Investigation of microbial diversity in an acid tar lagoon and the effect of low pH on two microorganisms isolated from an acid tar lagoon

Leanne Sunter

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Department of Molecular Biology and Biotechnology
University of Sheffield

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Abstract

Acid tar lagoons are heavily polluted, man-made environments found in several locations around the world, including the United Kingdom, Germany and the United States of America. Each lagoon is unique due to the different methods of tar production resulting in a specific waste tar composition. There is also a great deal of heterogeneity within each lagoon as a result of the viscous and mobile nature of the tar waste, alongside a combination of attempted remediation efforts and/or illegal fly tipping.

The work presented in this thesis concerns one particular acid tar lagoon situated in the North West of England which arose as a result of deposition of waste from benzole refining. The effects on microbial diversity of a combination of low pH and high levels of organic pollutants were investigated using samples taken from various locations within this lagoon. A variety of techniques including classical microbiology, DGGE and T-RFLP, were used to examine the microbial diversity, which was found to be much lower in lagoon samples than pristine environments and appears to have similarities with the microbial communities present in the Rio Tinto, Spain.

Following microbial diversity analysis a detailed examination of two specific organisms isolated from lagoon samples was carried out in order to understand some of the mechanisms of survival and tolerance to such an extreme environment. An acidotolerant unicellular alga which appears to form a large biofilm in several areas of the lagoon, *Euglena gracilis* G46, and an acidophilic bacterium, *Acidocella* 29, were the focus of this aspect of the work. It appears that *E. gracilis* G46 maintains a near-neutral pH under acidic conditions, *Acidocella* 29 is thought to have an unusually low intracellular pH.

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Abbreviations

Abbreviations used in this thesis, not including elemental symbols and SI units are as follows:

a_i: concentration inside the cell

AMD: Acid Mine Drainage

a_o: concentration outside the cell

APS: Ammonium persulphate

ARDRA: Amplified Ribosomal DNA Restriction Analysis

BAC: Bacterial Artificial Chromosome

BSA: Bovine Serum Albumin

BTEX: Benzene, Toluene, Ethylbenzene and Xylene

CCCP: Carbonyl cyanide 3-chlorophenylhydrazone

Chl: Chlorophyll

DCMU: Diuron; 3-(3,4-dichlorophenyl)-1,1-dimethlyurea

DGGE: Denaturing Gradient Gel Electrophoresis

DMSO: Dimethylsulphoxide

dpm: Disintegrations per minute

ECV: extracellular volume

EDTA: ethylenediaminetetraacetic acid

ICV: intracellular volume

NAD: nicotinamide adenine dinucleotide (oxidised form)

NADH: nicotinamide adenine dinucleotide (reduced form)

OD: Optical density

PAH: Polycyclic aromatic hydrocarbons

PCR: Polymerase Chain Reaction

pH_i: Internal pH

pH_o: External pH

rRNA: ribosomal Ribonucleic Acid

TAE: Tris Acetate EDTA

TEMED: N,N,N',N'-Tetramethylethylenediamine

TGGE: Temperature Gradient Gel Electrophoresis

T-RFLP: Terminal Restriction Fragment Length Polymorphism

T-RF(s): Terminal Restriction Fragment(s)

Chapter One: General Introduