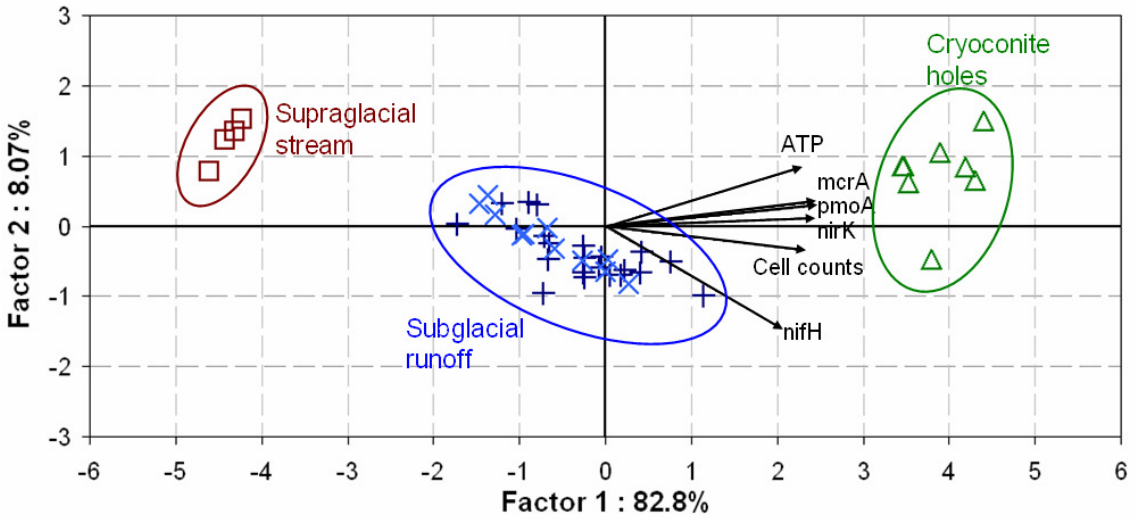


*Figure 7-4. PCA analysis of different sample types using real-time PCR (nifH, nirK, pmoA and mcrA), ATP data and cell counts. The subglacial runoff samples are labelled as: + for morning samples and* X *for afternoon samples. The arrows show the projection of the biomarkers. The ovals are placed arbitrarily to distinguish among the different sample types.*

## 7.4. Discussion

Very little is known about the microbial concentration, diversity or activity in subglacial environments (Christner *et al.* 2008), and the studies that have been completed tend to concentrate on the microbial diversity of these subglacial communities (Christner *et al.* 2000, Bhatia *et al.* 2006, Kaštovská *et al.* 2007, Mikucki *et al.* 2009). A few quantitative analyses have been conducted, however no attempts have previously been made to explore temporal changes either between or within a melt season. To aid in the understanding of globally important yet remote environments such as the Greenland Ice Sheet, temporal changes in glacial runoff were studied. In-field analysis was selected as it reduced the logistical requirements for sample transportation needed to collect such data, however the main advantage provided by in-field analysis in this study was the ability to develop sampling strategies and protocol in the field, as the concentrations of microbial targets were unknown prior to the field campaign. To address these unknowns preliminary experiments were conducted, these also derisk the application of new techniques to unfamiliar samples, *e.g.* by confirming sufficient sample is being collected for the analytical technique. Which, in turn, leads to more efficient scientific return for given resources as initial data can be collected, and results gained can be applied to subsequent analyses in the same field season. Simple changes such as changing the concentration of casein in the blocking solution could be easily made.

Combination of these preliminary analyses, previous experience (*e.g.* subglacial meltwater AS samples, Engabreen in Chapter 5), practicalities concerning how the analytical techniques are performed and time required (only a single person was directly involved in sample collection, processing and analysis) lead to the choice to take a single samples, process them daily then after short term storage conduct analyses in batches. The in-field storage time was a balance among the desires to limit the differences among extraction batches, the reagents required to analyse separate standard curves, the length of time of in-field storage of the individual samples and the time for information feedback (*e.g.* if biomarker concentration falls below the detection limit of the assay, this could lead to increasing volume of sample processed).

High variability is seen among the samples in the real time PCR data, as exemplified by pmoA gene in Figure 7-2 where concentration changed by 7 Ct units between day 208 and day 211. As the pattern of some of these changes are also visible in ATP concentration, although a lower magnitude (lows on 209 and 211), then they are likely to reflect real changes in microbiology of the samples. The higher relative changes in real-time PCR data could be because PCR detects the copy number of a gene which can vary among genes and between species, and frequently greater than one for microbial cells (Farrelly *et al.* 1995, Klappenbach *et al.* 2000). Also, ATP is sensitive to microbial activity as well as size and concentration (Fairbanks *et al.* 1984), therefore cells with low activity, would have a lower apparent ATP change. When the changes are observed in only one analytical technique this could be an artefact of sample processing strategy. Future field campaigns could address these issues by collecting multiple samples from the same time, pooling of samples from different collection points along river length or multiple extractions of same sample. The high variability observed in analyses here may mean that one-off sampling from these types of environments may not be satisfactory for extrapolation to large-scale microbial concentration or activity estimates in subglacial environments (Priscu *et al.* 2008, Wadham *et al.* 2008), especially if using highly sensitive techniques like real-time PCR.

It was necessary to analyse some of the samples for ATP concentration and cell counts after the field campaign. In-field storage at -13°C appeared to be insufficient to maintain the stability of reconstituted ATP assay reagents between analysis batches. As the activity of the ATP assay reagents dropped so that they were no longer usable a few days after reconstitution from freeze dried state, it is likely that there was some freeze/thaw of reagents; this drastically reduces activity. Therefore for subsequent field campaigns cooler storage could be required

for the ATP assay reagents, these reagents could be aliquoited and lypholised in smaller volumes or equivalent, more thermostable reagents need to be identified. Additionally some samples for cell counts were conducted after the field campaign due to the time that this technique took. This timescale was magnified as the fluorescence dye used in analyses started to fade during some counts within the time it took to count 25 fields of view. It was concluded that this was likely to be due to the temperature sensitive anti-fade reagent used in this study. Hence cell counts needed to be conducted when ambient temperature was below 22°C, which limited them to be completed during the late evening.

The short term storage and batch analysis lead to 'rounds' of sample collection. In the field it was observed that LPS concentration increased after 2$^{nd}$ (on Day 205) and 3$^{rd}$ (between Day 212 and 213) analysis batch (Figure 7-2). As the supernatant was tested, this increase in available LPS is most likely due to cell lysis during storage. Occasional drastic changes were observed associated with DNA extraction batches for real-time PCR (*e.g.* after first six samples in Figure 7-2), and probably due to inconsistent cell lysis during the initial 10 minute vortexing step with beads. For future campaigns the batch to batch extraction repeatability should be investigated, along with the use of field portable vortex mixer. These observations could only be tested statistically upon completion of the dataset, and revealed significant correlation. The variation during storage observed over short timescales here would probably be amplified significantly if storage was longer, especially considering microbial metabolism has been observed down to -20°C (Price & Sowers 2004, Junge *et al.* 2006). Limiting such changes are particularly important if looking at quantitative or relative changes and community structure as addressed here.

An awareness of these limitations in the dataset aids in more robust interpretation of the dataset in terms of microbial. Therefore, LPS and correlated real-time PCR results were excluded from multi variant analysis applied to interpret the biomarker data in terms of temporal changes and contextual information comparing sample types. Although no statistical difference exists between the ATP and sediment concentration data (Factor 2 in Table 7-3 and Figure 7-3), the increase in the ATP concentration cannot directly be attributed to an increase in sediment concentration as the relative changes are different (654% and 36% increase from minimum to maximum value, respectively - Figure 7-2). Also if the sediment concentration caused an increase in microbiological concentrations in the runoff then an overall trend would be expected in the real-time PCR, cell counts and rFC assay data, which is not seen in Figure 7-2. The correlation between a small increase in sediment load and ATP concentration could

be coincidental, an increase in overall microbial activity, or the addition of a number of significantly more active microbes during this time.

PCA represented in Figure 7-4 clearly separates the subglacial and two supraglacial sample types. As all of the biomarkers positively correlate with Factor 1 in Figure 7-4, Factor 1 is likely to be dominated by changes in microbial concentration. The similar statistical behaviour of mcrA and pmoA genes could be due to them both showing the ability to oxidise different carbon compounds. The subglacial runoff samples have comparatively higher levels of nifH gene than the supraglacial samples. The nifH gene encodes for a section of the nitrogenase reductase protein which is involved in the biological fixation of nitrogen. The increased presence of this gene in subglacial runoff suggests a nitrogen limited and anaerobic environment under the glacier. The nifH gene has been found in sediments from a southern hemisphere glacier (Foght *et al.* 2004). Nitrogen fixation is sensitive to oxygen concentrations and energy intensive hence it tends to occur when there is no alternative nitrogen source available and is most common in an anaerobic environment (Schlesinger 1997, Jjemba 2004). Its presence is consistent with the widespread evidence for anaerobic conditions and microbial activity at the base of glaciers including Alpine and Greenland subglacial environment (Bottrell & Tranter 2002, Sheridan *et al.* 2003, Wadham *et al.,* 2008).

## 7.5. Conclusions

In-field analysis allowed for collection of quantitative data on the microbiology of Leverett Glacier, Western Greenland, where previously little was known about microbial concentrations. Preliminary analyses provided data to select sampling strategy and to refine sample processing protocols in the field, and allowed for the collection of high-temporal resolution data within one field campaign. The biomarker detection techniques comprised of real-time PCR with primer pairs targeting sequences bacterial and archaeal phylogeny and five functional genes, ATP bioluminescence, LPS detection and fluorescence cell counts.

Samples were collected daily or twice daily and showed variations over an order of magnitude during the three week collection period. Only ATP and sediment concentration show a trend during this time, in this case an overall increase. The dataset collected on these samples shows a complex system with many influences; including microbial concentration, community structure and sample processing. Some of the sample processing influences were detected and therefore this data was not included in subsequent interpretation, including a potential increase in activity in the latter part of sampling time. However the future challenge lies in

limiting these influences and hence providing detailed understanding on the changes in microbial community and structure.

## Acknowledgements

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# 8. Towards the Integration of Multiple Life Detection Assays in a Field Deployable, Fluidic Device: Initial use with ATP and LPS Assays

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## Abstract

Portable detection devices based on biomarker detection technology have been previously developed for in-field (non-laboratory) use. Such technologies are generally formatted to analyse only one type of biomarker target using one type of assay. For in-field biomarker detection, it is desirable to have multiple, independent approaches to enable multiple biomarker detection to aid in scientific interpretation of analytical outputs. To simplify in-field detection, the integration of multiple biomarker detection approaches into single analytical systems is clearly desirable in term of field logistics. To address this, a system has been investigated where a subset of life-detection techniques can be incorporated in a common format, and so can be detected by a single instrument. For both initial demonstration, and potential future manufacture, simple fluidic devices have been fabricated by a combination of laser cutting and room-temperature lamination of polymer sheet materials. The devices rely upon capillary action for fluid movement to avoid the complexity of actively pumped systems. Optical readout is compatible with a range of commercial off-the-shelf portable and bench-top instruments, and bespoke instrumentation is being developed to provide a single detection instrument. Two single-step commercial, biomarker detection assays, both with optically readable endpoints, were identified: adenosine triphosphate (ATP) bioluminescence and recombinant Factor C (rFC) endotoxin detection. The assay reagents were integrated into devices by freeze-drying water soluble assay reagents into laser-cut glass-fibre pads, which were subsequently incorporated into assembled devices. ATP detection using an ATP bioluminescence assay has

been demonstrated in the devices and developments made to incorporate rFC assay for detection of lipopolysaccharide (LPS). The work provides a basis for the refinement and future manufacture of these devices.

Keywords

Portable devices, ATP bioluminescence, rFC endotoxin detection

## 8.1. Introduction

On Earth, wherever there is liquid water there appears to be life. This observation has led to increasing research into trying to place limits of the ability of life to survive on Earth, including extremes of temperature, pressure and pH (Price & Sowers 2004, Amend & Teske 2005, Koch *et al.* 2006 Laybourn-Parry 2009). These extreme environments are often remote, making the logistical costs to study them high. One approach to reduce these costs is to improve the efficiency of studying life in these extreme environments, and can be achieved through in-field life detection experiments. This improved efficiency stems from the ability to develop hypotheses and sample processing protocols in the field, exploit serendipitous discoveries, protect scientific return and increase spatial and temporal resolution.

The fast, sensitive detection of organisms can be achieved by detection of biomarkers, which can be specific to the presence, abundance or activity of a particular microorganism or group of microorganisms. A number of biomarkers can be detected by portable, commercially available systems that include the readout instrumentation and consumables containing the reagents. Systems for the detection of single biomarkers, in both clinical and environmental settings, can be detected by systems such as: HY-LiTE, (Merck & Co., Inc, NJ, USA) and systemSURE II and Ultrasnap (Hygiena, CA, USA) for ATP; Endosafe PTS® (Charles River Laboratories International Inc., MA, USA) for lipopolysaccharide; pregnancy test kits for pregnancy hormones; and CyScope® Malaria (Partec GmbH, Görlitz, Germany) for malarial biomarkers. Instrumentation for the detection of multiple biomarkers, include RAZOR™ (Idaho Industries, UT, USA) for nucleic acids; Triage® MeterPro (BioSite Inc, CA, USA) for proteins; and i-STAT® (Abbott Point of Care Inc., NJ, USA) for blood components. These instruments have been developed for detection of biomarkers in the health, food, defence and pharmaceutical industries. As these instruments are designed to analyse samples different to those in extreme environments, the in-built sample processing is unlikely to be optimal. Therefore the application of these commercial techniques in extreme environments is limited by these constraints.

An alternative to using complete commercial off the shelf systems is the application of less restrictive commercially available assay kits that can be used in conjunction with more appropriate sample processing. Two test-tube based commercial assays were used to provide biochemical reagents to detect the biomarkers adenosine triphosphate (ATP) and lipopolysaccharide (LPS). ATP is present in all known cells, and can be detected by the ATP bioluminescence assay. This assay relies on the enzyme (luciferase) mediated reaction of ATP and luciferin that is present in many organisms, including the tails of fireflies. The product of the ATP and luciferin reaction is in an excited state and emits visible light, proportional to ATP concentration, as it relaxes.

LPS is a major component of the cell wall of Gram-negative bacteria. Fractions of the hemolymph of horseshoe crabs (*Limulus*) naturally clot in the presence of LPS. This property has been developed into the *Limulus* amoebocyte lysate (LAL) assay, where commercial preparations will give a change in turbidity or colour in proportion to LPS concentration. The LPS activated enzyme (Factor C) has been identified, isolated and a recombinant form produced (Nakamura *et al.* 1986, Ding & Ho 2001). This recombinant Factor C (rFC) has subsequently been developed into a fluorescence based assay for the quantification of LPS.

The overall aim of this study was to test a system capable of detection multiple biomarkers with a consistent format for future integration into a single device. This system consisted of fluid-based devices constructed from three polymer sheets and used assay reagents from commercial kits for the detection of ATP and LPS. A fluid based system was used so that devices and instrumentation can be constructed without the need for external fluid control by pumps and valves. Rapid manufacture and testing of new designs was possible by lamination of three layers cut from commercially available polymer sheets, where the central sheet was used as a spacer layer to create a system of chambers and linking channels and ports and the top or base layer had equivalent ports to allow for the addition of sample solutions. This was addressed by the following objectives. (i) Test that three layered polymer sheets are a suitable format for construction of fluidic devices. (ii) Test various channel designs to assess the effect of different fluidic designs on the ability of liquid sample to homogenously fill the glass fibre pad. (iii) Incorporate ATP bioluminescence assay reagents into devices. (iv) Test the performance of the ATP devices in terms of adaptability, assay response and short term storage. (v) Modify the devices for the incorporation of rFC assay.

## 8.2. Materials and Methods

The described protocols for the manufacture of the fluidic devices and the incorporation of assay materials were developed using ATP bioluminescence assay reagents. The general fabrication methods described were established in the lead-authors' laboratory prior to commencement of the current work, but did require minor modifications to fluidic design, laser settings and assay reagents for the devices described here.

### *8.2.1. Fluidic design, materials and assembly*

All fluidic patterns were designed in A9CAD (software version 2.2.1) (A9 Tech Inc., WA, USA) 2D CAD drawing package. The devices consist of a three layer fluidic system (Figure 8-1). The external dimensions of the design were dictated by the dimensions acceptable by the assay readout instrumentation, in the case of Figure 8-1 to fit inside a test tube with rounded base. Each layer incorporates holes to allow for alignment during assembly. The spacer layer also contains a circular area to initially receive liquid sample, a chamber which approximates to a rectangle and which accepts the glass fibre pad and narrow connecting channels. The top layer contains a large circular aperture to accept the sample and a small vent hole to eliminate trapped air and allow capillary filling of the assembled device with liquid sample. The glass

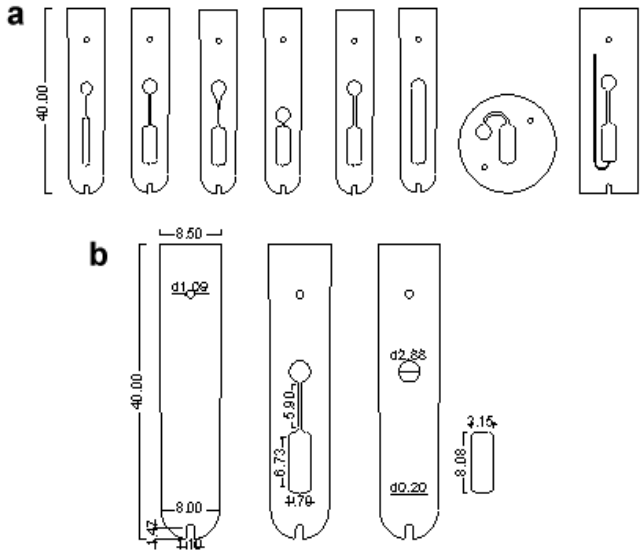


*Figure 8-1. CAD drawings, a) outlines of all the spacer layers used within this study. From left to right: initial design, original design, more filleting, no connector, 3 x channel width (standard design), maximum channel width, microtitre plate adaptation and rFC devices, b) dimensions in mm of the three layers and glass fibre pad of the standard device as it was used in the majority of the development. From left to right; base layer, spacer layer, top layer and glass fibre pad.*

fibre pad contains the biochemical reagents for the assay. Each layer was cut using a $CO_2$ laser marker (Fenix Laser Marker, Sinrad Inc., WA, USA), controlled by WinMark Pro v2.1.0.4065 software, the settings used are in Table 8-1.

The appropriate materials for ATP bioluminescence devices were selected from a variety of sheet polymer materials and glass fibre samples used in previous related laboratory work – details are given in Table 8-1.

*Table 8-1. Materials used and corresponding laser marker settings for materials for the development of ATP bioluminescence fluidic devices. There was a 5 second pause between each pass.*

| Layer | Material | Laser setting |
|---|---|---|
| Top | Melinex® ST725 – 250 µm thick – optically transparent polyester sheet | 5 passes at 90% power |
| Spacer | ARcare® 90485 – 178 µm thick clear polyester film coated on both sides with AS-110 pressure sensitive acrylic adhesive | 4 passes at 90% power |
| Base | Melinex ST725 – 250 µm thick – optically transparent polyester sheet | 5 passes at 90% power |
| Pad | Pall type A/C binder free borosilicate glass, nominal 1 µm pore size – 216 to 292 µm thick | 4 passes at 10% power |

After cutting, the top and base layers were cleaned with acetone to remove laser cutting debris, this was not appropriate for the spacer layer due to the presence of backing material protecting the adhesive. The top and base layers were treated, making them hydrophilic to allow for filling via capillary action. This was achieved using helium plasma in a plasma chamber (EmiTech K1050X, Quorum Technologies Ltd, East Grindstead, UK) at 25 watts for 10 minutes. Early tests demonstrated that it was not necessary to plasma treat the spacer layer. Devices were assembled using the alignment holes, and in order to ensure consistent lamination the devices were compacted with a simple hand-pump workshop hydraulic press. Two devices were pressed at a time to 3.5 metric tonnes force and were visually inspected to confirm total lamination around the chambers and connecting channels before use.

### *8.2.2. Assay reagents and their incorporation*

For the development of ATP bioluminescence devices, ATP Bioluminescence Assay Kit HSII and ATP Bioluminescence Assay Kit CLSII (Roche Diagnostics, Burgess Hill, UK) were used. Both reagent kits are supplied freeze dried and were reconstituted to the desired concentration with autoclaved reverse osmosis water. The HSII assay kit has peak

luminescence at time zero before decaying rapidly, whereas the CLSII assay kit gives a constant luminescence for 30 minutes before decaying. However the CLSII assay kit is an order of magnitude less sensitive than the HSII. ATP standards were reconstituted in autoclaved 100 mM Tris, 4 mM EDTA buffer.

To detect LPS, recombinant Factor C (rFC) endotoxin detection system (Cambrex Lonza Biologics Plc, Slough, UK) was used. It is supplied as three components that are mixed immediately before use to make a working reagent; enzyme, buffer and fluorescent substrate. LPS standard and endotoxin free water are also supplied.

To incorporate a constant volume of assay reagent into each device, 8.5 µl of HSII or CLSII assay reagent was pipetted into the centre of a glass fibre pad and then frozen in liquid nitrogen. The glass fibre pads containing assay reagents were then freeze dried (CoolSafe$^{TM}$, ScanVac) for minimum of 4 hours. The rFC assay working reagent was not incorporated into pads, however the stability of the reagents during freeze drying was tested in borosilicate glass mini-cuvettes (Turner BioSystems Inc., CA, USA), where a total working solution of 25 µl was used and placed in freeze drier as liquids and dried overnight.

### *8.2.3. Buffer cocktails*

Two cocktail buffers were tested to assess their suitability to improve the dissolution of the ATP assay reagents. Both cocktails were developed for other applications to freeze dry biochemical assay reagents in the lead author's laboratory. The sucrose cocktail consists of 10% (w/v) sucrose, 0.05% (w/v) BSA and 0.01% (w/v) Thimerosal in 100 mM HEPES (pH 7.4) buffer. The blue dextran cocktail consists of 0.1% (w/v) blue dextran, 1% (w/v) BSA and 0.34% (w/v) ascorbate in 100 mM HEPES (pH 7.4) buffer.

### *8.2.4. Detection instrumentation*

During development of the devices commercially available instrumentation was used to detect the optical readout of the devices. For the ATP bioluminescence assay a hand-held tube luminometer (HY-LiTE 2, Merck KGaA, Darmstadt, Germany) and benchtop plate reader (Varioskan Flash, Thermo Scientific, MA, USA) were used. The benchtop plate reader was used in luminescence mode and an alternative device was designed to fit a 12-well microtitre plate where the internal dimensions of the chamber and size of inlet port remained as for the standard device in Figure 8-1, however the outer dimensions were altered. The output for both instruments is in relative light units (RLU), although they are not directly comparable.

The rFC assay was detected using a handheld cuvette fluorometer (*Pico*Fluor, Turner BioSystems Inc., CA, USA) with fluorescence excitation at a wavelength centred at 365 nm and fluorescence emission in the wavelength range 440-470 nm. A benchtop fluorometer (Cary Eclipse, Varian Inc., CA, USA) was used to measure emission fluorescence for the different materials described in Section 8.3.5.1. The output for both instruments is in relative fluorescence units (RFU), although they are not directly comparable.

## 8.3. Experimental

Two commercially available biomarker detection assays were selected for incorporation into the devices. ATP bioluminescence and rFC endotoxin detection were chosen as they fulfilled the following two criteria: (i) the assay needed to be able to be performed in a single step, *i.e.* the sample can be added to the assay reagents to give the optically readable endpoint without further processing and (ii) the endpoint of the assay had to be optically readable. Development with the ATP bioluminescence assay reagents was conducted first.

### *8.3.1. Testing three layered polymer sheet construction*

Construction of three layers for fluidic assembly and reagent incorporation into glass fibre pads, was previously used for antibody assay reagents, therefore it was tested for suitability for the incorporation of ATP bioluminescence reagents by constructing devices with a preliminary design. Three different concentrations of CLSII reagents, freeze dried into glass fibre pads, were tested with 8 µl ATP standards between $10^{-4}$ and $10^{-9}$ M ATP. Figure 8-2 shows a positive correlation between ATP concentration and bioluminescence values hence demonstrating these materials and methods can be used to incorporate ATP bioluminescence assay. However, the results in Figure 8-2 do highlight the requirement for improvements in device to device repeatability and sensitivity, *i.e.* increase the bioluminescence values for equivalent ATP concentration.

Two approaches were made to improve the ATP devices; refinement of the fluidic design and modification of the contents of the pad, which include the addition of ingredients not involved in the ATP bioluminescence assay (excipients).

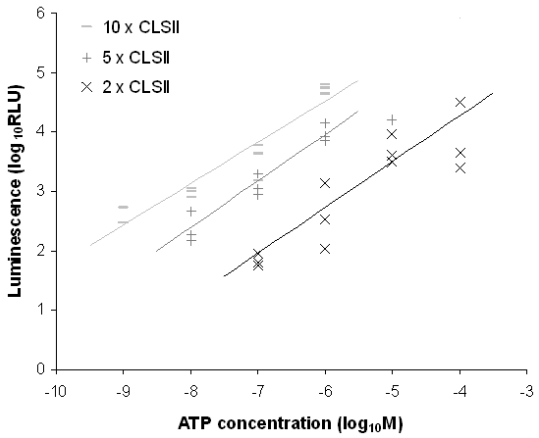


*Figure 8-2. Standard curves produced with CLSII reagent at three concentrations incorporated into glass fibre pads within fluidic single use devices. Luminescence was measured 1 minute after the added liquid reached the glass fibre pad. Data points are the individual values and the lines show least square linear regression of log-log plot of the mean values. Blank values for x 2 were 10, 13 and 12, for x 5 were 54, 98 and 110 and for x 10 were 370 and 180.*

### *8.3.2. Effect of fluidic device channel design*

After the initial investigations, the size and shape of the glass fibre pad and hence spacer layer was modified in conjunction with developments in the design of the bespoke reader, to give the size and shape of pad used in the standard devices in Figure 8-1b.

The concentration of the assay reagents affects the bioluminescence values, therefore uneven dissolution and distribution of reagents among the different devices of the same design could lead to the differences in bioluminescence values. To assess how the design of the spacer layer effects the final distribution of reagents in the glass fibre pads, five different channel shapes were designed and tested, where the degree of filleting and the length and thickness of the channel were modified (Figure 8-1a and Figure 8-3). Ten devices of each design were constructed to assess the variability of sample filling and distribution through the devices. To aid in visual examination, 0.5 $mg.ml^{-1}$ of a hydrophilic, water soluble dye (Allura Red) was added to 5 x CLSII concentration reagents in the pad prior to freeze drying. ATP test sample was added and the devices photographed at various intervals (time after 2 and 20 minutes is shown in Figure 8-3).

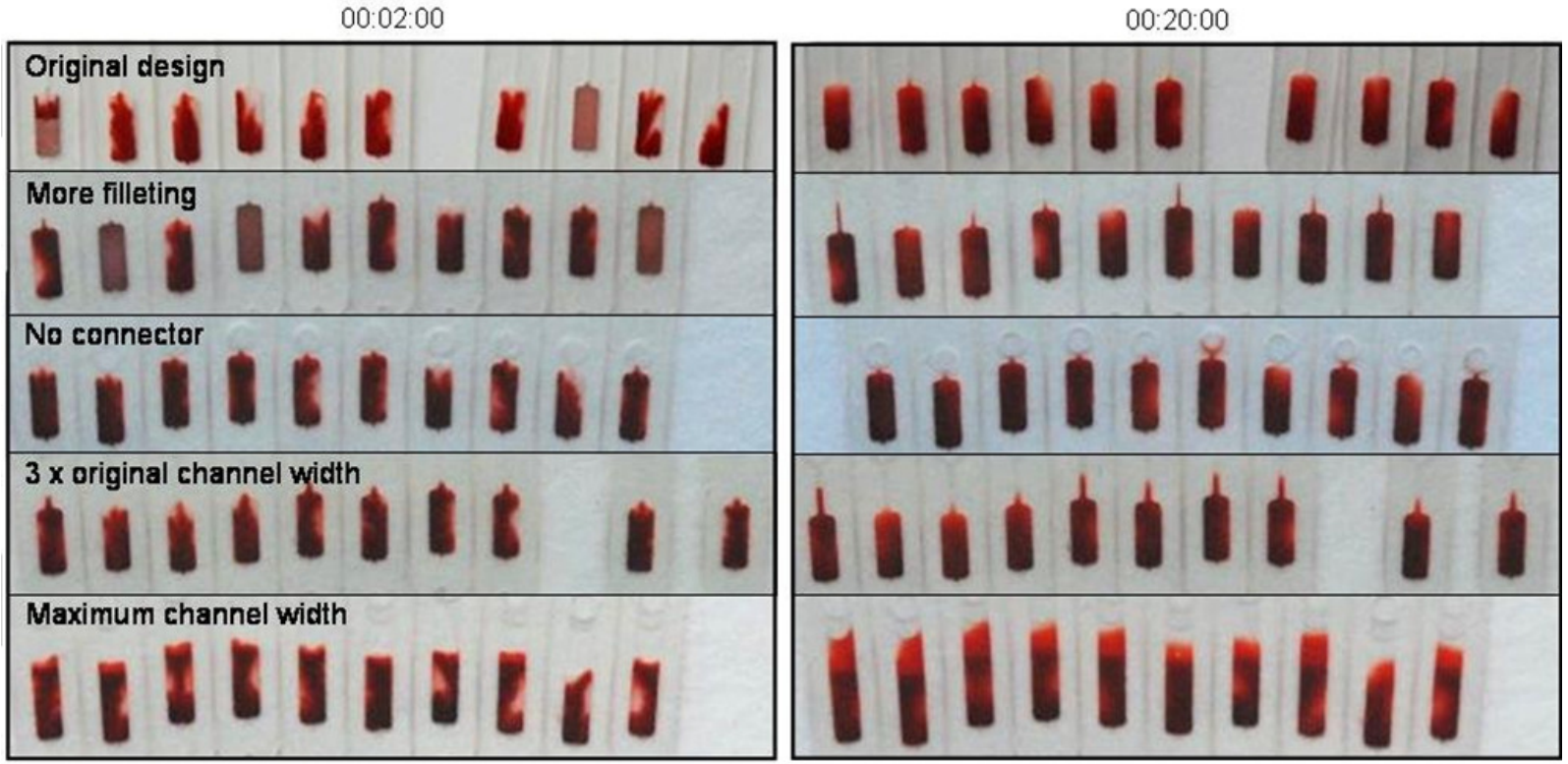


*Figure 8-3. Images of pads with 0.5 mg.ml$^{-1}$ Allura Red freeze dried with 5 x CLSII ATP assay reagents. Column one was taken after 2 minutes and column two after 20 minutes and after addition to last device in each set. Liquid was added to devices from left to right, 7 µl of $10^{-5}$ ATP was used in all samples except for maximum channel width where 10 µl was required for the liquid to reach the pad. Images were taken with Nikon D80, brightness and contrast have been modified to enhance images. Due to complications in experiment set up the ATP standard was added to the 10$^{th}$ device (from left) in original design after 105s, the 4$^{th}$ device (from left) in more filleting after 5 minutes and the 10$^{th}$ (from left) device in 3 x original channel width after 78s.*

The results in Figure 8-3 show that after 2 minutes the "original design" sample set has the greatest heterogeneity of the dye distribution after and that in some regions the tracer dye and therefore one assumes the ATP assay reagents are washed to and concentrated elsewhere in the pads. "Maximum channel width" sample set appears similar. The remaining three designs all appear to have similar behaviour after 2 minutes. After 20 minutes there appears to be tracer dye reappearing in regions of the pads seen after 2 minutes as being clear, therefore it is assumed that this represents diffusion of the tracer dye and one assumes ATP assay reagents in the presence of stagnant fluid flow. One can also see that for the "maximum channel width" samples, that the diffusion of the tracer dye back along the open channel is much more pronounced than for any of the other channel designs. In addition to the results seen in Figure 8-3 increasing the channel width by minimum of three (to 0.6 mm) was sufficient to reduce the time for reagent to reach the pad from between 5 and 242 seconds for the "original design" and between 1 and 168 seconds for "more filleting" to less than 2 seconds for the remaining designs. Few differences were seen between the "3 x original channel width" and "no connector" design, in terms of reduced liquid heterogeneity and more consistent time to reach the pad, however the presence of a connector would be useful for subsequent integration of multiple assays. It was therefore decided to pursue the "3 x original channel width" and hereafter is referred to as standard design, with dimensions in Figure 8-1b.

### *8.3.3. Incorporation of ATP assay reagents*

Two aspects of assay reagent incorporation were investigated to optimise bioluminescence values for a given ATP concentration and to improve the device to device repeatability. These involved the addition of extra reagents not involved in the ATP bioluminescence assay (excipients) and refining choice of ATP bioluminescence reagents and concentration.

#### *8.3.3.1. Addition of excipients for improved assay reagent dissolution*

Within the manufacturer's standard protocol for the CLSII ATP assay, the freeze dried ATP reagents are incubated for 5 minutes at 4ºC after the addition of water to allow for dissolution of reagents before the addition of a sample. As dissolution in the fluidic devices is conducted by the sample, this time to allow for complete dissolution is not available. Increasing the rate of dissolution is expected to lead to an improvement in peak bioluminescence values and consistency between devices. To increase the rate of dissolution the addition of excipients were investigated.

To reduce the dissolution of the assay reagents and their subsequent movement through the pads, and improve sensitivity additional ingredients not required for the ATP bioluminescence assay (excipients) were added to the assay reagents before freeze drying into the pads. All excipients were tested with the ATP bioluminescence assay reagents at 5 times the manufacturer's recommended concentration. Two sugars (5% (w/v) sucrose and fructose) and two cocktails (called sucrose and blue dextran and described in Section 8.2.3) were added. The sugars were added to 5 x CLSII assay reagents. 6.5 µl of $10^{-5}$ M ATP was added to the devices (volume was decreased from 8 to 6.5 µl to reduce leakage of ATP reagents from the outlet hole) and luminescence was recorded in the tube luminometer every 3 to 5 minutes for 1 hour (time zero was when ATP standard reached the pad). The results in Figure 8-4a show that the addition of the sugars to CLSII bioluminescence assay reagents improves the device to device assay reproducibility (CV reduced from 62% for CLSII to 15% with sucrose and 30% with fructose) and is likely to decrease the lower limit of detection (bioluminescence output increased to 169% with sucrose and 137% with fructose when compared to output without the excipients).

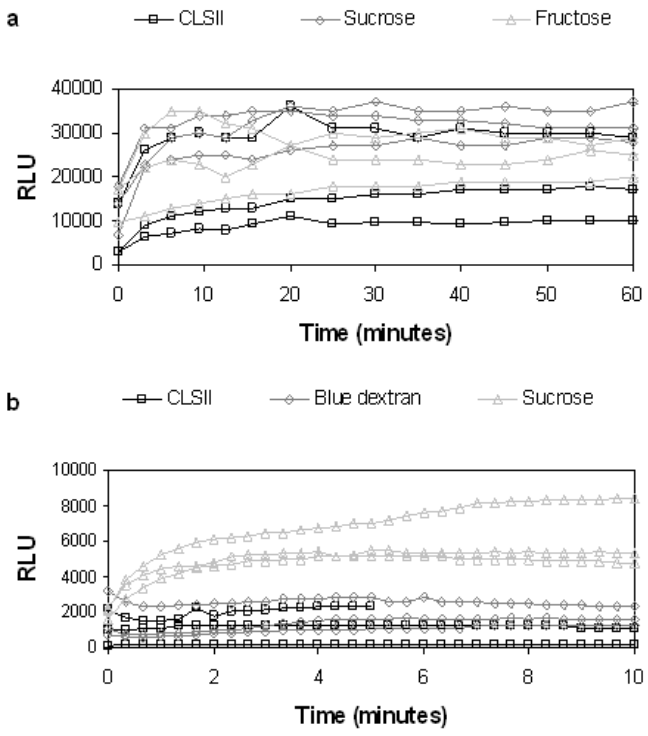


*Figure 8-4. Addition of excipients to improve repeatability and reduce lower limits of detection, a) effect of 5% (w/v) sucrose and fructose, b) effect of sucrose and blue dextran cocktails. Assay reagents at 5 x CLSII, 6.5 µl of $10^{-5}$ M ATP was added. Details of cocktail recipes are in Section 8.2.3.*

In addition to the single sugars described previously, two buffer cocktails, based on sugars with additional ingredients, were tested as they were being developed to improve the recovery of freeze dried biochemical assay reagents from glass fibre pads for other applications. Both sucrose and blue dextran cocktails were tested with 5 x CLSII and 5 x HSII, where 6.5 µl of $10^{-5}$ M ATP and $10^{-7}$ M ATP were added respectively (only the results from CLSII reagents are shown as representative). Readings were taken every 20 seconds for 10 minutes in the tube luminometer. Three devices with each buffer cocktail were tested. The results in Figure 8-4b show similar temporal behaviour with and without the addition of sucrose cocktail, both devices show an initial increase in bioluminescence values over the first 2 to 3 minutes, and then stabilise for the remaining time. The bioluminescence values increased with sucrose cocktail with both HSII and CLSII assay reagents by approximately 4 and 3-fold respectively compared to no cocktail. A different temporal evolution in the devices was observed with the blue dextran cocktail, the bioluminescence values decrease over the first 40 seconds before starting to increase slowly. The mean bioluminescence values approximately doubled when blue dextran cocktail was added to CLSII but halved when it was added to HSII. There was also an improvement in device to device variability with decreased mean CVs with both cocktails, from 87% with CLSII and no cocktail to 44% with blue dextran cocktail and 22% with sucrose cocktail.

#### *8.3.3.2. Effect of ATP bioluminescence assay reagent concentration*

The concentration of assay reagents was tested by constructing standard devices with 1 x, 2 x and 5 x CLSII and HSII assay reagents with sucrose cocktail. Readings were taken every minute for 30 minutes, and then after 35, 40 and 45 minutes in the tube luminometer. Three devices with each concentration of assay reagent were tested.

The bioluminescence values increased with increasing reagent concentration for both the HSII and CLSII assays, however this increase in luminescence is not directly proportional to the increase in reagent concentration. This observation is most prominent in the CLSII reagents, where the average bioluminescence values increase from 41000, 55000 to 56000 for 1 x, 2 x and 5 x CLSII reagents respectively. The equivalent mean bioluminescence values for the HSII reagents were 3600, 6100 and 7500, with CVs of 23.6%, 26.5% and 35.7%.

To finalise the ATP bioluminescence reagent choice and concentration, 1 x HSII, 2 x HSII and 1 x CLSII were selected due to the better device to device repeatability, and nine standard design fluidic devices of each assay reagent concentration were constructed with sucrose

cocktail. 6.5 µl of $10^{-7}$ M ATP for the HSII based devices and $10^{-5}$ M ATP for the CLSII based devices were added, the higher ATP concentration was required to maximise bioluminescence values in the devices with CLSII assay reagents. Readings were taken every minute for 30 minutes, and then after 35, 40 and 45 minutes in the tube luminometer. Even though the bioluminescence values are significantly higher using the CLSII reagents, a 100-fold higher concentration of ATP was used compared to the HSII reagents (Figure 8-5) making the HSII devices more sensitive and hence were used in the final devices. Of the two concentrations of HSII reagents used the 2 x had higher bioluminescence values, however the 1 x HSII reagents were used as there was only one outlier (a device that behaved differently from the others) compared to the three with 2 x HSII, *i.e.* the final selection was based upon reproducibility.

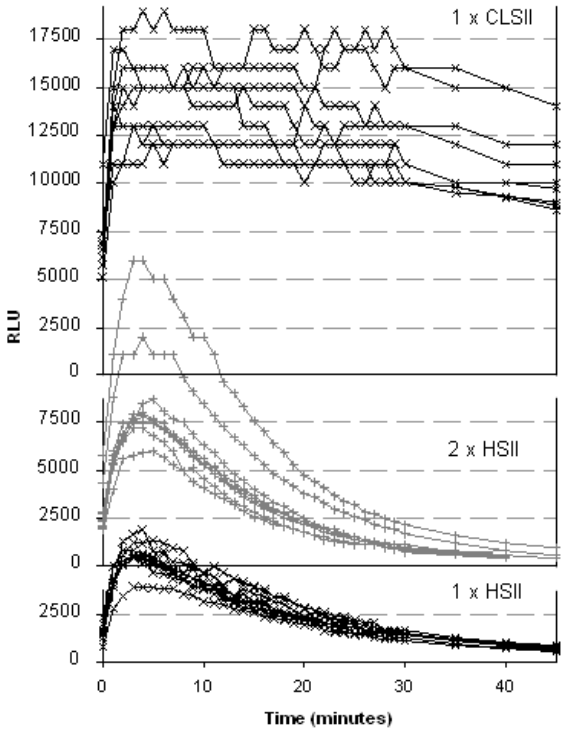


*Figure 8-5. The effect of assay reagent concentration on bioluminescence values. Standard devices and sucrose cocktail were used with the following reagents and concentrations 1 x CLSII, 2 x HSII and 1 x HSII. 6.5 µl of $10^{-5}$ M ATP was added to CLSII and 6.5 of µl $10^{-7}$ M ATP added to HSII. Measurements were made in the handheld tube luminometer.*

### *8.3.4. Testing ATP bioluminescence device performance*

Once the fluidic design, excipient ingredients and assay reagent concentration were refined, three additional aspects of the devices were tested; the adaptability, the response to different ATP concentrations and the short term storage. The former two aspects were tested by adapting devices for detection in a plate reader with various ATP standards. The effect of short term storage was tested at three different temperatures using tube luminometer.

#### *8.3.4.1. Adaptability and response of devices in plate reader*

The compatibility of the devices in the benchtop plate reader was tested with ATP standards. 6.5 µl of blank and $10^{-7}$, $10^{-6}$ and $10^{-5}$ M ATP standards were used to assess the response at different concentrations. The devices were modified so the pad was centred on a circle with diameter of 21 mm to fit the 12-well microtitre plate used (Figure 8-1a). To maintain a similar length connector from the inlet, the connector was curved. The pads contained 1 x HSII in sucrose buffer cocktail. The ATP standards were added to the devices from highest to lowest ATP concentration, readings were taken as soon as possible (less than 2 minutes from addition of standard) then every minute for 1 hour and in the same order as the standards were added. Twelve devices were constructed, and three devices of each ATP concentration were tested.

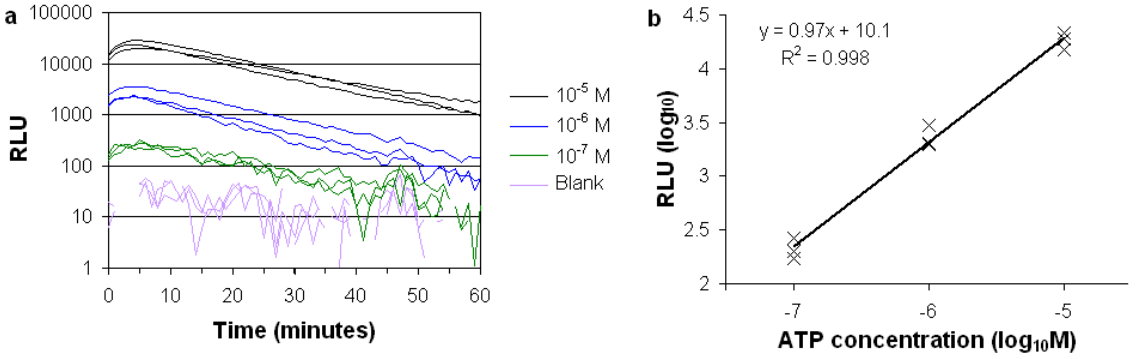


*Figure 8-6. Bioluminescence values of fluidic devices adapted to be compatible with the benchtop plate reader, a) with three ATP standards and blank. Some of the bioluminescence values from the blank are negative, and due to the log scale they have been omitted from the graph. b) standard curve taking bioluminescence values after 4 minutes.*

Using the devices modified for compatibility with the plate reader, discrimination is possible between blanks, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M ATP standards (Figure 8-6). The different standards and blanks follow a similar time evolution; there is an initial increase in bioluminescence which

peaks between 3 and 5 minutes and then the bioluminescence values begin to decrease. The noise (as a fraction of signal value) increases at low bioluminescence values. Using the bioluminescence values of each ATP concentration at 4 minutes, a linear correlation between mean bioluminescence values and ATP concentration is observed, with linear least squares fit gradient of 0.998 and $R^2$ of 0.999 (Figure 8-6b).

### *8.3.4.2. Short term storage of devices*

To test the short term stability of assembled standard design fluidic devices with sucrose cocktail, three storage temperatures were investigated; -20°C, +4°C and +18°C. Three devices were tested immediately with $10^{-7}$ M ATP solution then the remaining devices were stored at the specified temperature in a sealed container with silica gel and in the dark. Three of each device was then tested after 7 and a further 3 after 14 days (Figure 8-7).

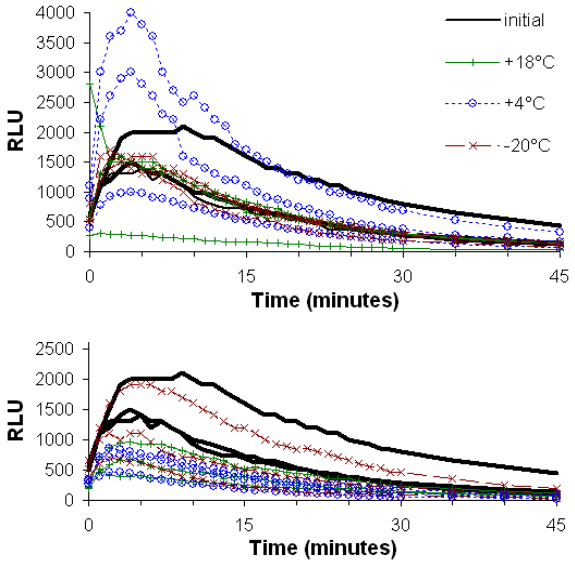


*Figure 8-7. Testing the stability of the fluidic devices in different temperature storage conditions. a) after 7 days, b) after 14 days.*

The results in Figure 8-7 show that storage decreased bioluminescence values, and the warmer the storage temperatures the greater the decrease in bioluminescence values. The mean bioluminescence value decreases by 20% after 7 days and 33% after 14 days at -20ºC. At +18ºC the equivalent values are 35% and 60%.

### *8.3.5. Modifications for incorporation of rFC assay*

Development of the ATP bioluminescence devices demonstrated that using laminated polymer sheets and glass fibre pads for the incorporation and assessment of assay devices is

possible. However to allow for development of the devices to detect multiple targets in a single device, an additional life detection assay needed to be shown to be able to work using this system. LPS detection using the rFC assay was chosen for development.

Adaptations to the design of the standard fluidic devices were made so they are compatible with the hand-held fluorometer (Figure 8-1a). The position of the pad was adjusted to be centred on the emission from the fluorescence excitation LED and external shape modified to fit diagonally across the cuvette holder (width of 13mm). A thinner connector was added to the outlet port to prevent leaking of reagents through the outlet hole when devices were upright.

Devices to incorporate the rFC assay were made following the same procedure of laser cutting and cleaning as the ATP bioluminescent devices and the three components of the rFC assay were mixed and freeze dried into pads as in Section 5.2.2. During initial testing, no increase in fluorescence values was observed during the four hour incubation with these devices after the addition of 10 $EU.ml^{-1}$ standard. Therefore additional modifications were required to the rFC devices.

#### *8.3.5.1. Device Materials*

The material used for the base and top layer in the ATP bioluminescence devices (Melinex® ST725) fluoresced at wavelengths needed the rFC assay, excitation maximum 380 nm and emission maximum 440 nm. Therefore alternative materials were needed. ARcare® 90485 (*i.e.* the ATP bioluminescence devices spacer layer), TEFLON® FEP (DuPont, DE, USA), ARseal™ 90404 with and without adhesive (development product Adhesives Research Ireland Ltd, Ireland) were tested, and glass was used as a standard (Figure 8-8). Fluorescence was measured in the benchtop fluorometer; excitation was fixed at the optimum for the rFC assay and emission was measured every 1 nm from 400 to 500 nm (the emission maximum for rFC assay was 440 nm).

ARcare® 90485 without adhesive returned the lowest fluorescence emissions of the polymer sheets tested. RFU value of 15.4 at 440 nm is only slightly higher than glass at 14.9 RFU and a significant improvement over Melinex® ST725 at 258 RFU (Figure 8-8). Meaning that the ARcare® 90485 material suitable as material for the incorporation of rFC assay into devices.

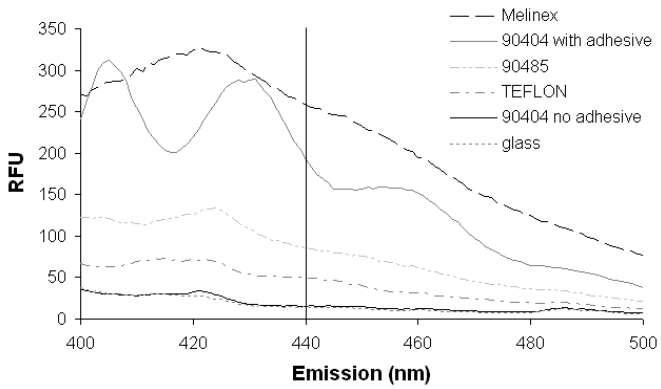


*Figure 8-8. Emission spectra for five materials tested for top and base layers for the rFC assay, glass was used as standard. ARseal™ 90404 was tested with and without adhesive. Excitation was at 380 nm, and the emission maximum for rFC assay is 440 nm. The nominal thicknesses of the materials varied; Melinex® ST725 – 250 µm, ARcare® 90485 – 178 µm, TEFLON® FEP – 250 µm, ARseal™ 90404 – 150 µm and glass – 130 to 170 µm.*

### *8.3.5.2. Incorporation of assay reagents*

Due to the problems with materials for the fluidic devices interfering with excitation and emission wavelength, pre-mixing and freeze drying of rFC reagents was tested in borosilicate glass mini-cuvettes (Turner BioSystems Inc., CA, USA) and adapter for the handheld fluorometer. The reagent volume (total 50 µl vs. 8.5 + 6.5 µl) and path length (3 mm vs. 178 µm) were greater in the glass cuvettes than for the standard design of fluidic devices. These factors need to be considered when using the results from the mini-cuvette experiments to guide the incorporation of rFC reagents into the fluidic devices.

The rFC assay is supplied in three components that are mixed to make the working reagent prior to adding to sample. To test the stability of the three components (rFC enzyme, assay buffer and fluorescent substrate) in dried form as required for the intended fluidic devices, they were placed in the freeze drier in mini-cuvettes and dried overnight as liquids, as shock freezing and subsequent thawing caused ~90% loss in activity. Seven different combinations of dried reagents were tested; these were three cuvettes with individual components, three cuvettes with combinations of two components and one cuvette with all three components. The total volume of working reagent was 25 µl. The dried reagents were reconstituted in the appropriate volume of endotoxin free water, when needed the other assay components were added to the cuvettes and then 25 µl of 10 $EU.ml^{-1}$ LPS standard added. The reagents were

incubated at room temperature in the dark. Fluorescence was recorded at time zero and then after every 30 minutes for 4 hours. Assays were conducted in triplicate.

Drying the components individually or mixed as two components did not show clear improvement over drying all three components premixed (data not shown). Figure 8-9 shows the dried working reagent show a different time evolution to the non-dried working reagent, however this is pronounced in the two samples where air bubbles were formed during the addition of the standard. Thus drying the reagents demonstrates a method suitable for the inclusion of the rFC assay reagents in the devices, potentially in a similar format to the ATP reagents.

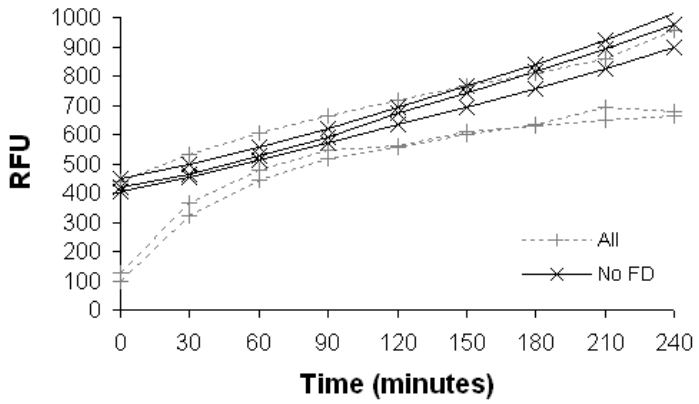


*Figure 8-9. The effect of drying the working reagent on the time evolution of the rFC assay. Reagents were dried directly in cuvettes. The low values at time zero in two of the three dried cuvettes was due to the formation of air bubbles during the addition of standard.*

## 8.4. Discussion

### *8.4.1. Preliminary testing of three layered polymer sheet construction*

A system consisting of three laminated polymer sheets and freeze drying biochemical reagents into glass fibre pads was successfully used to develop and rapidly manufacture fluidic-based devices for the detection of the biomarker ATP. In initial studies and using the initial design, higher concentrations of ATP standard resulted in higher bioluminescence values (Figure 8-2), however the variation between the triplicates was high with CVs up to 112% (x 2 CLSII, $10^{-6}$ ATP). To make the devices suitable for use, the variability had to be reduced, and ideally the sensitivity of the devices increased, *i.e.* higher bioluminescence values for the same ATP concentration. The origin of the high CVs was probably due to a combination of inconsistencies in the flow of sample through the fluidic devices and

differences in the rate of dissolution of the freeze-dried reagents from the pad. To improve this, two aspects of the fluidic devices were investigated further: the basic design of the fluidic path via design of the fluidic device's spacer layer and the composition of the reagents freeze dried into the pad.

### *8.4.2. Effect of fluidic device channel design*

As the ATP bioluminescence assay output is sensitive to reagent concentration, uneven distribution in the pads caused by irregular fluid flow would lead to different bioluminescence values for the same ATP concentration. Five different spacer layers were designed to investigate the effect channel design on fluid flow and subsequent reagent dissolution, specifically the degree of filleting and the length and width of the connector (Figure 8-3). Increasing the channel width by three to 0.6 mm was sufficient to reduce the time for reagent to reach the pad from between up to 4 minutes for the "original design" to between 1 and 2 seconds. The extra width meant that any small restrictions in the channel did not cause significant blockages, these are likely to arise from small amounts of adhesive squeezing into the edges of the channels during lamination of the devices. Also, the "3 x channel width" led to a visually assessed reduction in the movement of reagents within the pads. Inconsistencies in the "maximum channel width" are likely to result from the formation of 'fronts' of different shapes as the liquid moves through the pad, *e.g.* the liquid flowed more along one edge or the fastest flow was in the centre. Therefore the 3 x channel width was used in subsequent development with glass fibre pad excipients and testing of the devices.

### *8.4.3. Effect of excipients and reagents in the glass fibre pad*

To further improve the consistency between repeats and sensitivity of the ATP bioluminescence assay, excipients were added to the assay reagents prior to freeze drying. Fructose and sucrose both increase bioluminescence values and reduced mean CVs (Figure 8-4), sucrose having the greater effect. Both of these sugars have high solubility, therefore their inclusion in the freeze dried matrix should increase the solubility of the assay reagents as some of the active ingredients will be dried onto the sugars instead of directly to the glass fibre in the pads. Different excipients are selected for their different properties, such as the high solubility of sugars, therefore a cocktail can be created depending on the requirements. Two buffer cocktails were being developed by colleagues for other, similar applications: referred to as sucrose cocktail and blue dextran cocktail (Figure 8-4). The improvement in the repeatability of the assay reagents was similar with sucrose alone and sucrose buffer. However, mean bioluminescence values were higher with the sucrose cocktail than the

sucrose alone. The additional ingredients in the sucrose buffer cocktail could provide protection of the reagents during freezing and subsequent drying (Tamiya *et al.* 1985, Roy & Gupta 2004). When blue dextran cocktail was added to CLSII reagents, the mean bioluminescence value was less than half the mean bioluminescence value with just CLSII assay reagents. More significantly the time evolution of the assay was different, as the bioluminescence values decreased initially before starting to increase. This is likely to be due to interference with the bioluminescence reaction, as the blue chromophore on blue dextran can bind to the ATP binding site of the luciferase (Rajgopal & Vijayalakshmie 1982). Therefore as the blue dextran dissolves out of solution it starts to reduce the luciferase available for the ATP reaction, hence reducing bioluminescence values.

In addition to the use of excipients, the concentration of active ingredients was investigated. Increasing the concentration of the CLSII and HSII assay reagents increased the bioluminescence values. However, this increase is not directly proportional to assay concentration, *i.e.* a 5-fold increase in ATP reagent concentration does not result in a 5-fold increase in the bioluminescence values. This could be due to the incorporation of reagents in the devices, however high concentrations of luciferin induce conformational changes in luciferase which leads to a temporary reduction in activity rate (Lembert & Idahl 1995), hence providing some inhibitory effect as concentration increases. Although the higher concentration of reagents increased the bioluminescence values it also increased the number of outliers (the 2 x HSII concentration in Figure 8-5 has three outliers compared to 1 with 1 x HSII). Therefore 1 x HSII concentration was used in the glass fibre pad and used with sucrose cocktail to test the devices.

### *8.4.4. Testing ATP bioluminescence device performance*

The final ATP bioluminescence design required ~100 times the ATP concentration to produce the same bioluminescence values than when following the manufacturer's stated protocol, effectively making the assay two orders of magnitude less sensitive. One order of magnitude is likely to stem from the lower reagent and sample volume used in the fluidic devices (total fluid volume used in the final devices was 15 µl compared to 200 µl in the manufacturer's stated protocol). The rest of the decrease is likely to be due to a combination of the loss of the initial 'flash' of luminescence with the HSII reagents during dissolution (for the HSII reagents there is a ~30% reduction 2 minutes after addition of ATP, this drops to ~50% after 5 minutes), loss of activity during freezing and subsequent drying processes (~80% of original

reactivity during one freeze thaw process as stated by the manufacturer) and interference from the pad.

Testing the adaptability of the devices they were modified for detection with a benchtop plate reader, the required modifications to the design were the external shape, position of pad and position of inlet hole. These changes were quick to implement due to the laser cutting based manufacturing process. Adapting the devices to the different readout instrumentation was simple as no modifications to the assay reagents in the pad or the assembly protocol were required. Testing the adapted ATP bioluminescence devices in the plate reader, a linear correlation was observed between log bioluminescence values and log ATP concentrations of $10^{-5}$, $10^{-6}$ and $10^{-7}$ M ATP and these bioluminescence values were all distinguishable from the blank (Figure 8-6). However, to establish the actual limits of detection of the devices higher and lower ATP concentrations would need to be tested in future work.

The other aspect of the devices that was tested was their short term stability (after one and two weeks storage), where a reduction in the bioluminescence values was observed at the three temperatures used in this study. The lower bioluminescence values of the devices stored at +18ºC after 1 week and +4ºC after two weeks is likely to be due to loss of activity of the ATP assay reagents. As the manufacturer states the reagents, once reconstituted in liquid form, are stable for one day at room temperature (+18ºC), one week at +4ºC, and significantly longer at -20ºC. Even though the assay reagents were freeze dried in the pads and stored at -20ºC there was still a reduction in the bioluminescence values at this storage temperature (Figure 8-7). In the glass fibre pads, the reagents have a higher potential to interact with the atmosphere during storage compared to the sealed vials the original reagents are supplied in, hence allowing for more opportunities to degrade reagent activity. Future studies will involve storage in controlled atmospheres – *e.g.* in heat sealed foil pouches with desiccant and inert (oxygen-free) atmosphere.

### *8.4.5. Preliminary work to incorporate rFC assay into the devices*

The incorporation of rFC assay into the fluidic devices is preliminary work and details of required further work are included in Section 8.5.

When incorporating the rFC assay reagents into the devices two addition challenges were encountered, the first being that the rFC assay reagents are supplied as three different liquids and from initial testing it was discovered the materials used in manufacture of ATP bioluminescence devices fluoresces at the wavelengths used in the rFC assay. To be able to

incorporate the rFC assay reagents into the devices in a similar fashion to the ATP bioluminescence assay they need to be able to survive drying. However for rFC assay, the reagents were dried, due to loss of activity during shock freezing, therefore an alternative method to incorporate these reagents into the devices would be required, either by drying reagents into glass fibre pads, other materials or directly into the devices. The results in Figure 8-9 demonstrate that the assay reagents can be dried and retain subsequent assay activity, however the time evolution of the assay may be slightly different between the dried reagents and the standard reagents. If each component of the rFC assay has different stability to drying and / or different dissolution rates, the final composition of working reagent will be modified and this composition could change during the time evolution of the assay.

To be able to construct fluidic-based devices to incorporate the rFC assay in the devices, a different polymer sheet material was needed. Removing adhesive from ARcare® 90485 had the lowest fluorescence at the excitation and emission wavelengths used in the rFC assay for the polymers tested and was similar to glass. These materials can be used in future work for the assembly of rFC assay devices.

## 8.5. Conclusions and Future Work

Laminating laser cut polymers sheets and incorporating ATP bioluminescence assay reagents by freeze drying into glass fibre pads provides a method for the rapid design and manufacture of simple devices for life detection. The early designs indicated this system was suitable as increasing the ATP concentration increased the corresponding bioluminescence values, however improvements to the sensitivity and repeatability were required. Refining the fluidic design and the addition of a sucrose buffer cocktail meant the devices could be used to distinguish among the blank and $10^{-5}$, $10^{-6}$ and $10^{-7}$ M ATP standards with CVs of 18%, 2.9% and 8.8% respectively (bioluminescence values read after at 4 minutes Figure 8-6).

Attempts to incorporate the rFC assay for LPS detection into the devices required alternative polymer sheets for manufacture, which have been identified.

This work provides a good basis for the development of integrated life detection assays, which are suitable for laboratory and in-field analysis. The future developments involve:-

**Refinement of ATP bioluminescence devices**

This encompasses the reduction of the lower limit of detection, improvement in the stability of reagents during storage and further reduction in the repeatability of the devices. To reduce

the lower limit of detection three methods could be used; (i) the incorporation of a more sensitive ATP bioluminescence assay into the devices, (ii) increase the concentration of ATP reagents, without increasing the CVs and / or (iii) allow for the detection of the 'flash' of the HSII reagents by developing methods to dissolve the ATP reagents faster *e.g.* refining the buffer cocktail or use a sugar matrix to incorporate the reagents into the device rather than glass fibre pads. Improvements to the short term stability of the reagents are needed; potentially by modifying the cocktail to include preservatives and / or to design individual cover slips to seal off reagents from atmospheric gases. Also a detailed scrutiny of the devices may lead to the visual identification of the outliers and, by changing quality control accordingly, improve the repeatability of the devices.

**Incorporation of rFC assay into devices**

Having identified suitable polymer sheet materials, the rFC assay needs to be incorporated into the devices, either in glass fibre pads or directly in the devices. These adaptations may require an increase the pathlength for the fluorescence. Also the excipients and buffer cocktails from the ATP bioluminescence devices could be added prior to freeze drying to improve dissolution of reagents.

**Integration of multiple life detection assays**

Using the benchtop plate reader, both luminescence and fluorescence can be detected in the same device, making it suitable to test the integration of the ATP bioluminescence and rFC assays.

**Establish other life detection techniques into the devices**

To keep the same format these techniques would need to be single step assays with optically readable endpoints. Examples include DNA quantification with dsDNA specific fluorescent stains (Fierer *et al.* 2005), hydrolytic enzyme activity (Marx *et al.* 2001).

**Testing with bespoke reader in laboratory and field settings**

Bespoke instrumentation is also being developed that can measure luminescence and fluorescence and can be transported to the field. The current design of the bespoke reader interfaces with the devices with a glass slide sized cassette which accepts the devices and fits into the bespoke instrumentation. The instrumentation includes a photomultiplier tube for low light detection, a CCD camera capable of imaging and possibility of excitation.

**Development of extraction protocol.**

To be able to detect ATP and LPS in environmental samples, these targets have to be released from samples and any interfering components have to be removed. The development of extraction protocol has to be compatible with field deployments and considerations made a single extraction technique for the combined devices.

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# 9. Overall Discussion, Conclusions and Future Work

## 9.1. Summary and Discussion

The overall discussion is structured in terms of the original thesis objectives outlined in Chapter 1 Section 1.4 and an additional section on the interpretation of the data acquired during the thesis in terms of the microbiology of subglacial environments.

### *9.1.1. Techniques for in-field life detection and characterisation*

- Identify a number of complementary techniques that have the potential to detect and characterise life at the low target concentrations that are typical of glacial environments.

Reviewing the current techniques that have been used to study microbial life in icy environments has identified twenty five techniques that have been applied to detect or characterise life in samples from icy environments (Chapter 2). Approximately half of these techniques have been used in the field, both in icy environments and other disciplines, however these techniques have only been used in a few studies and covering a narrow range of environments or sample types, meaning their full potential may not yet have been reached. These techniques can be divided into four categories that are based on microscopy, cell physiology and biochemistry, molecular biology and physical sensors. To select the complimentary techniques for in-field life detection, the techniques were selected from three of the four categories and include some of the techniques described in Chapter 2. Of the seven techniques used within this thesis: ATP bioluminescence has already been used in the field in icy environments; real-time PCR and fluorescence cell counts have been used in the field but not in icy environments; LAL-based (or specifically recombinant Factor C (rFC)) assay and immunoassays have been applied to samples from icy environments but not yet in the field; and hydrolytic enzyme activity and colorimetric respiration measurements have not yet been applied to samples from icy environments. ATP bioluminescence, rFC assay and fluorescence cell counts were used to show the presence and abundance of microorganisms, hydrolytic enzyme activity, respiration measurements and ATP bioluminescence (in combination with other techniques) could be used to demonstrate activity of microorganisms and real-time PCR and rFC assay (in combination with other assays) could be used to show the diversity of microbial communities.

- Identify suitable commercially available assay reagents, instrumentation and consumables and additional laboratory equipment that can be used to apply these techniques and that are compatible with field deployment.

To apply the above techniques, suitable assay reagents (as commercially available kits or from well established protocols), instrumentation, consumables and general laboratory equipment (*i.e.* pipettors, beakers) are required. For most of the techniques selected for use for in-field detection of life in the field, there is a choice of commercially available assay reagents, instrumentation, consumables or equipment, providing opportunities to select the most suitable for field use. To assemble a field laboratory, the individual components should be robust and with minimal mass, volume and power requirements. An additional consideration for instrumentation and general laboratory equipments is whether any particular item could be used for more than one analytical technique. The techniques used here allowed for the same instrument to be used for rFC assay and hydrolytic enzyme activity (fluorometer) and the absorbance reader for immunoassays and colorimetric respiration measurements (however not in the same field campaign). All of the associated general laboratory equipment was shared among the techniques. The only modification that was required to the instrumentation from the manufacturer's standard was the correct choice of filters for the fluorescent microscope. The excitation LED was the same, however an alternative emission bandpass filter was required (made by the manufacturer). An additional requirement for the assay reagents was the stability, particularly under fluctuating temperature, as the majority of the assay reagents involve biochemicals which are generally temperature sensitive.

The final selection of the instrumentation and reagents was a combination of the preceding criteria and practicalities including availability, cost and opportunity, and are listed below. ATP bioluminescence reagents and equipment (Merck HY-LiTE 2) were selected due to loan of luminometer by the UK distributor (VWR International Ltd., Lutterworth, UK) and previous use in a local non-icy field environment. The rFC assay used here (Cambrex PyroGene® Lonza, UK) is currently the only commercial LAL-based assay using this modified pathway, and the fluorometer (*Pico*Fluor, Turner BioSystems, CA, USA) was chosen as it had suitable excitation and emission wavelengths, it was light (400g) and it could be powered by 4 x AA batteries. The microscope (CyScope, Partec UK, UK) with which the fluorescence cell counts were conducted was build for field use (primarily for detection of specific diseases in mobile hospitals in Africa), and constructed such that it was compatible

with ‘plug in’ excitation LEDs and emission filters supplied by the manufacturer. The real-time PCR instrument (MiniOpticon™ Bio-Rad Laboratories) and PCR reagents were loaned and donated by the manufacturer for the November 2008 field campaign, and PCR primers were selected from published literature. The GroEL immunoassay was used as it was being developed for other extreme environment applications, hence reagents were available, and the detection instrumentation could be identified that was portable (BioTek Manual Microplate reader). Hydrolytic enzyme activity was tested as could use instrumentation and reagents that were already available. This is the same case for the colorimetric respiration measurements.

- Consider and test modifications to assay reagents, assay protocols, instrumentation and consumables that may be necessary for in-field use.

No modifications were made to the assay reagents themselves, however some of the reagents were aliquoted prior to transport to the field. As the instrumentation and consumables were selected for their compatibility with field use, no additional modifications were required beyond those necessary to complete the technique, *i.e.* the modified emission bandpass filters with the fluorescence microscope. The main area of modification, either pre-field or in-field, was to the assay protocols, and originated from a desire to minimise any additional equipment that needed to be transported to the field. The levels of modifications varied among the different techniques, including no modifications required to apply the fluorescence cell counts and real-time PCR protocols in the field. Minor modifications were made to hydrolytic enzyme activity (where buffer was changed from methanol to ethanol due to the knowledge that a supply of ethanol was present at the field site from previous field campaign) and to ATP bioluminescence (where the bioluminescence value of ATP bioluminescence reagents pre- and post- addition of the sample to confirm lack of contamination in the reaction tubes). The rFC assay and colorimetric enzyme activity required the most modifications. For the rFC assay, the manufacturer’s protocol states incubation at 37°C, which was deemed impractical for field use, therefore modifications to the protocol involving greater incubation time, and additional measurements were developed, tested and implemented in the field. Details of the modification and subsequent discussion are outlined in Chapter 4. One of the main applications of the colorimetric respiration measurement is to measure respiration in soil samples, and is used in systems such as MicroResp™ (Macaulay Land Use Research Institute, Aberdeen, UK), where 96-well microtitre plate is sealed on top of soils samples with different incubation conditions. As

respiration rates in ice samples are likely to be much lower than rates in soils an alternative experimental set-up was devised involving a larger, airtight sample containers (400 ml each) to maximise the chance of measurable respiration, in addition these containers could be sterilised by autoclave. Blocks of debris-rich ice or sterile water control were sealed into containers for 5 days and incubated in the dark in the bedrock tunnels by the ice cave in Engabreen. Measurable colour differences, caused by a pH sensitive dye embedded in agar reacting to changes in $CO_2$ concentration, were seen between the two samples however further investigations involving calibration would be required to pursue this further.

### *9.1.2. Sample processing*

- Identify suitable published protocols and commercially available kits for the extraction of targets of each technique and that are compatible with field deployment.

The same considerations to minimise mass, volume and power consumption are true of any equipment required for in-field sample processing. These, along with a desire to minimise the time to develop and conduct the technique, mean that simple, established protocols were desired. For cell counts and hydrolytic enzyme activity only minimal sample processing was required, *i.e.* melting ice or snow samples. A commercially available DNA extraction kit was used to prepare samples for real-time PCR. A variety of published protocols were identified to extract the targets for the ATP bioluminescence, rFC assay and immunoassays. For the colorimetric respiration measurement, sample processing involved breaking ice into suitable sizes blocks and sterilising the outer surface with ethanol then removing this layer with hot sterile water by melting.

- Test these protocols and any adaptations that are necessary for use in-field in institutional laboratories and in the field.

For cell counts, hydrolytic enzyme activity and colorimetric respiration measurements minimal sample processing was sufficient to analyse the samples. Modifications to DNA extraction kit protocol involved replacing the vortex mixing step with manual shaking and all centrifuge steps were performed at 7200 x g rather than 10000 x g. The combination of these modifications allowed for the detection of the PCR products via real-time PCR, however some difference among the extraction batches were observed in the real-time PCR results from Greenland, where statistical correlation was observed ($P<0.05$) between results and extraction batch for three of the seven primer pairs. For some of the targets there is not the same commercial demand for extraction kits as for DNA, however published protocols may

exist. Therefore for the extraction of the remaining targets, a variety of published protocols were tested. For rFC assay direct analysis was found the best out of direct analysis and two alternative protocols (details in Chapter 4), although the direct analysis was modified by the addition of a centrifuge step for the Greenland samples to reduce sediment concentration in the sample aliquot. A typical protein extraction protocol involving sonication, and cooling in a buffer of Tris saline was applied to samples prior to the GroEL immunoassay. No GroEL was detected in samples tested in Engabreen including basal ice, ambient bedrock tunnel sediments and subglacial meltwater, therefore due to the ability to detect ATP and LPS by the ATP bioluminescence and rFC assay with protocols tested in the same field campaign this technique and hence need for sample extraction was not perused. For the ATP bioluminescence assay a variety of buffer based sample extraction techniques were tested, including boiling Tris EDTA, bicarbonate and sodium phosphate. Boiling Tris EDTA gave the most consistent results and was applied in the field, however issues were encountered with the subglacial meltwater and debris-rich ice samples (Chapters 3 and 5), and probably due to the presence of sediments that adsorbed free ATP.

- Develop suitable target extraction protocols if the published and commercially available ones are unsuccessful.

Due to the issues found when using ATP bioluminescence assay in samples with clay minerals in Chapter 3 and 5, a protocol for ATP extraction based on the 'blocking' of adsorption sites and 'boiling' to extract the ATP extraction protocol was developed in Chapter 6. Of the thirteen chemicals that were tested to block or modify the adsorption sites on model clay, protein was the most successful. The protocol developed in Chapter 6 was then applied to the samples during the Greenland field campaign.

### *9.1.3. Field testing*

- Identify appropriate field sites in icy environments appropriate to field testing of techniques.

Two field sites were used during the course of this thesis; Engabreen and Greenland. The Svartisen Subglacial Laboratory underneath Engabreen had mains electricity, running water (pumped from a local subglacial source), permanent laboratory space and internal laboratory temperature controlled in the region of +18 to +22 °C, and therefore was a benign environment for the in-field testing and development of techniques, sample processing protocols and sampling strategies. The Engabreen field site was used as a 'stepping stone' to

a more extreme field site, in this case Greenland. Greenland allowed for testing of the techniques developed in and applied to Engabreen without mains electricity, running water, permanent laboratory space and controlled laboratory temperature.

The dominant bedrock geology surrounding Engabreen (Svartisen Ice Cap) and Leverett Glacier (Greenland Ice Sheet) is metamorphic, either gneiss or schist, with some granitic intrusions near Leverett Glacier. However the Greenland Ice Sheet is significantly larger than Svartisen Ice Cap, with a larger volume subglacial drainage (Dowdeswell 2006, Hanna *et al.* 2009), meaning Leverett Glacier is more significant to global biogeochemical cycles in the cryosphere.

- Test the target extraction, sampling strategies and the analytical techniques at field sites, making necessary in-field adaptations to protocols.

All seven techniques were analytically possible in the Engabreen field site (*i.e.* successful detection of positive controls) however GroEL could not be detected using the GroEL immunoassay in any of the glacial and periglacial ice and water samples. As the specifics of target extraction, sampling strategies and analytical techniques are contained in the specific chapters, the remainder of this discussion concentrates on the additional adaptations required in the Greenland field campaign. The four techniques that were applied systematically in Engabreen (Chapter 5) could also be used in the field in Greenland and on subglacial and supraglacial samples. To apply these four techniques in Greenland no additional adaptations were required to instrumentation or the general laboratory equipment, however adaptations to the sample treatment were made, *i.e.* sample processing and sample analysis. The changes to the sample processing were minimal and included the implementation of the ATP extraction protocol described in Chapter 6 and a centrifuge step was added prior to testing the supernatant for the rFC assay, to reduce interference from particulates in the meltwater. Modification to the assay protocol was required to conduct cell counts in Greenland, as the high temperature of the laboratory (up to +37ºC) caused fluorescence dye to fade within counting timescale, therefore all cell counts had to be completed when the ambient tent laboratory temperature was below +22°C. Also worth noting are issues concerning cross contamination of tubes and short term stability of reconstituted reagents for the ATP bioluminescence assay. Once reconstituted from their freeze dried state the ATP assay reagents were only stable for a few days in the in-field freezer (set at -13°C, but occasionally temperature rose by a few degrees due to low battery). These issues could be overcome by

more stringent cleaning regime or use of disposable ATP tubes and aliquoting into smaller reagent volumes, prior to a similar length field campaign.

### *9.1.4. Bespoke analytical devices*

Conducting analysis with individual sample preparation and dedicated equipment for each technique is time consuming and requires a large number and combined mass of instrumentation, hence there is a desire for bespoke instrumentation. The discussion of the desire for bespoke instrumentation and developments towards such an instrument are addressed in Chapter 8, however concerning the overall objectives from Chapter 1 are as follows.

- Consider a method to integrate a number of life detection techniques into a single system.

Fluidic based devices, prepared by the lamination of three polymer sheets allowed for incorporation of assay reagents into chambers within the central layer. Each layer is cut using high-powered $CO_2$ laser and new designs can be quickly implemented through designs in 2D CAD software package. Modifications to the design of chambers and associated connectors would allow for the incorporation of different assay reagents.

- Test the system by integrating commercially available assay reagents that were identified as suitable for in-field detection of life in icy environments.

Life detection assays were selected from those that were being adapted for in-field life detection using commercially available reagents and instrumentation. The ATP bioluminescence assay was successfully integrated by freeze drying reagents along with sucrose based buffer cocktail into glass fibre pads. This allowed for the detection of ATP over three orders of magnitude and the devices could be modified for reading by a handheld luminometer or benchtop plate reader with 12-well microtitre plates. The incorporation of the rFC assay into the same format devices is under progress having identified suitable materials for assembly of the devices and progression into techniques for incorporation of the rFC assay reagents.

### *9.1.5. Scientific understanding of microbial life in subglacial environments*

Although this is not an original objective, during the development and testing of in-field life detection techniques, data was collected on the microbiology of subglacial environments. In terms of microbial life in icy environments this thesis has added two more subglacial environments where signs of life has been detected; Engabreen in Northern Norway and

Leverett Glacier in Western Greenland. Engabreen shows distinct biomarkers among different sample types with statistically significant difference between the debris-rich and debris-poor ice. The data collected during the Greenland field campaign showed distinct biomarkers in the subglacial and supraglacial environments, and changes in the biomarker concentration in subglacial meltwaters of the Leverett Glacier over diurnal and longer timescales. The details of the interpretation of what these data means in terms of the microbiology of icy environments are discussed in Chapters 5 and 7 and focussed on spatial resolution in Engabreen and temporal resolution in Greenland respectively. Comparing the datasets acquired from Engabreen and Greenland field campaigns and putting them into context of other findings on microbiology in subglacial environments are discussed in the following section (with some additional calculations from the results not included in Chapters 3 to 5 and 7). The focus concerns the abundance and distribution among sample types, the activity levels and the diversity.

Regarding the abundance and diversity, higher microbial populations are associated with sediment in glacial environments. Foght *et al.* (2004) reported viable cell counts of 2 and $7x10^6$ cells.ml$^{-1}$ in debris-rich ice from a southern hemisphere glacier where the concentration of microorganisms was about 3 to 4 orders of magnitude higher in debris-rich than glacial ice. Mader *et al.* (2006) also found $10^2$ to $10^3$ cells.ml$^{-1}$ in debris-poor glacier ice from a High Arctic glacier. This distribution agrees with findings from Engabreen where fluorescence cell counts were $3x10^4$ to $1x10^6$ cells.ml$^{-1}$ in debris-rich ice and $2x10^2$ to $1x10^3$ cells.ml$^{-1}$ of debris-poor glacial ice, the debris-rich ice sample displaying concentrations at the lower limits of published data (Foght *et al.* 2004). In the Greenland samples the cell counts were nearly two orders of magnitude higher in the subglacial runoff ($1x10^5$ to $4.3x10^5$ cells.ml$^{-1}$) compared to the supraglacial stream ($4.3x10^3$ to $8.6x10^3$ cells.ml$^{-1}$).

Regarding activity, subglacial environments are now widely believed to be microbiologically active (Christner *et al.* 2008). As indicators of microbial activity, ATP was the dominant marker; however detection of enzymes in debris-rich ice samples from Engabreen using hydrolytic enzyme activity also shows active microbial population. An indicator of activity explored in this study was the apparent ATP concentration per cell, in Engabreen the apparent ATP per cell has a much broader range in the surrounding environment (0.03 to 63.4 fgATP.cell$^{-1}$), than the Engabreen glacial samples (0.06 to 2.35 fgATP.cell$^{-1}$ - Figure 3–4), where the highest ATP per cell are found in Snow samples and therefore possibly due to the presence of larger eukaryotic microorganisms. The subglacial meltwater samples in

Greenland (not calculated in Chapter 7) had low apparent mean ATP per cell (0.026 fgATP.cell$^{-1}$) and lower than the supraglacial samples (0.18 fgATP.cell$^{-1}$ for supraglacial meltwater and 0.19 fgATP.cell$^{-1}$ for cryoconite holes). No other studies using ATP concentration and cell counts have been conducted on subglacial environments, however studies on cultured microorganisms shows ATP per cell can range from 0.16 to 2.25 fgATP.cell$^{-1}$ (Fairbanks *et al.* 1984). The apparent ATP per cell values in the Greenland subglacial meltwaters are below this value and hence there is likely to be a contribution from non-active cells to the fluorescence cell counts. To support this, the lowest apparent ATP per cell in the Engabreen glacial samples was meltwater, likely to have some subglacial origin (Side Stream). Therefore the apparent ATP per cell concentration does not necessarily indicate actual amount per cell.

The majority of the assessment of microbial diversity is from the real-time PCR data. However the detection of LPS shows the presence of Gram-negative bacteria. With the exception of the debris-rich ice samples from Engabreen, LPS were detected when cell counts was greater than about $10^3$ cells.ml$^{-1}$ (Engabreen Snow samples and the majority of the Glacier Ice samples - Table 5–2). As the lowest field standard of 0.1 EU.ml$^{-1}$ also represents approximately $10^3$ Gram-negative bacterial cells.ml$^{-1}$ (Mueller *et al.* 2004 and La Ferla *et al.* 2004), then it is likely that Gram-negative bacteria are a significant proportion of the subglacial microbial population. Kingdom level classifications for bacteria, archaea and fungi were developed by selection and optimisation of primer pairs for real-time PCR. Selected samples were analysed with fungal primers, however unsatisfactory melt curves were observed and therefore not applied to all samples. Both bacterial and archaeal primer pairs were applied to all samples from Engabreen 2008 and 2009 and Greenland field campaigns and were found in all samples and the bacterial concentration was greater. Using the difference between the Ct values for bacteria and archaea to represent relative archaeal abundances, the greatest difference was the in the Greenland cryoconite hole samples ($2^{11}$ fold difference). The difference between bacterial and archaeal concentration was significantly narrower in all other sample types: Engabreen Debris-rich Ice (9.3 ± 1.9), Engabreen Snow (9.2 ± 3.7), Engabreen Alex's Stream water samples (8.4 ± 1.0), Greenland subglacial meltwaters (7.9 ± 1.8), Greenland supraglacial meltwaters (7.8 ±1.0), Engabreen Spiral Tunnel water (6.6 ± 0.7) and Engabreen Glacier Ice samples are the lowest at 6.3 ± 1.3 (errors are ± 1 SD from all samples in group). Hence the sample type seems to have a greater effect on the relative abundance of archaea than location. Using the functional pathway genetic targets detecting DNA sequences, evidence for the potential involvement of

subglacial microbial populations in carbon, sulphur and nitrogen cycles in Engabreen and Greenland and agrees with previous findings (Sharp *et al.* 1999, Skidmore *et al.* 2000, Bottrell & Tranter 2002, Gaidos *et al.* 2004, Wynn *et al.* 2007). Most of previous studies have concentrated on chemical analyses of meltwaters (Sharp *et al.* 1999, Wadham *et al.* 2004), studies on cultured microorganisms (Skidmore *et al.* 2000) or comparing DNA sequences to known cultured microorganisms, hence inferring similar metabolic pathways (Mikucki *et al.* 2004). Only Yergeau & Kowalchuk (2008) have used primer pairs for functional genes in glacial environments, in this study reverse transcription real-time PCR was used to detect mRNA, however this study focused on samples of mineral soils. Hence the use of real-time PCR with functional pathways provides an additional method to study metabolism in subglacial environments, and as it is high throughput, it can be used to study changes both spatially (Engabreen) and temporally (Greenland). An extension to detection of RNA would lead to ability to detect changes in the active metabolic pathways.

## 9.2. Conclusions

The following conclusions can be made based around the initial aims of the thesis:

- It has been demonstrated that in-field detection of microorganisms using multiple techniques in icy environments is possible (Chapters 2 through 5 and 7) and through this, unique and more efficient scientific studies can be conducted (emphasised in Chapter 7).
- That in-field detection and characterisation of microorganisms in the field in icy environments can be achieved with a suite of readily commercially available techniques (Chapters 5 and 7).
- The logistical feasibility of the in-field detection of microorganisms using multiple techniques in icy environments has been demonstrated at two logistically challenging glacial field sites: Engabreen, Norwegian Arctic and Leverett Glacier, Western Greenland.
- Having demonstrated the detection of multiple biomarker in subglacial and surrounding environment, the next stage is requires the addition of techniques to relate them to specific microorganisms, such as DNA sequencing.

The following conclusions are summarised from preceding chapters or can be drawn from the discussions in this chapter.

**Techniques**

- Multiple techniques (ATP bioluminescence, real-time PCR, fluorescence cell counts and LPS endotoxin detection) can be used to detect life in samples from icy environment.
- Commercially available instrumentation and reagents can be, and already have been, used to conduct some of these techniques in the field.
- Further modifications to assay protocols are required to some assay protocols to be compatible with field use.

**Sample processing**

- For sample processing to be compatible for in-field detection in icy environments it needs to be simple, quick and can be used on samples with low biomass.
- Two issues that were addressed by using sample processing prior to analysis were low concentration of target in sample (concentration of targets was increase by filtrating samples onto filter membrane), and the interference of sample matrix, as typified by the debris-rich ice samples from Engabreen. Chemical modifiers were added to extraction buffer to block ATP adsorption sites on clay and casein was selected as it had the highest ATP recovery of all the modifiers tested.

**Field testing**

- In-field detection of life by multiple techniques in icy environments is possible in both of the chosen field sites (ATP bioluminescence, real-time PCR, fluorescence cell counts and LPS endotoxin detection).
- Applying the same life detection techniques in the two field sites, adaptations were required to the sample processing protocols. These adaptations have two causes, either due to variations in the sample (*e.g.* change filter volume) or due to variations in the conditions that the process is being conducting (*e.g.* higher laboratory temperature required completion of cell counts to be conducted when the Greenland laboratory was less than +22°C).
- Due to desire to minimise the equipment that is transported to the field, conducting the same life detection techniques in the field is more labour intensive than institutional based analysis for some techniques, *e.g.* the requirement to individually

measure fluorescence for each samples for the rFC assay, rather than automated measurements that could be conducted by a plate reader.

**Bespoke instrumentation**

- The three polymer sheet system provides a suitable platform for the rapid manufacture of bespoke analytical devices.
- This system has the potential to detect different microbiological targets in a single instrument, with potential for integration of several biomarkers.

**Scientific understanding of icy environments**

- As suspected there is life in subglacial environments under Engabreen and Leverett Glacier, and this likely to be active.
- Having demonstrated the techniques and advantages of in-field analysis, these techniques provide tools that can be used to address biogeochemistry questions concerning microbial life in icy environments.

## 9.3. Future work

Arising from the work contained within this thesis and conclusions of the thesis as a whole, there are some general areas of future work and that are in addition to the specific work outlined in the relevant chapters.

### *9.3.1. Development of techniques used and addition of new in-field life detection techniques*

**The techniques already used in this thesis**

There are three techniques that have been used in the field but have not optimised: colorimetric respiration measurements, hydrolytic enzyme activity and immunoassay. Prior to subsequent field development each of these techniques would benefit from additional research. The colorimetric respiration measurements would benefit from thorough testing, including seal of the containers, stringent controls to minimise and equalise the headspace in each sample. Hydrolytic enzyme activity could be expanded using a greater number the enzymes, looking into sample processing protocols including the possibility to concentrate targets to increases the lowest limit of detection of some of the less abundant enzymes. GroEL immunoassay would require the development of a suitable sampling strategy and optimisation of processing protocol, testing under non-temperature controlled conditions, and explore potential other targets that can be detected by immunoassays.

**Techniques that could be used with same detection instrumentation**

The detection instrumentation used during this study has applications for other techniques and include the measurement of total DNA quantification using fluorescent dyes with suitable excitation and emission spectra and handheld fluorometer. The fluorescent microscope could be used by changing excitation LED and emission filters with dye sets that stain live and dead bacteria with different fluorescence wavelengths, the possibility for sequence specific dyes using FISH or for chlorophyll autofluorescence. By using RNA extraction kit and reverse-transcription stage prior to real-time PCR amplification, the real-time PCR instrumentation can be used to study RNA. This will confirm whether the gene encoded by the primer pair is active, hence providing more information into the involvement of these microorganisms in biogeochemical cycles. The development of other immunoassays with colorimetric endpoint could be applied to detect the target in the field and endpoint detected with the manual strip reader, particular if targets are already detectable via ELISA immunoassay. Considerations for appropriate sample preparation and storage would be required for all of these techniques, in particular RNA as it is less stable and therefore harder to extract than DNA.

**Other techniques that have been identified in Chapter 2**

More techniques have been used in the field in other environments as identified and discuses in Chapter 2 Section 2.4 onwards. These and similar techniques could be developed for applications in the field in icy environments by applying what has been learnt by adapting life detection techniques for in-field use in this study. Of particular interest is the use of physical and chemical techniques to integrate with more traditional microbiological ones used in this study, *e.g.* SERS and automated sampling of chemical species.

### *9.3.2. Refining in-field sample treatment*

The long term experiment in Greenland highlighted that refining the short term in-field storage of samples for the rFC assay and the DNA extraction protocol would improve the robustness of the interpretation of the results regarding microbiology of Leverett Glacier, Greenland. Investigations into the optimal storage temperature and time for LPS standards and model systems with Gram-negative bacteria would help refine the in-field temperature storage of samples to be analysed by the rFC assay. For the DNA extraction protocol an in-field alternative to a vortex mixer could be sought and investigations into the effect of temperature on extraction efficiency would be required.

Reductions to the time taken for in-field sample preparation could be done in a combination of 'shortening' the time needed to complete the existing techniques and by convergence of sample processing techniques, or stages of sample processing. Convergence of sample processing has already been demonstrated by using the same filter membrane for samples analysed by ATP bioluminescence assay and real-time PCR. Completely converged techniques would be required for the analysis of multiple targets in the bespoke instrumentation.

### *9.3.3. Use of in-field microbiological techniques to answer geomicrobiology questions*

Before the full benefits of applying multiple in-field life detection techniques can be realised, data processing, development of sampling strategy and selection of the combination of specific techniques needs to be refined to prove that the changes in the biomarkers used here are due to changes in microbiology or their community structure. Together these can be used to focus on specific science questions. To illustrate this, a few examples of further work in terms of an overall aim and some potential objectives are outlined below and based on the sampling strategies conducted at the Engabreen and Greenland field sites respectively.

Can microbiological populations be used to determine glacial hydrology? Objectives: (i) Accurate characterisation of end member influences of meltwaters such as snow, debris-rich ice and debris-poor ice. (ii) Conduct the same analyses on the meltwater sample of interest. (iii) Conduct statistical analyses to objectively assess the relationships among the samples.

What affects meltwater microbial populations during a glacial melt season? Objectives: (i) In a field campaign similar to Leverett Glacier in Greenland, in terms of length of time and subglacial drainage, maintain a base level of sampling and analyses meltwaters for microbiological markers from onset of glacial melting. (ii) Allow for the capacity for more frequent sampling, which could be triggered by changes in physical measures such as river level, turbidity or weather conditions which are continuously monitored. (iii) Collect parallel samples to conduct chemical analyses of species and organic matter. (iv) Integrate the microbiological and chemical data to elucidate any whether these track each other over the full melt season as well as effects of shorter temporal events such as rainfall, or sudden changes in subglacial drainage.

How do psychrophilic and psychrotolerant microorganisms' response to natural changes in external conditions? Objectives: (i) Identify multiple, independent techniques to assess microbial activity, *e.g.* hydrolytic enzyme activity and reverse transcription real-time PCR.

(ii) Monitor external conditions *e.g.* temperature, oxygen concentrations and pH. (iii) Use the techniques to measure the responses of microorganisms under these external conditions. (iv) Use statistical analysis to assess relationships between external conditions and response.

### *9.3.4. The application of in-field life detection to other environments*

The techniques developed here as well as future developments to in-field microbiological detection have multiple future applications. Regarding icy environments the more extensive environments tend to be more remote, *i.e.* ice sheets of Antarctica and Greenland. Due to their larger size these environments have the potential for a greater impact on global biogeochemical cycling. Along with the ice sheets themselves being in remote places some of the 'sub' environments within these are also difficult to access. Growing interest has been shown in the possibility of microorganisms in subglacial lakes in Antarctica (Pearce 2009). In-field detection techniques for applications such as proposed probe for drilling into Subglacial Lake Ellsworth (Siegert *et al.* 2007) can either be used via development of a field deployable life detection technique with the probe or testing for contamination of instrumentation prior to probe deployment. Recently alternative subglacial environments within Antarctica have shown to contain active microbial communities and include ice slurries (Mikucki *et al.* 2004, Mikucki & Priscu 2007) and ice streams. Although some of these microbial analyses have only been conducted on cores originally collected for other purpose, hence initially not treated to minimise potential for microbial contamination and growth (Lanoil *et al.* 2009), which could lead to changes in community structure. Liquid water has also been found under Greenland Ice sheet at sediment ice interface of Northern Greenland Ice Core Project (NGRIP) core (North Greenland Ice Core Project members 2004). Active microorganisms have been found associated with basal clay in Greenland Ice Sheet Project (GISP2) core (Tung *et al.* 2006), demonstrating a potentially large reservoir of microbial life, Hence, being able to analyse deep or shallower core samples more rapidly for microbial content would give a much more accurate representation of abundances of microorganisms, metabolic state and diversity. Study of surface icy environments would also benefit from in field analysis, again due to ability to preserve and hence measure the field microbial community structure, increasing our understanding of temporal and spatial variations in surface snow (Amato *et al.* 2007, Segawa *et al.* 2005) and cryoconite holes (Stibal *et al.* 2008).

Along with additional icy environments the advantages of in-field sample processing and analysis suits remote field locations, especially where sample transportation of large samples

would be required and necessary if samples cannot be returned for analysis, like current planned missions to Mars or future exploration of extraterrestrial icy environments on Mars, Europa and Enceladus. In-field analysis is particularly advantageous in environments where little is understood about the microbiology in that particular environment as preliminary analysis can be used to develop sampling strategies and sample processing technique.

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THE END

... of the beginning!

... but the start of who knows?

# Appendix A – Photographs Depicting Aspects of Field Work

## Engabreen, Svartisen Icecap, Northern Norway

Engabreen from the far side of the snow covered Proglacial Lake (Engabrevatnet). The location of the tunnel entrance is circled, March 2009.

The entrance to the underground tunnel system. Engabreen is behind the snowdrift in the bottom right of image and the rest of the Svartisen Ice Cap is off to the right, November 2008.

Satellite laboratory inside of the tunnel system. The laboratory equipment for this field campaign is contained within the two backpacks and cooler in the foreground and includes analytical equipment, consumables and reagents, November 2008.

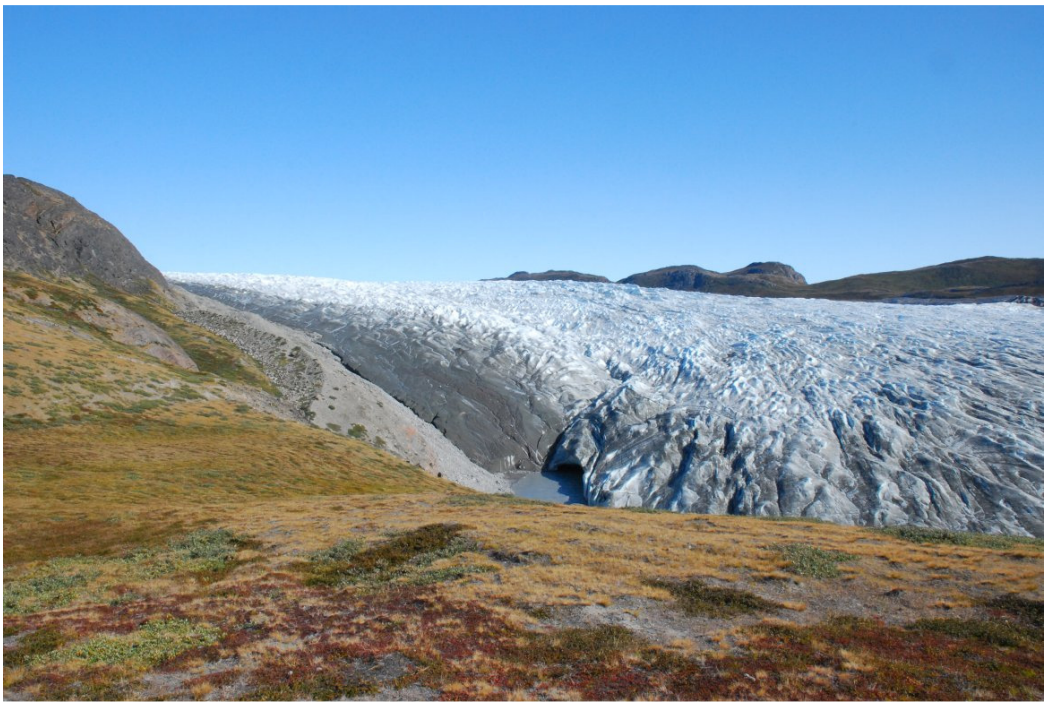
Sample preparation in the tunnel system. Samples were filtered and the membranes split in the tunnel and then transported back to the satellite laboratory to conduct analysis, March 2009.

Hose used in hot water drilling of the ice cave and the ice cave which allows for access to pristine subglacial samples. The hose support is about 90 cm high, November 2008.

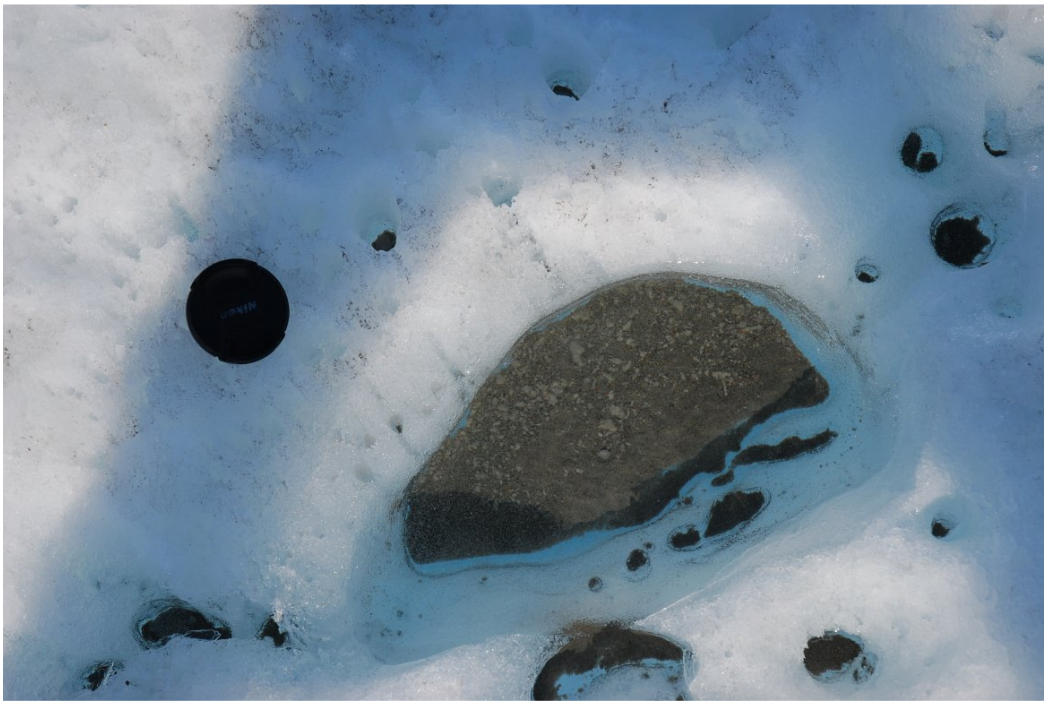
Removing samples from the ice cave, showing the clear transition between the debris-rich (brown) and the debris-poor (blue) ice. The chisel was wiped in ethanol prior to use, November 2008.

## Leverett Glacier, West Greenland

Overview of laboratory tent with generator and the autoclave being attended to. Leverett Glacier is in the background and the sample location of the meltwater stream off to the right of the image.



Exit portal for the subglacial river. Image also shows typical shrubland environment in the foreground. The access to the glacier was on the left side of the glacier from this view.



Typical cryoconite hole, approximately 10 cm deep. Lens cap 53 mm diameter. Approximately 1.2 km from the snout.

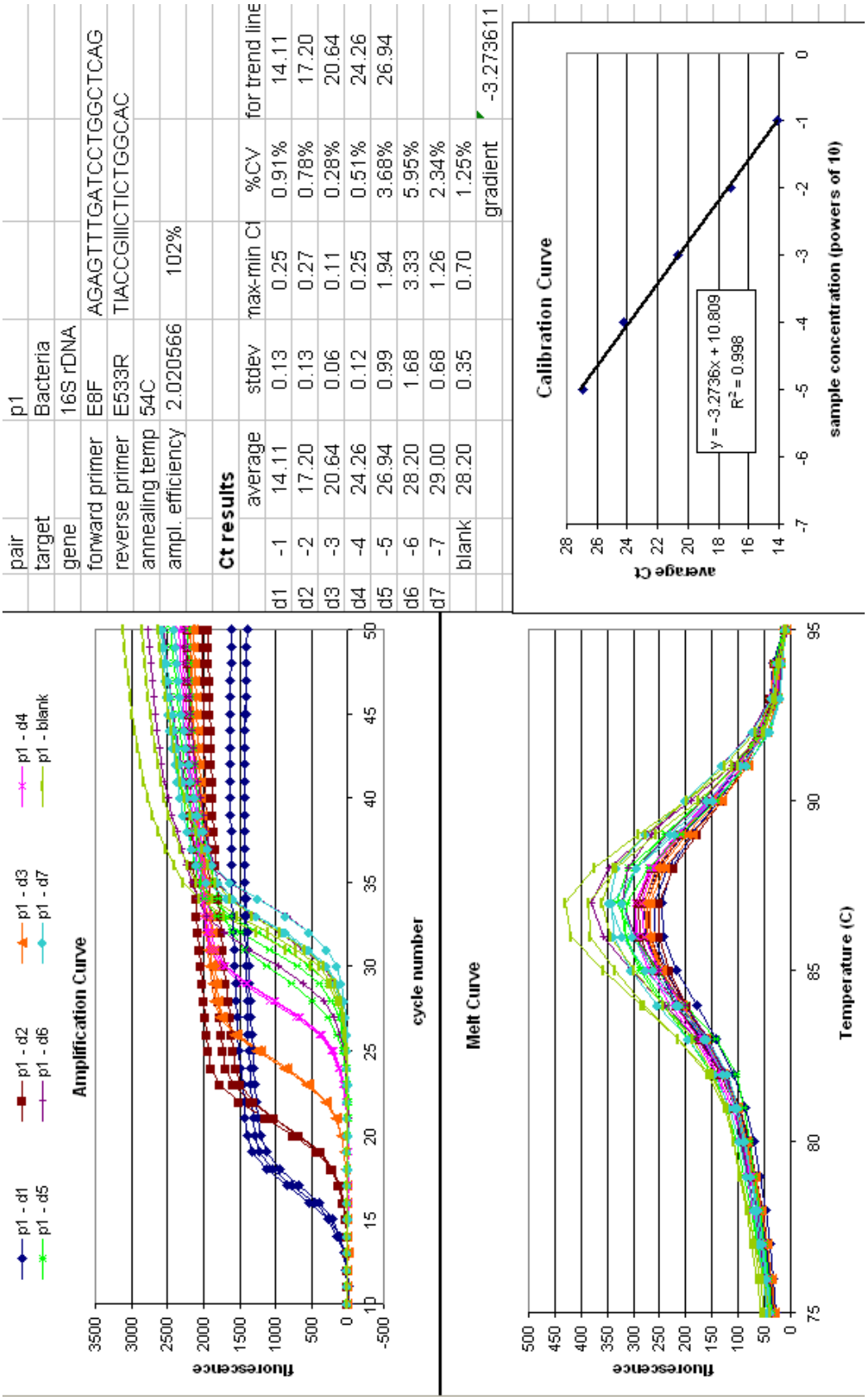
Sampling of the supraglacial meltwater stream, 1.2 km from the snout.

All images were taken with Nikon D80, except third and fifth Engabreen images, which were taken with Pentax Optio S7.

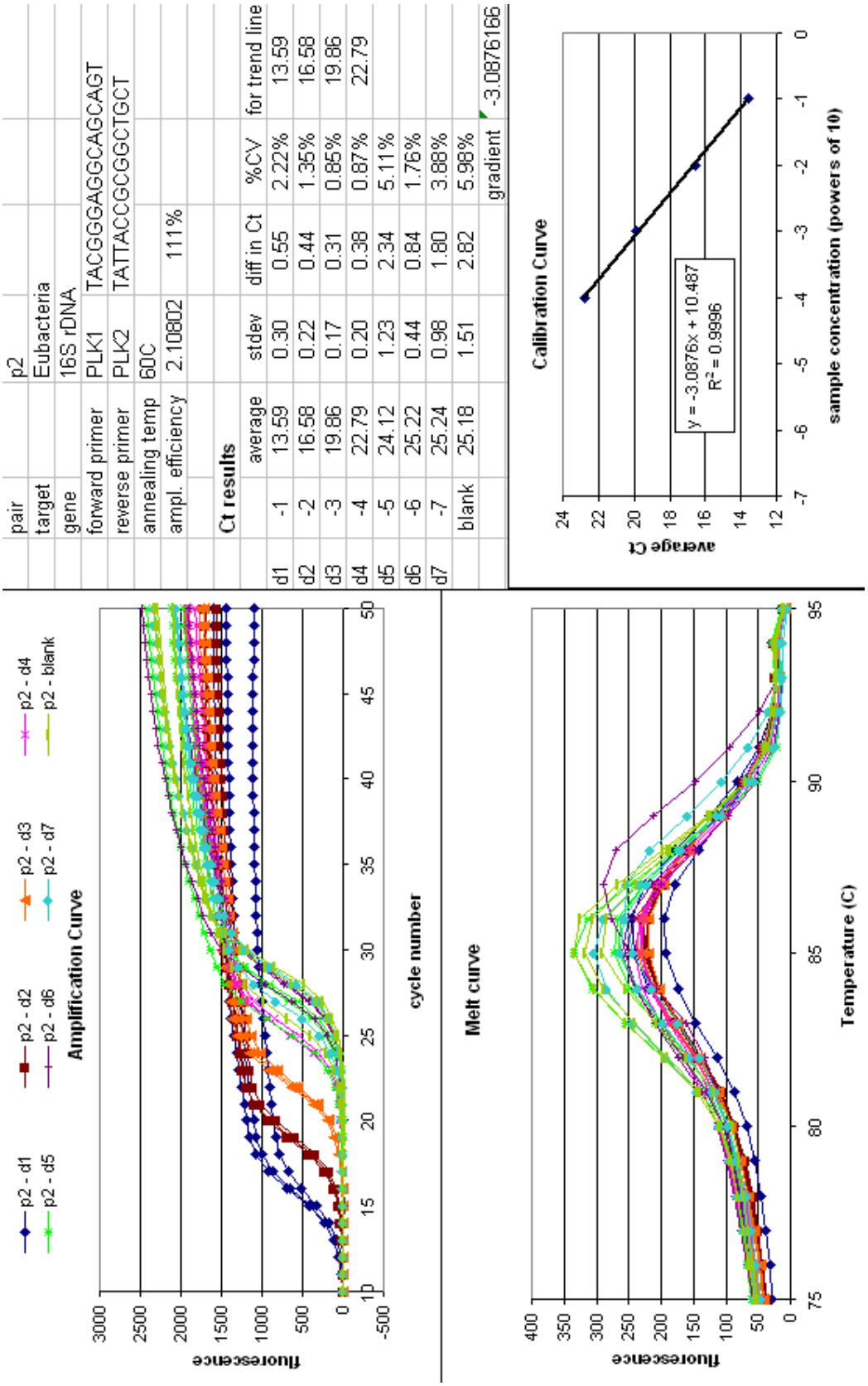
# Appendix B – Optimisation of Real-Time PCR Primer Conditions

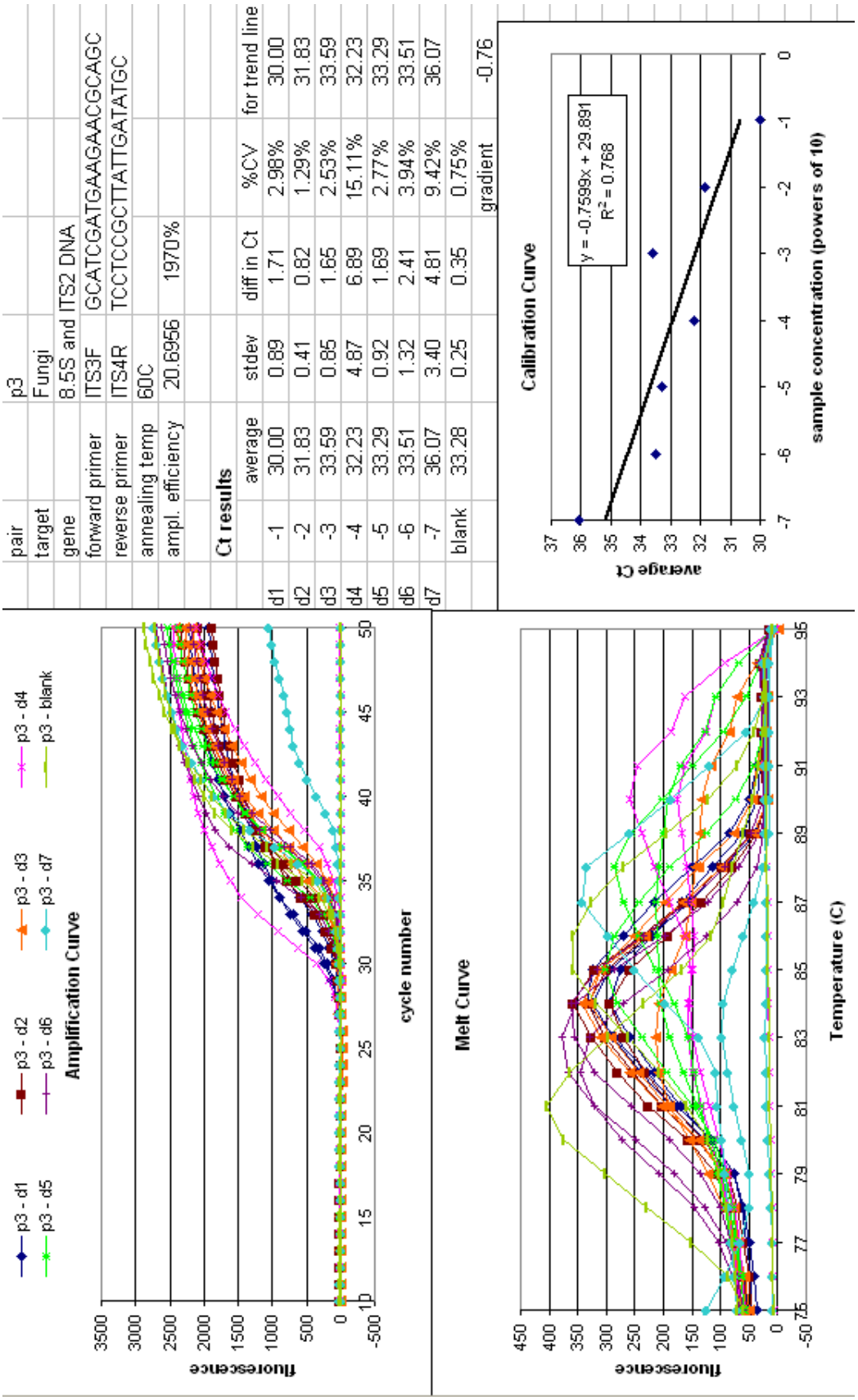
- To optimise the real-time PCR conditions, a template of DNA extracted from activated sludge was used.
- To select the annealing temperature a 500 mM of forward and reverse primer[†] was added to 100 and 10000 fold dilution of the DNA stock extraction with a temperature gradient from 50 to 62°C. The annealing temperature was then selected using a combination of lowest Ct values and lowest CV value from triplicates.
- Each primer pair was then amplified with $10^1$ to $10^7$ fold dilutions of stock solution and assayed in duplicate.
- The subsequent pages show the amplification curves, melt curves and calibration curves for the different target concentrations for each primer pair, as well as the following information:-
    - Target
    - Gene
    - Forward primer name and sequence
    - Reverse primer name and sequence
    - Annealing temperature
    - Amplification efficiency = $10^{(-1/\text{gradient of calibration curve})}-1$
- Ct values are where the amplification curves cross an arbitrary threshold, graphically a horizontal line. This line is as close to background level as possible, and in the initial exponential increase in fluorescence.
- Calibration curves for p4 (fungi) was conducted at two different temperatures.
- There is no p16.

[†] For p14, cyanobacteria the final forward / reverse primer concentration was 300 nM and 900 nM respectively and was selected to reduce the primer dimer formation

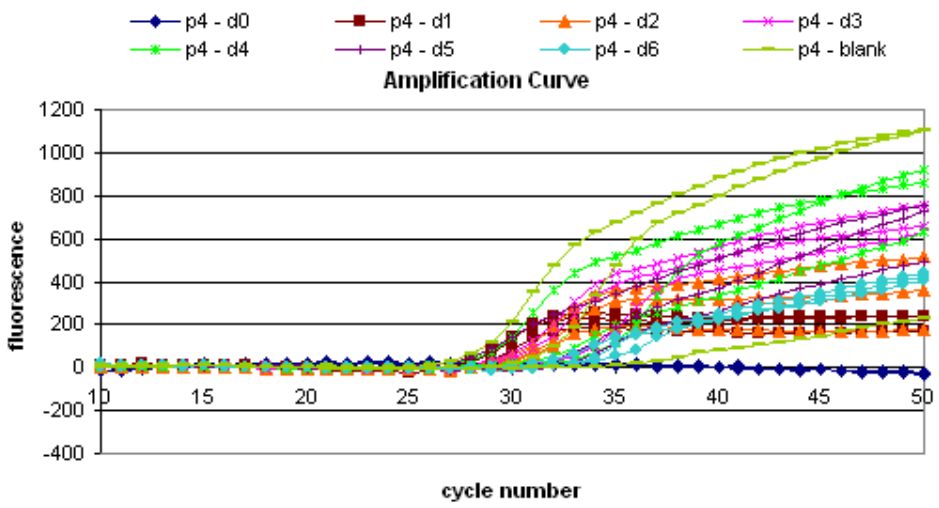


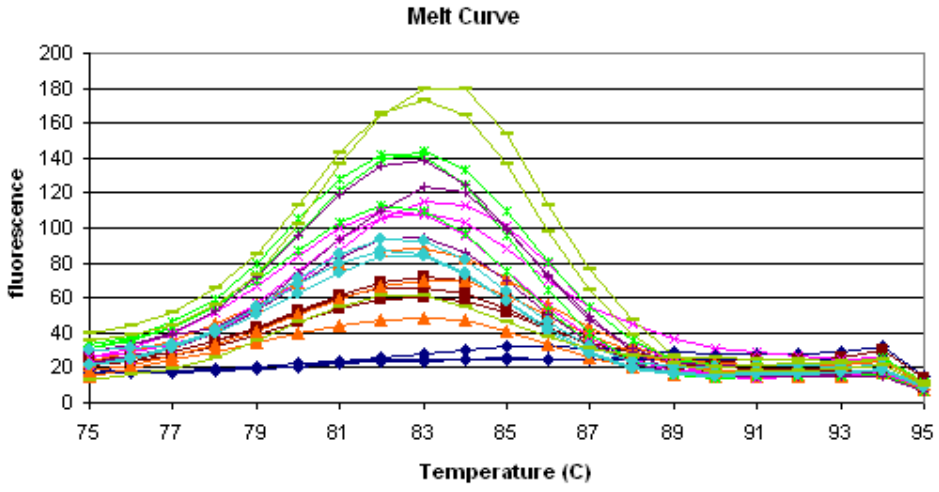

| | pair | | p1 | | | | |
|---|---|---|---|---|---|---|---|
| | target | | Bacteria | | | | |
| | gene | | 16S rDNA | | | | |
| | forward primer | | E8F | AGAGTTTGATCCTGGCTCAG | | | |
| | reverse primer | | E533R | TIACCGIIICTICTGGCAC | | | |
| | annealing temp | | 54C | | | | |
| | ampl. efficiency | | 2.020566 | 102% | | | |
| | **Ct results** | | | | | | |
| | | | average | stdev | max-min Ct | %CV | for trend line |
| d1 | -1 | | 14.11 | 0.13 | 0.25 | 0.91% | 14.11 |
| d2 | -2 | | 17.20 | 0.13 | 0.27 | 0.78% | 17.20 |
| d3 | -3 | | 20.64 | 0.06 | 0.11 | 0.28% | 20.64 |
| d4 | -4 | | 24.26 | 0.12 | 0.25 | 0.51% | 24.26 |
| d5 | -5 | | 26.94 | 0.99 | 1.94 | 3.68% | 26.94 |
| d6 | -6 | | 28.20 | 1.68 | 3.33 | 5.95% | |
| d7 | -7 | | 29.00 | 0.68 | 1.26 | 2.34% | |
| | blank | | 28.20 | 0.35 | 0.70 | 1.25% | |
| | | | | | | gradient | -3.273611 |


pair p2
target Eubacteria
gene 16S rDNA
forward primer PLK1 TACGGGAGGCAGCAGT
reverse primer PLK2 TATTACCGCGGCTGCT
annealing temp 60C
ampl. efficiency 2.10802 111%
Ct results
average stdev diff in Ct %CV for trend line
d1 -1 13.59 0.30 0.55 2.22% 13.59
d2 -2 16.58 0.22 0.44 1.35% 16.58
d3 -3 19.86 0.17 0.31 0.85% 19.86
d4 -4 22.79 0.20 0.38 0.87% 22.79
d5 -5 24.12 1.23 2.34 5.11%
d6 -6 25.22 0.44 0.84 1.76%
d7 -7 25.24 0.98 1.80 3.88%
blank 25.18 1.51 2.82 5.98%
gradient -3.0876166
Calibration Curve
y = -3.0876x + 10.487
R² = 0.9996
average Ct
sample concentration (powers of 10)
Amplification Curve
p2 - d1
p2 - d2
p2 - d3
p2 - d4
p2 - d5
p2 - d6
p2 - d7
p2 - blank
fluorescence
cycle number
Melt curve
fluorescence
Temperature (C)

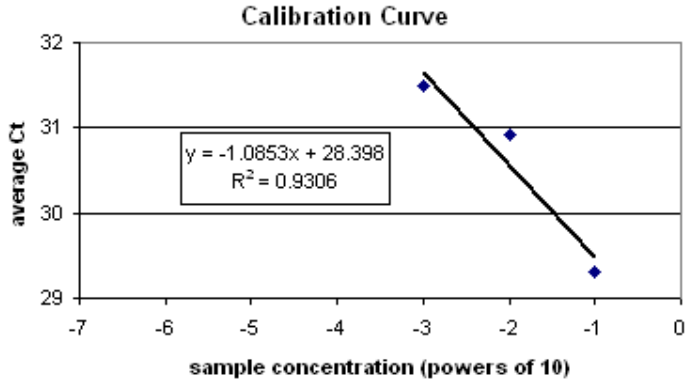


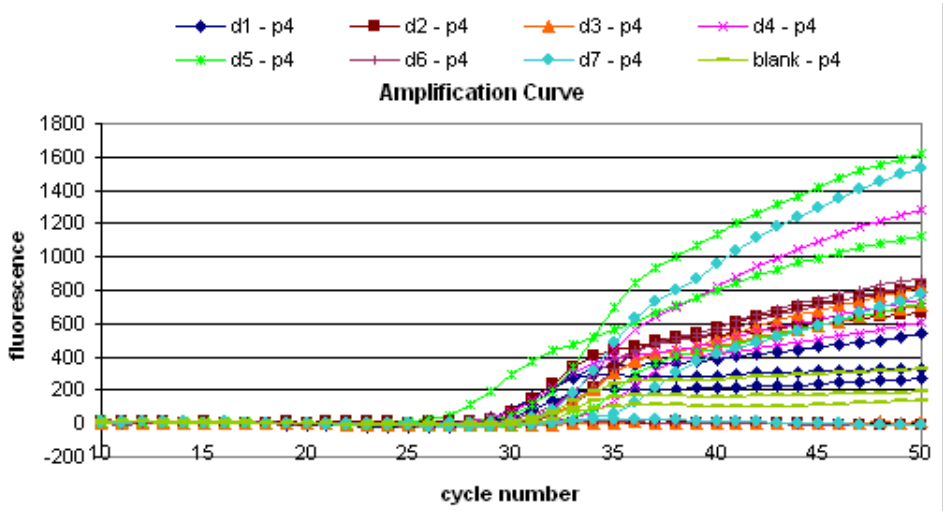

| | pair | | p3 | | | | |
|---|---|---|---|---|---|---|---|
| | target | | Fungi | | | | |
| | gene | | 8.5S and ITS2 DNA | | | | |
| | forward primer | | ITS3F | GCATCGATGAAGAACGCAGC | | | |
| | reverse primer | | ITS4R | TCCTCCGCTTATTGATATGC | | | |
| | annealing temp | | 60C | | | | |
| | ampl. efficiency | | 20.6956 | 1970% | | | |
| | **Ct results** | | | | | | |
| | | | average | stdev | diff in Ct | %CV | for trend line |
| d1 | | -1 | 30.00 | 0.89 | 1.71 | 2.98% | 30.00 |
| d2 | | -2 | 31.83 | 0.41 | 0.82 | 1.29% | 31.83 |
| d3 | | -3 | 33.59 | 0.85 | 1.65 | 2.53% | 33.59 |
| d4 | | -4 | 32.23 | 4.87 | 6.89 | 15.11% | 32.23 |
| d5 | | -5 | 33.29 | 0.92 | 1.69 | 2.77% | 33.29 |
| d6 | | -6 | 33.51 | 1.32 | 2.41 | 3.94% | 33.51 |
| d7 | | -7 | 36.07 | 3.40 | 4.81 | 9.42% | 36.07 |
| | | blank | 33.28 | 0.25 | 0.35 | 0.75% | |
| | | | | | | gradient | -0.76 |

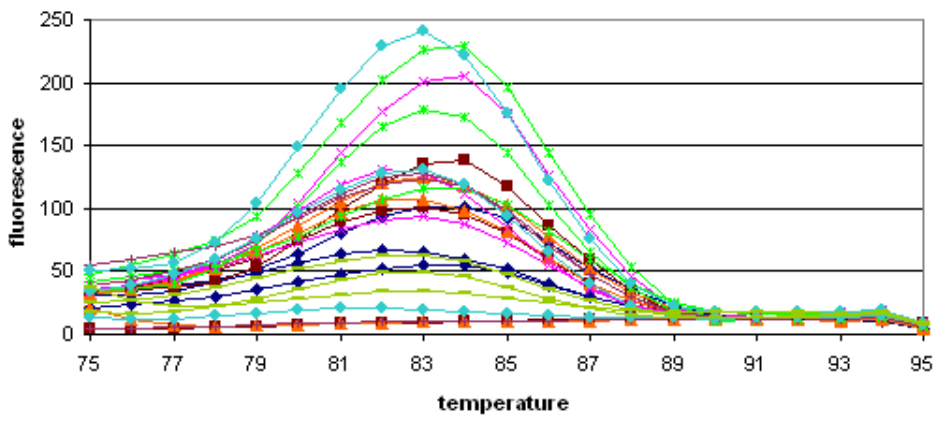


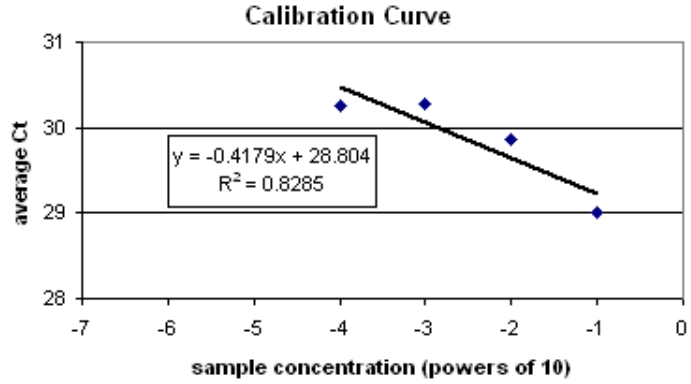

| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p4 | | | |
| | target | | Fungi | | | |
| | gene | | 18S rDNA | | | |
| | forward primer | | nu-SSU-0817 | TTAGCATGGAATRRAATAGGA | | |
| | reverse primer | | nu-SSU-1196 | TCTGGACCTGGTGAGTTTCC | | |
| | annealing temp | | 51C | | | |
| | ampl. efficiency | | 8.345262845 | 735% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d0 | 0 | #DIV/0! | #DIV/0! | 0.00 | #DIV/0! | |
| d1 | -1 | 29.31 | 0.43 | 0.84 | 1.48% | 29.31 |
| d2 | -2 | 30.91 | 0.52 | 0.99 | 1.67% | 30.91 |
| d3 | -3 | 31.48 | 1.75 | 3.24 | 5.55% | 31.48 |
| d4 | -4 | 31.85 | 2.63 | 5.13 | 8.25% | |
| d5 | -5 | 32.01 | 2.93 | 5.52 | 9.15% | |
| d6 | -6 | 34.44 | 1.25 | 2.51 | 3.64% | |
| | blank | 33.13 | 5.84 | 11.34 | 17.63% | |
| | | | | | gradient | -1.0852578 |

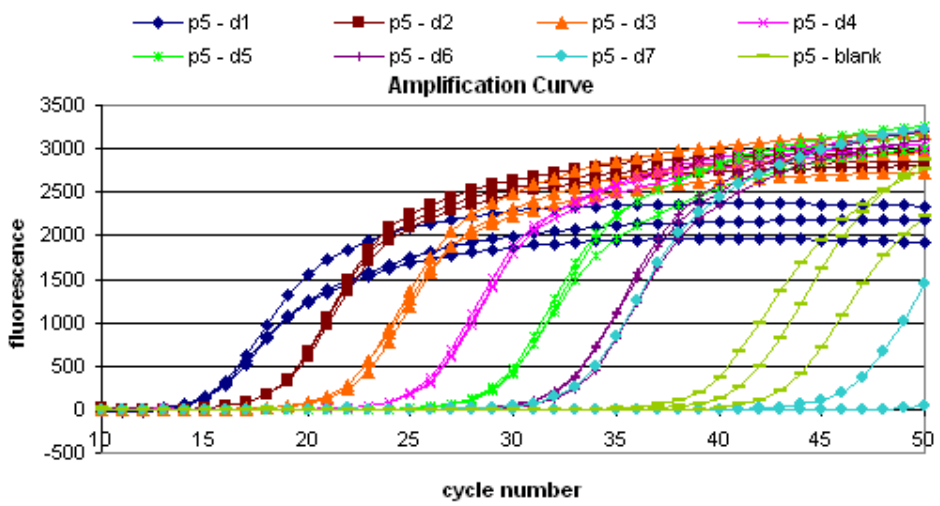


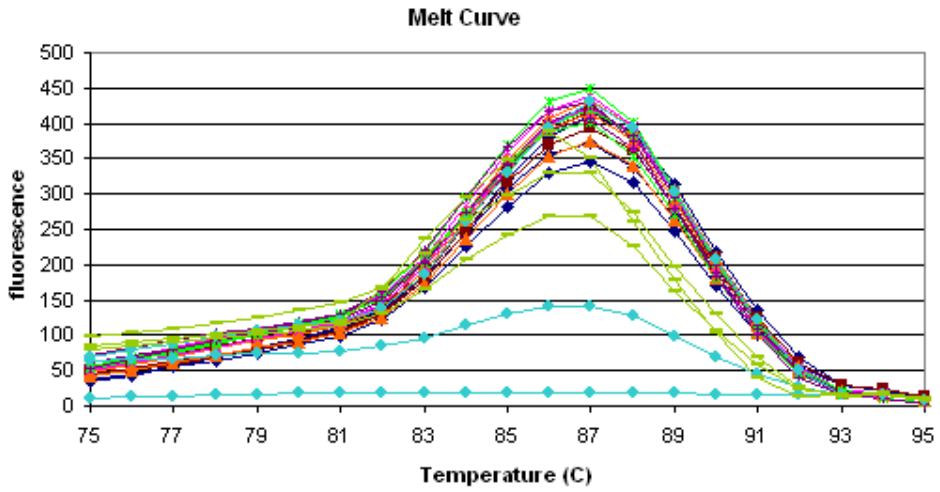


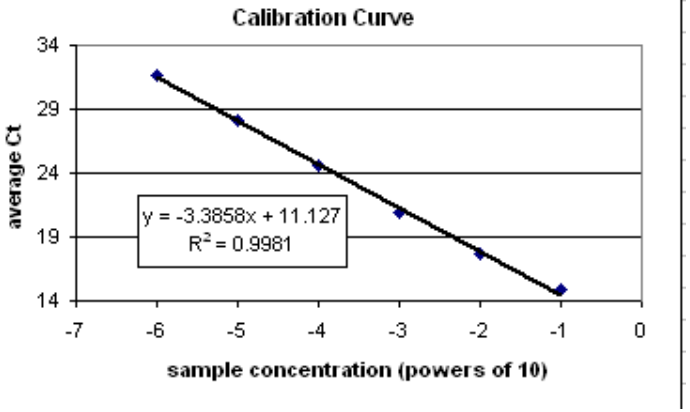

| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p4 | | | |
| | target | | Fungi | | | |
| | gene | | 18S rDNA | | | |
| | forward primer | | nu-SSU-08 | TTAGCATGGAATRRAATAGGA | | |
| | reverse primer | | nu-SSU-11 | TCTGGACCTGGTGAGTTTCC | | |
| | annealing temp | | 54C | | | |
| | ampl. efficiency | | 247.2576 | 24626% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | or trend line |
| d1 | -1 | 29.01 | 0.45 | 0.78 | 1.54% | 29.01 |
| d2 | -2 | 29.85 | 1.47 | 2.08 | 4.92% | 29.85 |
| d3 | -3 | 30.27 | 0.03 | 0.05 | 0.11% | 30.27 |
| d4 | -4 | 30.26 | 1.93 | 3.86 | 6.38% | 30.26 |
| d5 | -5 | 28.94 | 3.48 | 6.96 | 12.04% | |
| d6 | -6 | 30.66 | 0.92 | 1.30 | 2.99% | |
| d7 | -7 | 32.45 | 1.68 | 2.93 | 5.17% | |
| blank | | 30.93 | 0.70 | 1.21 | 2.25% | |
| | | | | | | -0.42 |

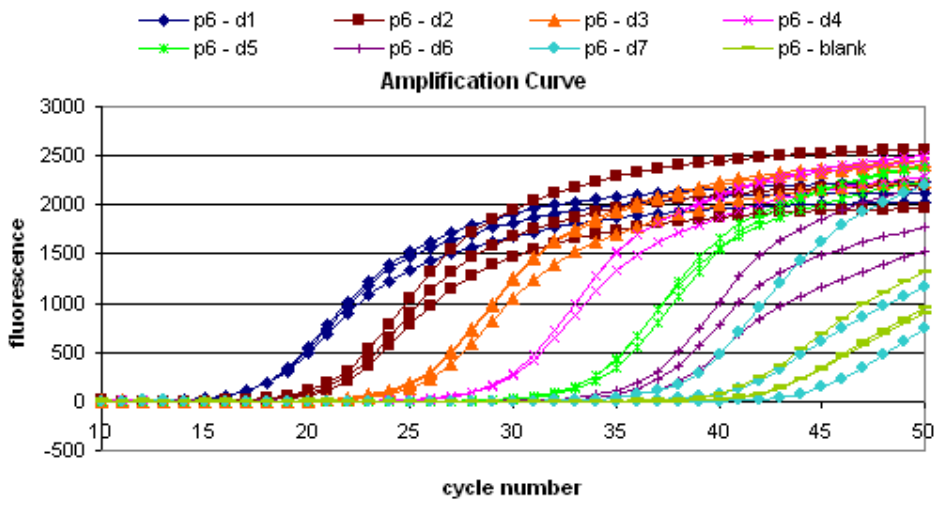


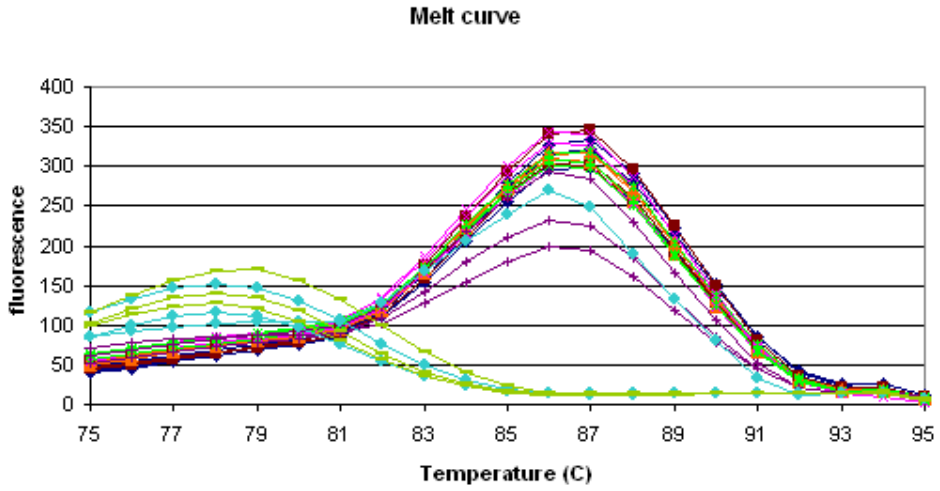


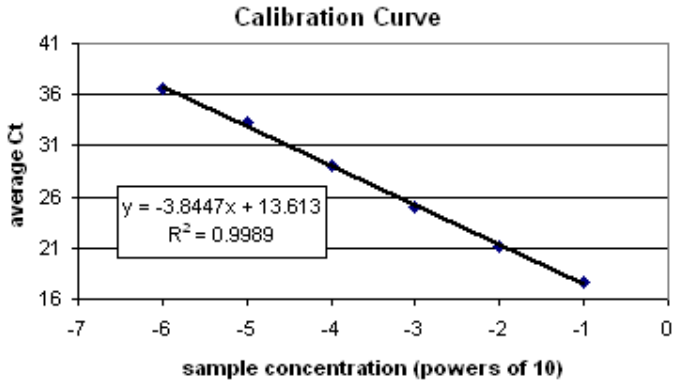

| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p5 | | | |
| | target | | Archaea | | | |
| | gene | | 16S rDNA | | | |
| | forward primer | | ARC787F | ATTAGATACCCSBGTAGTCC | | |
| | reverse primer | | ARC1059R | GCCATGCACCWCCTCT | | |
| | annealing temp | | 54C | | | |
| | ampl. efficiency | | 1.974014 | 97% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | or trend line |
| d1 | -1 | 14.93 | 0.10 | 0.20 | 0.70% | 14.93 |
| d2 | -2 | 17.62 | 0.08 | 0.16 | 0.46% | 17.62 |
| d3 | -3 | 20.97 | 0.16 | 0.28 | 0.75% | 20.97 |
| d4 | -4 | 24.60 | 0.11 | 0.21 | 0.46% | 24.60 |
| d5 | -5 | 28.15 | 0.09 | 0.19 | 0.33% | 28.15 |
| d6 | -6 | 31.59 | 0.44 | 0.82 | 1.40% | 31.59 |
| d7 | -7 | 38.67 | 9.45 | 13.37 | 24.45% | |
| | blank | 40.18 | 1.93 | 3.84 | 4.80% | |
| | | | | | gradient | -3.38581 |






| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p6 | | | |
| | target | | Archaea | | | |
| | gene | | 16s rDNA | | | |
| | forward primer | | ARCH349F | GYGCASCAGKCGMGAAW | | |
| | reverse primer | | ARCH806R | GGACTACVSGGGTATCTAAT | | |
| | annealing temp | | 60C | | | |
| | ampl. efficiency | | 1.8201049 | 82% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d1 | -1 | 17.67 | 0.05 | 0.09 | 0.30% | 17.67 |
| d2 | -2 | 21.11 | 0.36 | 0.72 | 1.71% | 21.11 |
| d3 | -3 | 24.92 | 0.32 | 0.61 | 1.28% | 24.92 |
| d4 | -4 | 29.00 | 0.12 | 0.24 | 0.41% | 29.00 |
| d5 | -5 | 33.20 | 0.21 | 0.39 | 0.63% | 33.20 |
| d6 | -6 | 36.51 | 0.91 | 1.79 | 2.50% | 36.51 |
| d7 | -7 | 41.51 | 3.68 | 7.35 | 8.85% | |
| | blank | 42.61 | 1.22 | 2.17 | 2.86% | |
| | | | | | gradient | -3.8447282 |




N

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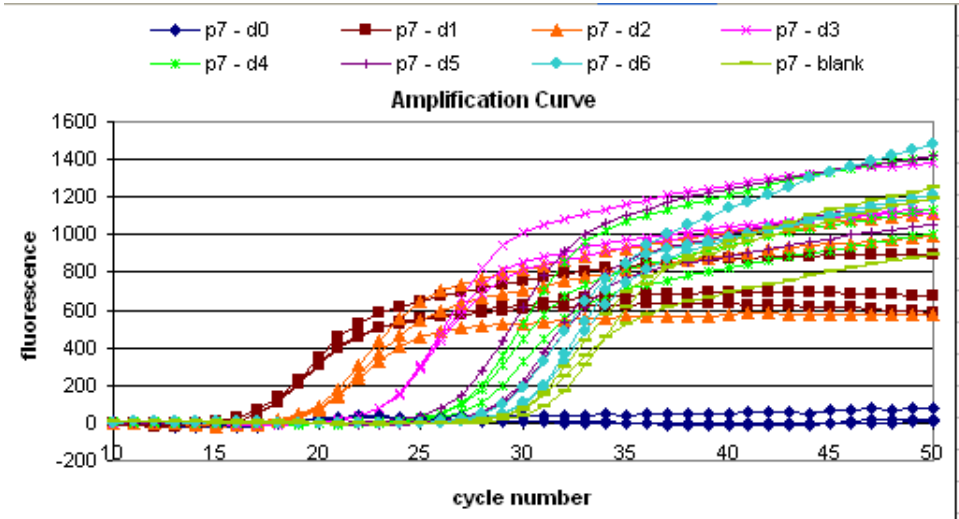
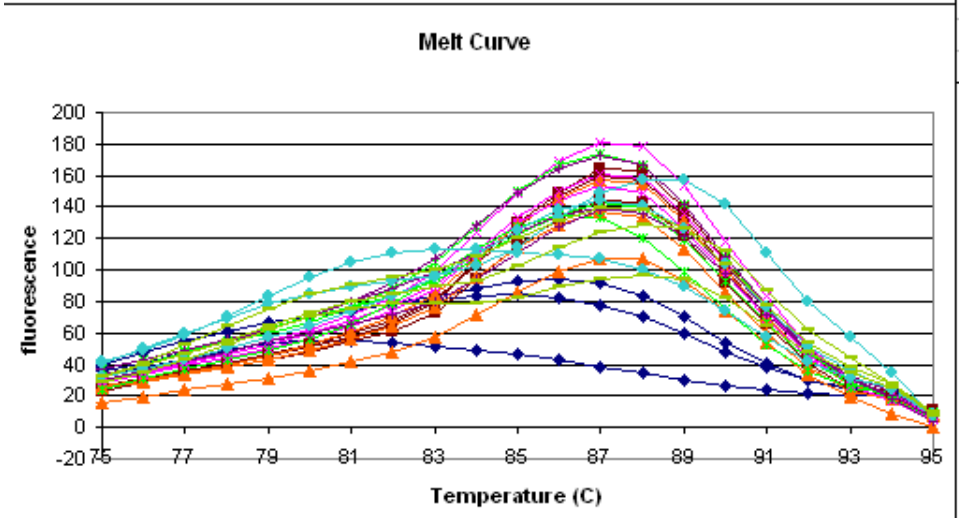
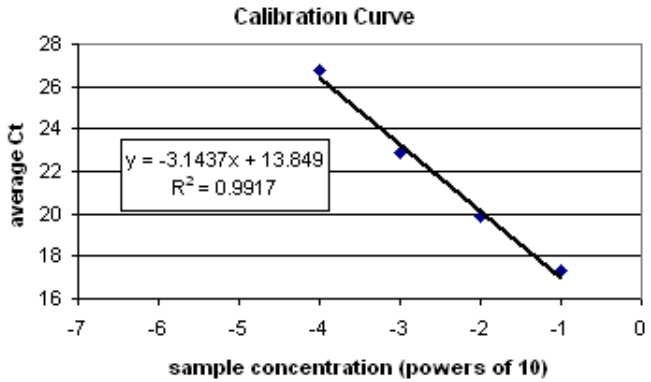
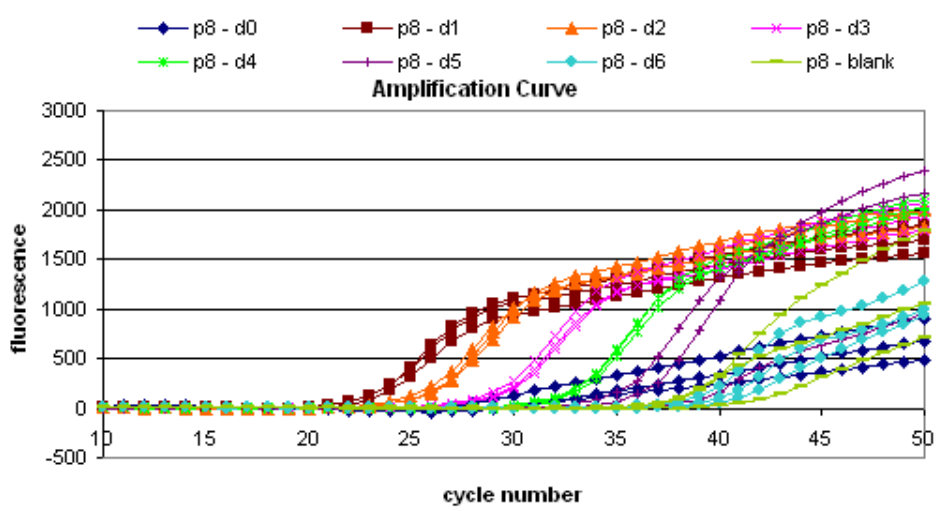


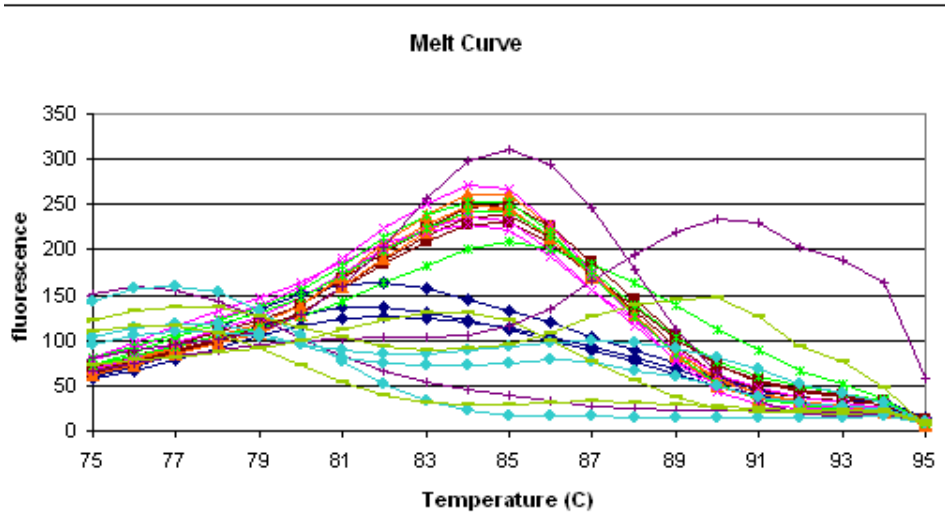



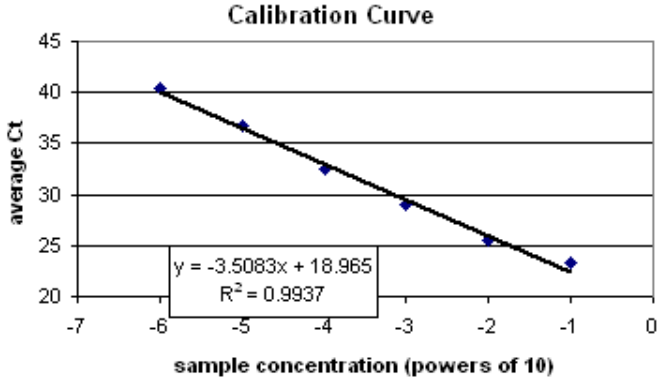

| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p7 | | | |
| | target | | Sulfate-reducing prokaryote | | | |
| | gene | | drsA | | | |
| | forward primer | | DSR1-F+ | ACSCACTGGAAGCACGGCGG | | |
| | reverse primer | | DSR-R | GTGGMRCCGTGCAKRTTGG | | |
| | annealing temp | | 51C | | | |
| | ampl. efficiency | | 2.080163 | 108% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d0 | 0 | 48.48 | #DIV/0! | 0.00 | #DIV/0! | |
| d1 | -1 | 17.30 | 0.18 | 0.33 | 1.01% | 17.30 |
| d2 | -2 | 19.87 | 0.18 | 0.36 | 0.93% | 19.87 |
| d3 | -3 | 22.91 | 0.07 | 0.12 | 0.29% | 22.91 |
| d4 | -4 | 26.76 | 0.45 | 0.85 | 1.67% | 26.76 |
| d5 | -5 | 27.55 | 1.41 | 2.55 | 5.10% | |
| d6 | -6 | 29.13 | 0.59 | 1.07 | 2.02% | |
| | blank | 30.22 | 0.50 | 0.94 | 1.66% | |
| | | | | | gradient | -3.143692 |






| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p8 | | | |
| | target | | Denitrifier | | | |
| | gene | | nirK | | | |
| | forward primer | | nirK 1F | GGMATGGTKCCSTGGCA | | |
| | reverse primer | | nirK 5R | GCCTCGATCAGRTTRTGGTT | | |
| | annealing temp | | 54C | | | |
| | ampl. efficiency | | 1.92768 | 93% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d0 | 0 | 32.93 | 2.47 | 4.73 | 7.49% | |
| d1 | -1 | 23.22 | 0.26 | 0.49 | 1.13% | 23.22 |
| d2 | -2 | 25.54 | 0.31 | 0.61 | 1.21% | 25.54 |
| d3 | -3 | 29.08 | 0.28 | 0.54 | 0.96% | 29.08 |
| d4 | -4 | 32.50 | 0.14 | 0.29 | 0.44% | 32.50 |
| d5 | -5 | 36.76 | 2.54 | 4.91 | 6.92% | 36.76 |
| d6 | -6 | 40.36 | 1.20 | 2.40 | 2.97% | 40.36 |
| | blank | 39.93 | 2.59 | 4.57 | 6.49% | |
| | | | | | gradient | -3.51 |




P

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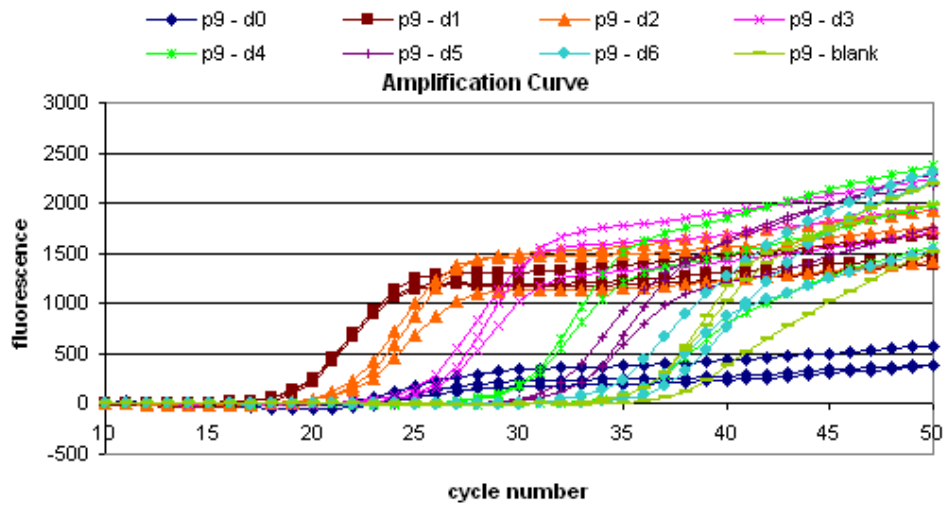
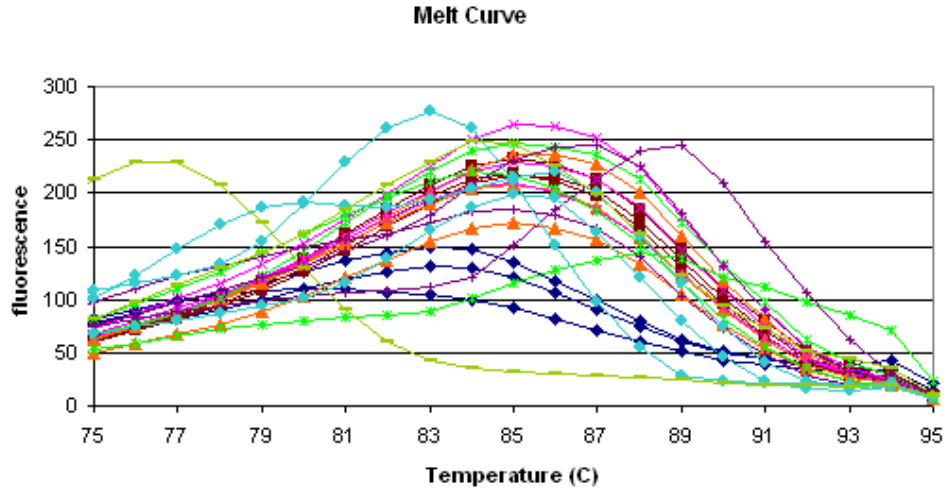
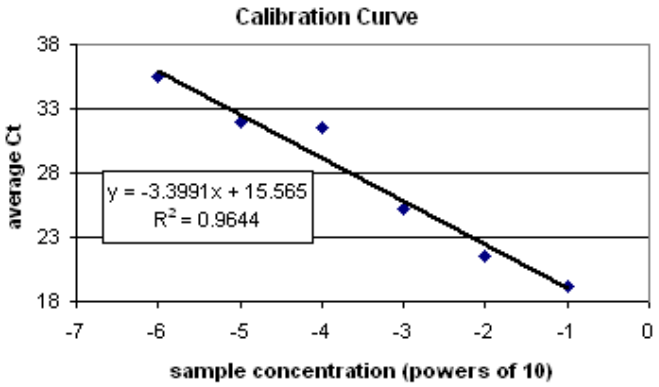
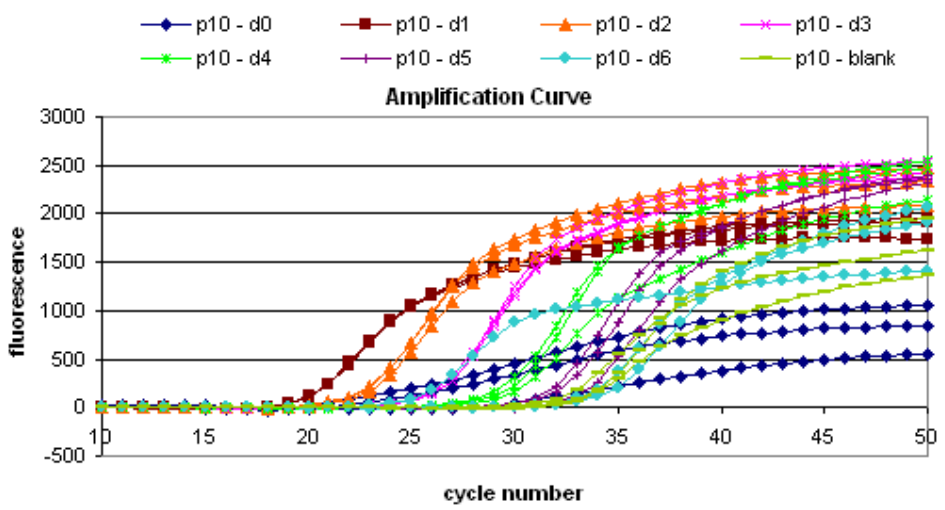


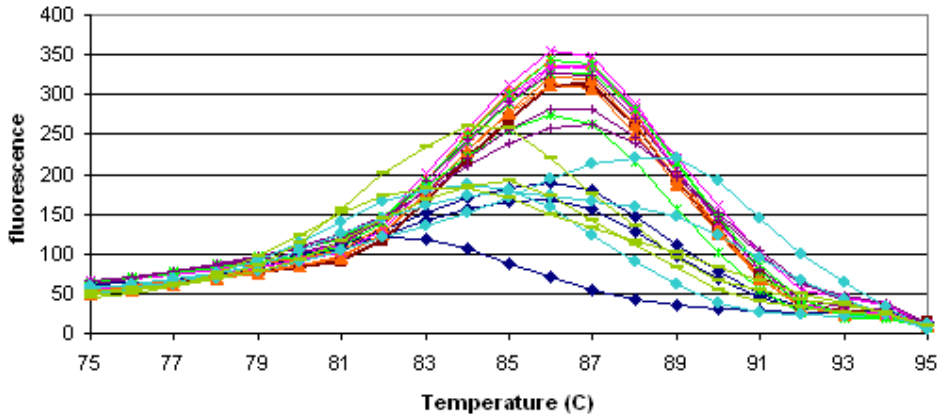



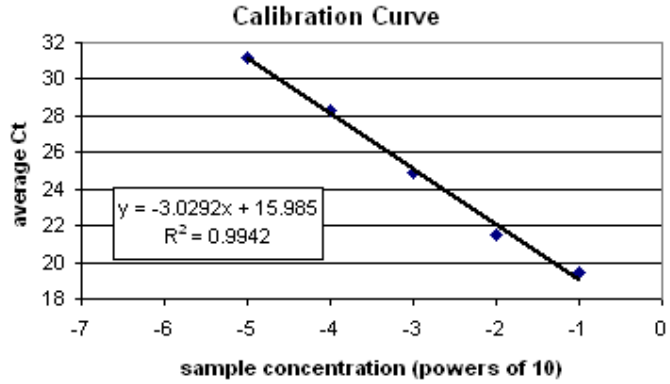

| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p9 | | | |
| | target | | Methanotroph | | | |
| | gene | | pmoA | | | |
| | forward primer | | A189 F | GGNGACTGGGACTTCTGG | | |
| | reverse primer | | Mb661 R | GGTAARGACGTTGCNCCGG | | |
| | annealing temp | | 54C | | | |
| | ampl. efficiency | | 1.96876 | 97% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d0 | 0 | 26.00 | 1.56 | 3.12 | 6.00% | |
| d1 | -1 | 19.14 | 0.20 | 0.39 | 1.07% | 19.14 |
| d2 | -2 | 21.52 | 0.34 | 0.68 | 1.59% | 21.52 |
| d3 | -3 | 25.22 | 0.50 | 1.00 | 2.00% | 25.22 |
| d4 | -4 | 31.55 | 3.56 | 6.27 | 11.29% | 31.55 |
| d5 | -5 | 31.91 | 0.54 | 0.96 | 1.70% | 31.91 |
| d6 | -6 | 35.43 | 1.31 | 2.56 | 3.69% | 35.43 |
| | blank | 36.52 | 1.44 | 2.55 | 3.93% | |
| | | | | | gradient | -3.40 |






| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p10 | | | |
| | target | | Aerobic methane consumption | | | |
| | gene | | mxaF | | | |
| | forward primer | | mxa1003f | GCGGCACCAACTGGGGCTGGT | | |
| | reverse primer | | mxa1561r | GGGCAGCATGAAGGGCTCCC | | |
| | annealing temp | | 51C | | | |
| | ampl. efficiency | | 2.138553 | 114% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d0 | 0 | 25.45 | 4.77 | 8.89 | 18.74% | |
| d1 | -1 | 19.44 | 0.04 | 0.08 | 0.23% | 19.44 |
| d2 | -2 | 21.50 | 0.23 | 0.45 | 1.08% | 21.50 |
| d3 | -3 | 24.93 | 0.12 | 0.22 | 0.47% | 24.93 |
| d4 | -4 | 28.30 | 0.42 | 0.84 | 1.48% | 28.30 |
| d5 | -5 | 31.19 | 0.95 | 1.83 | 3.04% | 31.19 |
| d6 | -6 | 30.15 | 4.78 | 8.64 | 15.84% | |
| | blank | 32.55 | 0.70 | 1.28 | 2.16% | |
| | | | | | gradient | -3.0292012 |




R

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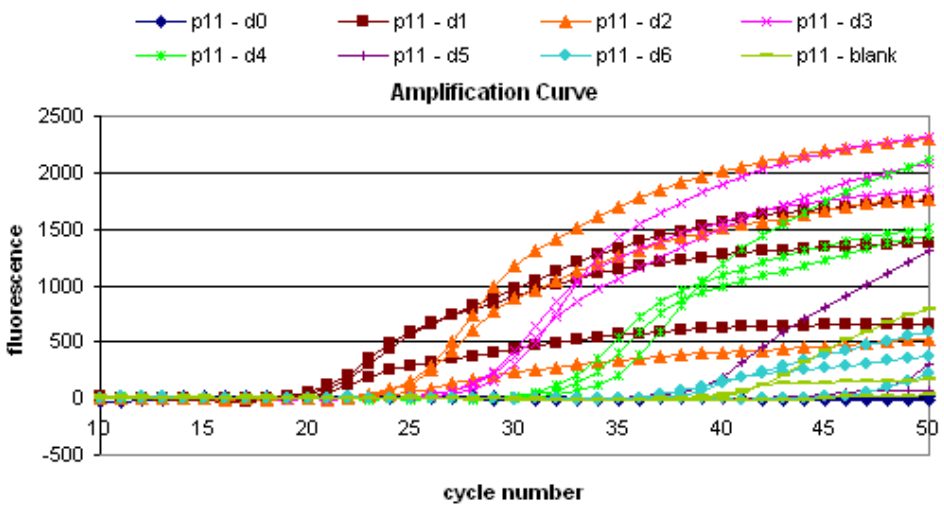
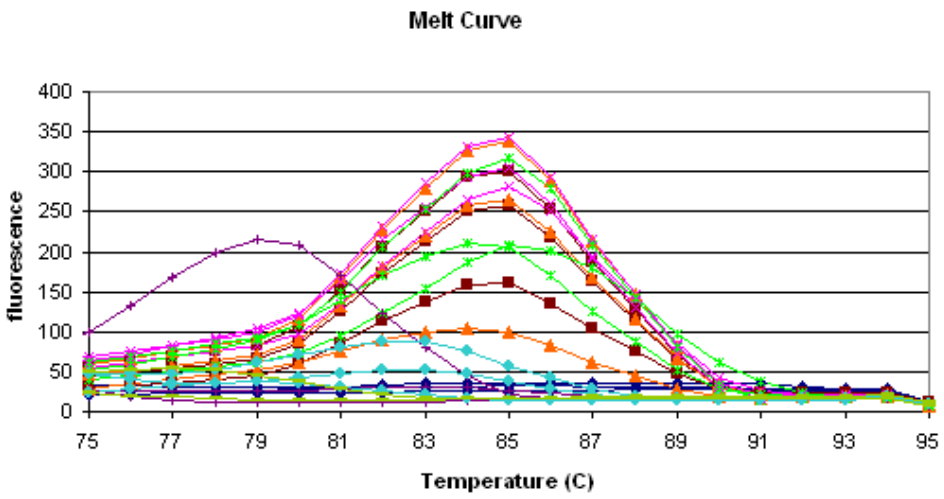
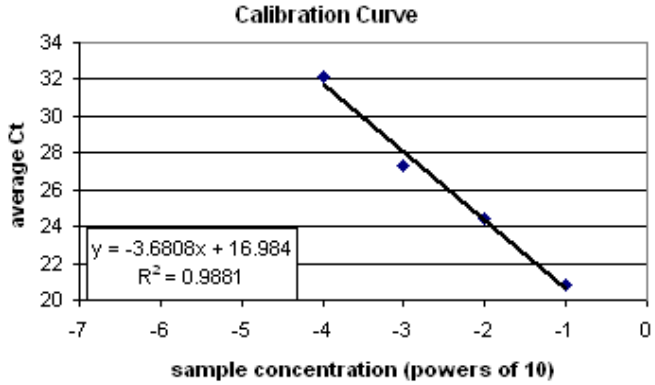
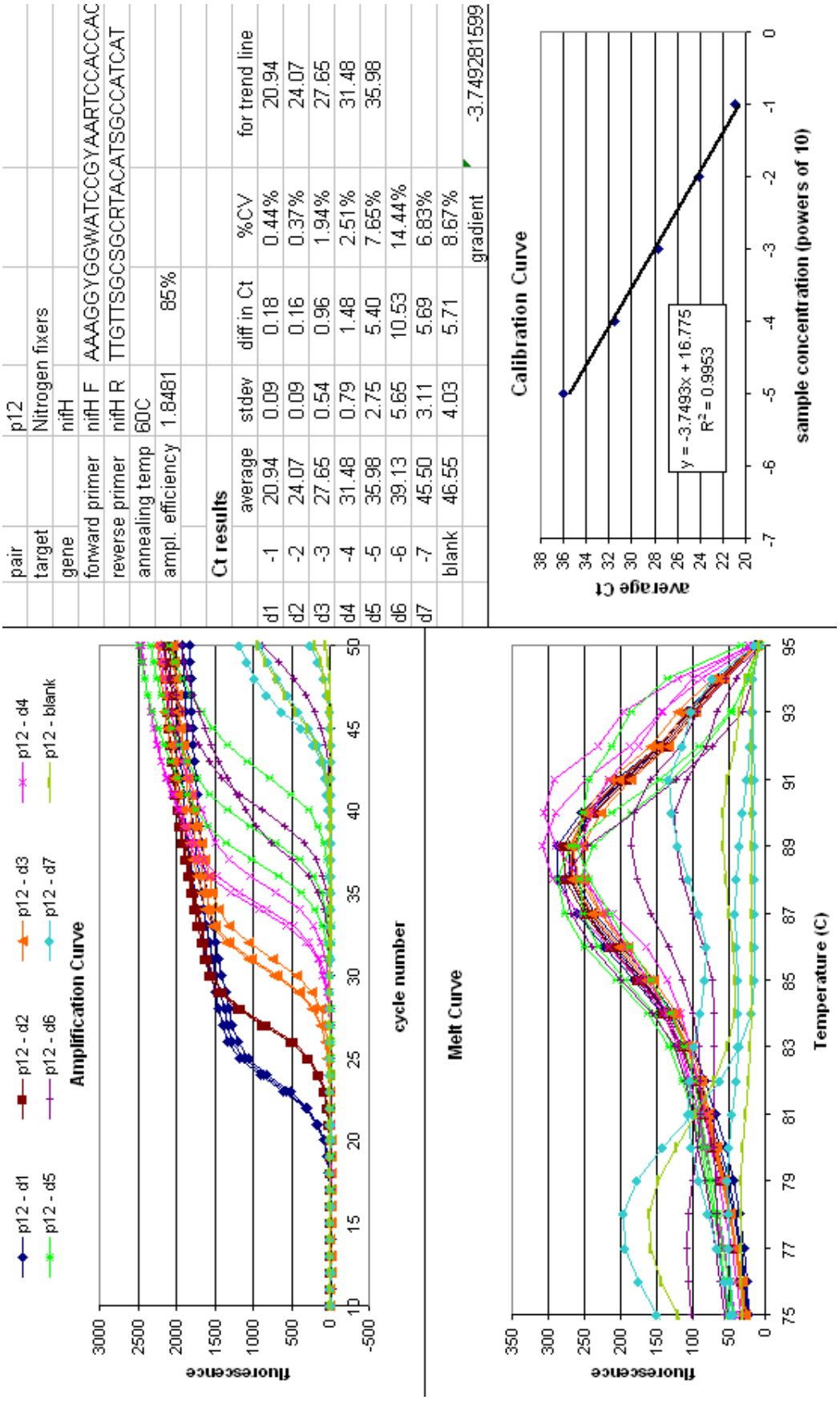
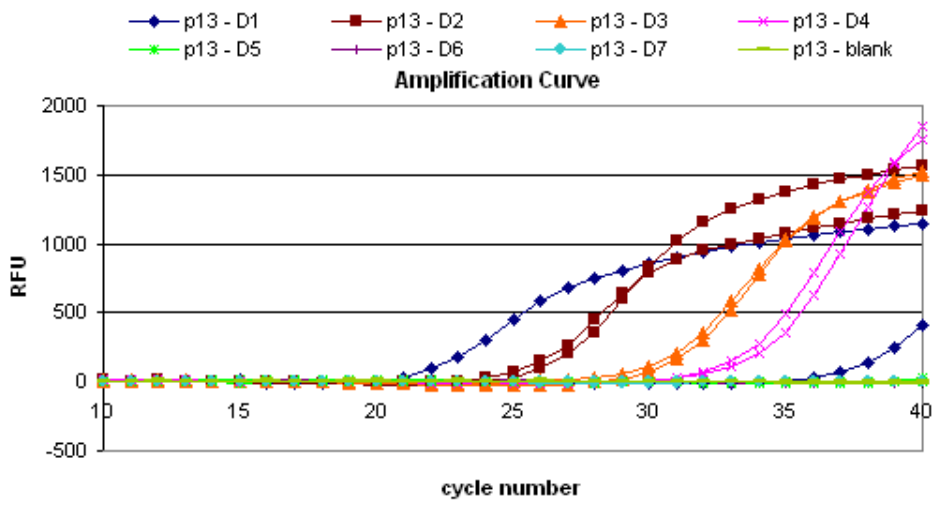


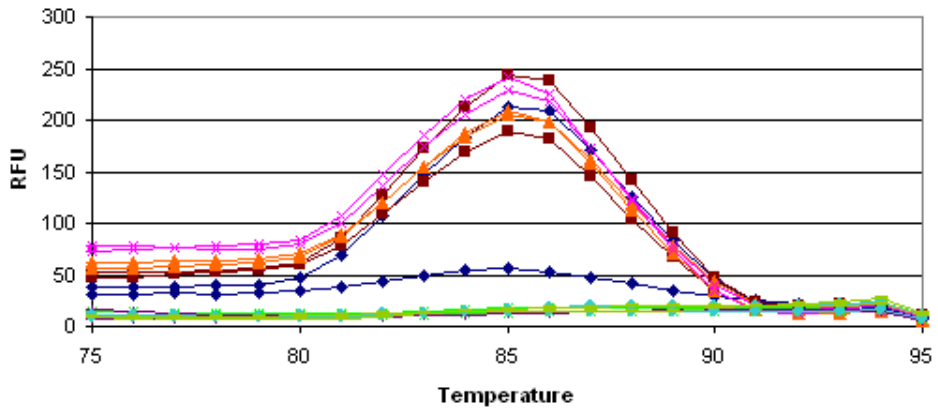

| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p11 | | | |
| | target | | Methanogen | | | |
| | gene | | mcrA | | | |
| | forward primer | | ME1f | CGMATGCARATHGGWATGTC | | |
| | reverse primer | | ME2r | TCATKGCRTAGTTDGGRTAGT | | |
| | annealing temp | | 51C | | | |
| | ampl. efficiency | | 1.86929 | 87% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d0 | 0 | #DIV/0! | #DIV/0! | 0.00 | #DIV/0! | |
| d1 | -1 | 20.83 | 0.49 | 0.94 | 2.36% | 20.83 |
| d2 | -2 | 24.47 | 0.92 | 1.66 | 3.78% | 24.47 |
| d3 | -3 | 27.28 | 0.32 | 0.56 | 1.18% | 27.28 |
| d4 | -4 | 32.16 | 0.88 | 1.76 | 2.75% | 32.16 |
| d5 | -5 | 42.98 | 6.42 | 9.08 | 14.93% | |
| d6 | -6 | 41.62 | 5.12 | 9.28 | 12.31% | |
| | blank | 41.13 | 0.17 | 0.24 | 0.41% | |
| | | | | | gradient | -3.6808368 |



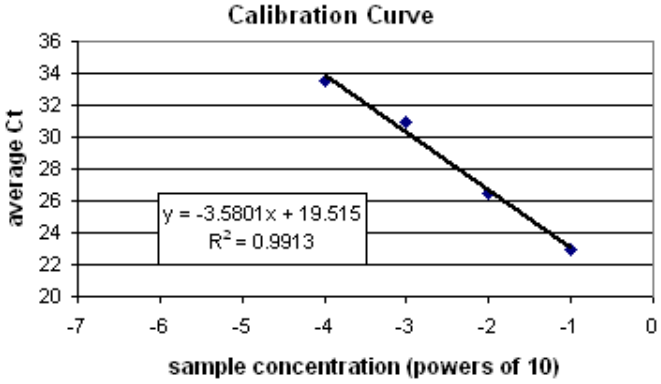



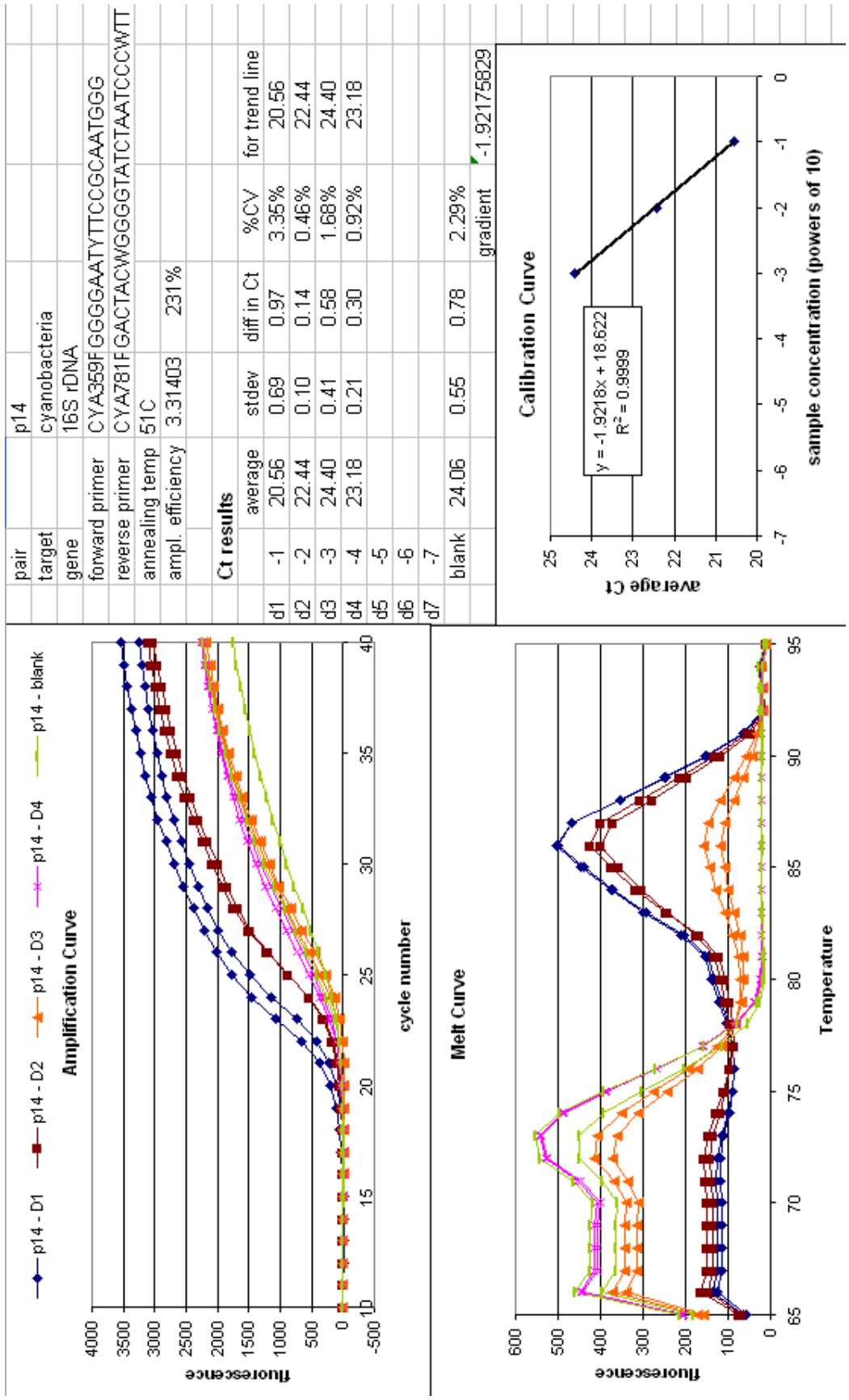

pair p12
target Nitrogen fixers
gene nifH
forward primer nifH F AAAGGYGGWATCCGYAARTCCACCAC
reverse primer nifH R TTGTTSGCSGCRTACATSGCCATCAT
annealing temp 60C
ampl. efficiency 1.8481 85%
Ct results
average stdev diff in Ct %CV for trend line
d1 -1 20.94 0.09 0.18 0.44% 20.94
d2 -2 24.07 0.09 0.16 0.37% 24.07
d3 -3 27.65 0.54 0.96 1.94% 27.65
d4 -4 31.48 0.79 1.48 2.51% 31.48
d5 -5 35.98 2.75 5.40 7.65% 35.98
d6 -6 39.13 5.65 10.53 14.44%
d7 -7 45.50 3.11 5.69 6.83%
blank 46.55 4.03 5.71 8.67%
gradient -3.749281599
Calibration Curve
y = -3.7493x + 16.775
R² = 0.9953
average Ct
sample concentration (powers of 10)
p12 - d1
p12 - d2
p12 - d3
p12 - d4
p12 - d5
p12 - d6
p12 - d7
p12 - blank
Amplification Curve
fluorescence
cycle number
Melt Curve
fluorescence
Temperature (C)

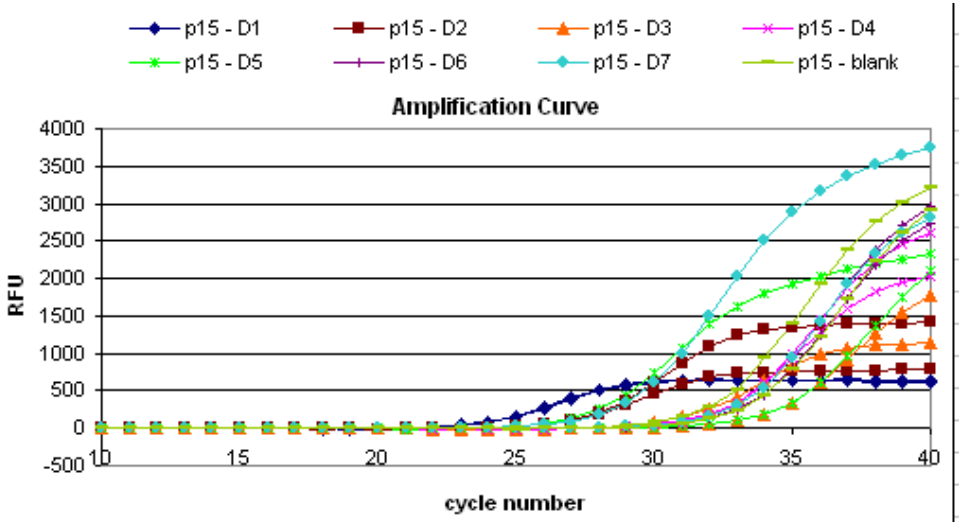


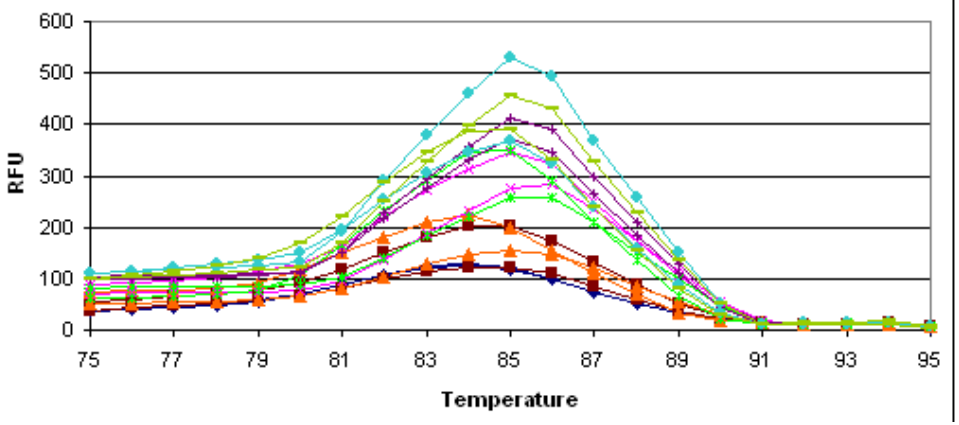




| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p13 | | | |
| | target | | Geobacteracea | | | |
| | gene | | 16S rDNA | | | |
| | forward primer | | Geo564F | AAGCGTTGTTCGGAWTTAT | | |
| | reverse primer | | Geo840R | ACTGCAGGGGTCAATA | | |
| | annealing temp | | 51C | | | |
| | ampl. efficiency | | 1.90248 | 90% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d1 | -1 | 23.00 | 10.86 | 15.36 | 47.24% | 23.00 |
| d2 | -2 | 26.50 | 0.38 | 0.54 | 1.43% | 26.50 |
| d3 | -3 | 30.88 | 0.29 | 0.41 | 0.94% | 30.88 |
| d4 | -4 | 33.47 | 0.36 | 0.50 | 1.06% | 33.47 |
| d5 | -5 | #DIV/0! | 0.00 | 0.00 | #DIV/0! | #DIV/0! |
| d6 | -6 | #DIV/0! | 0.00 | 0.00 | #DIV/0! | |
| d7 | -7 | #DIV/0! | 0.00 | 0.00 | #DIV/0! | |
| | blank | #DIV/0! | 0.00 | 0.00 | #DIV/0! | |
| | | | | | gradient | -3.58013141 |

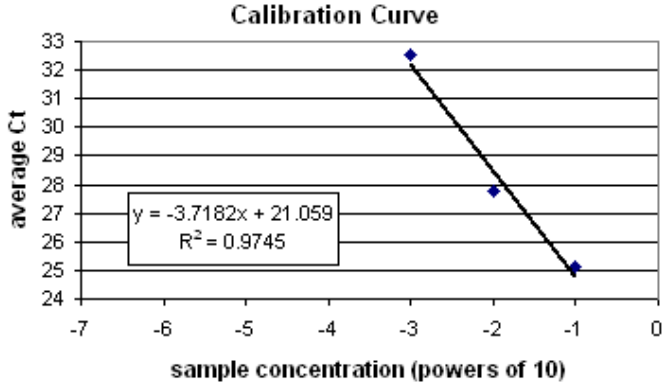


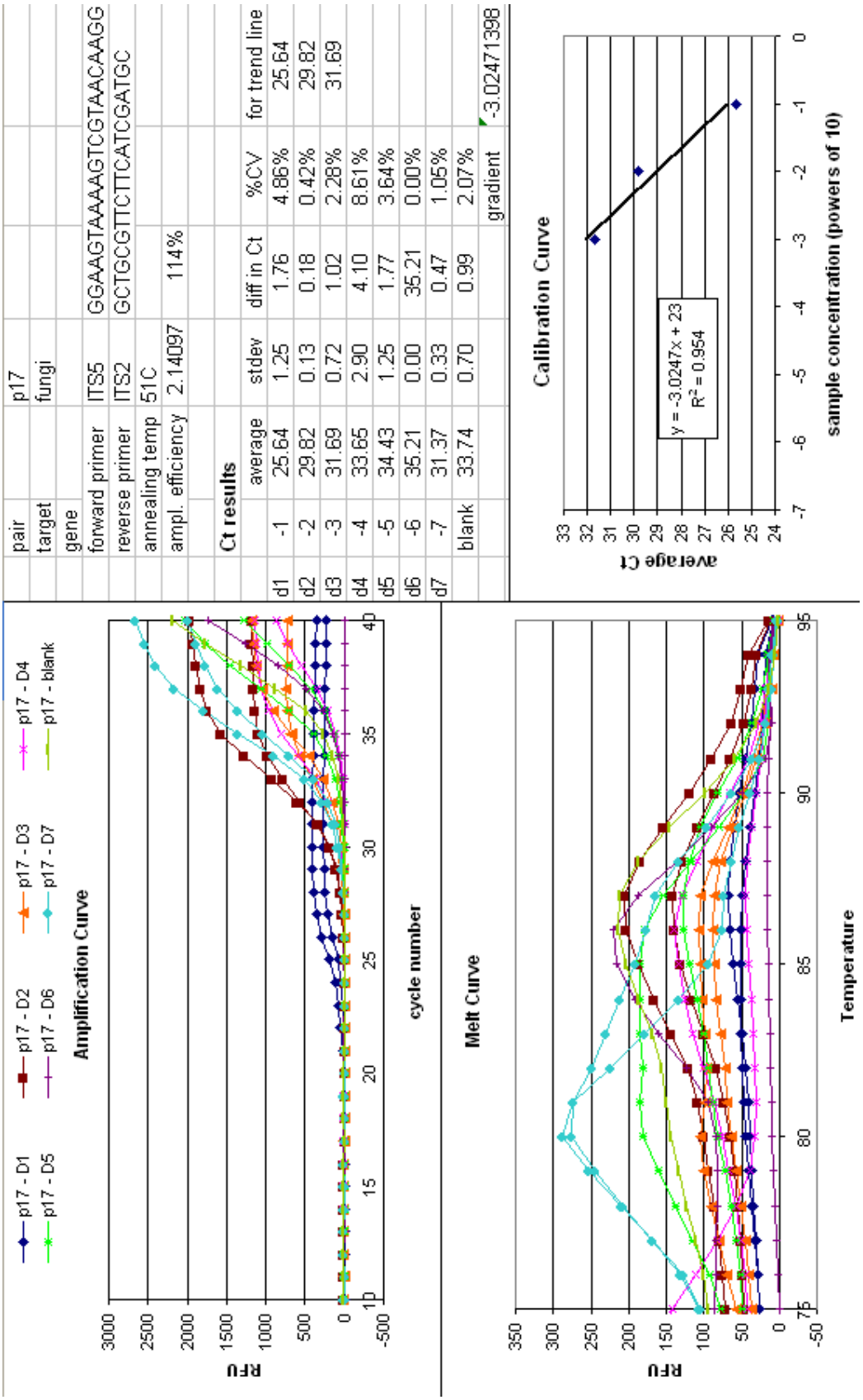

pair
target
gene
forward primer
reverse primer
annealing temp
ampl. efficiency
p14
cyanobacteria
16S rDNA
CYA359F GGGGAATYTTCCGCAATGGG
CYA781F GACTACWGGGGTATCTAATCCCWTT
51C
231%
3.31403
Ct results
average
stdev
diff in Ct
%CV
for trend line
d1 -1 20.56 0.69 0.97 3.35% 20.56
d2 -2 22.44 0.10 0.14 0.46% 22.44
d3 -3 24.40 0.41 0.58 1.68% 24.40
d4 -4 23.18 0.21 0.30 0.92% 23.18
d5 -5
d6 -6
d7 -7
blank 24.06 0.55 0.78 2.29%
gradient -1.92175829
Calibration Curve
y = -1.9218x + 18.622
R² = 0.9999
average Ct
sample concentration (powers of 10)
Amplification Curve
p14 - D1
p14 - D2
p14 - D3
p14 - D4
p14 - blank
fluorescence
cycle number
Melt Curve
fluorescence
Temperature




| | | | | | | |
|---|---|---|---|---|---|---|
| | pair | | p15 | | | |
| | target | | fungi | | | |
| | gene | | nuclear small rDNA | | | |
| | forward primer | | NS5 | AACTTAAAGGAATTGACGGAAG | | |
| | reverse primer | | NS6 | GCATCACAGACCTGTTATTGCCTC | | |
| | annealing temp | | 51C | | | |
| | ampl. efficiency | | 1.85757 | 86% | | |
| | | | | | | |
| | **Ct results** | | | | | |
| | | average | stdev | diff in Ct | %CV | for trend line |
| d1 | -1 | 25.12 | 0.05 | 0.07 | 0.19% | 25.12 |
| d2 | -2 | 27.80 | 0.12 | 0.18 | 0.45% | 27.80 |
| d3 | -3 | 32.56 | 1.81 | 2.56 | 5.55% | 32.56 |
| d4 | -4 | 31.98 | 0.03 | 0.05 | 0.11% | |
| d5 | -5 | 30.62 | 4.60 | 6.50 | 15.01% | |
| d6 | -6 | 32.24 | 0.19 | 0.27 | 0.59% | |
| d7 | -7 | 30.03 | 2.91 | 4.11 | 9.68% | |
| | blank | 31.78 | 0.93 | 1.31 | 2.92% | |
| | | | | | gradient | -3.7182446 |






| | | pair | | p17 | | | | |
|---|---|---|---|---|---|---|---|---|
| | | target | | fungi | | | | |
| | | gene | | | | | | |
| | | forward primer | | ITS5 | GGAAGTAAAAGTCGTAACAAGG | | | |
| | | reverse primer | | ITS2 | GCTGCGTTCTTCATCGATGC | | | |
| | | annealing temp | | 51C | | | | |
| | | ampl. efficiency | | 2.14097 | 114% | | | |
| | | **Ct results** | | | | | | |
| | | | average | stdev | diff in Ct | %CV | for trend line | |
| d1 | -1 | | 25.64 | 1.25 | 1.76 | 4.86% | 25.64 | |
| d2 | -2 | | 29.82 | 0.13 | 0.18 | 0.42% | 29.82 | |
| d3 | -3 | | 31.69 | 0.72 | 1.02 | 2.28% | 31.69 | |
| d4 | -4 | | 33.65 | 2.90 | 4.10 | 8.61% | | |
| d5 | -5 | | 34.43 | 1.25 | 1.77 | 3.64% | | |
| d6 | -6 | | 35.21 | 0.00 | 35.21 | 0.00% | | |
| d7 | -7 | | 31.37 | 0.33 | 0.47 | 1.05% | | |
| | blank | | 33.74 | 0.70 | 0.99 | 2.07% | | |
| | | | | | | gradient | -3.02471398 | |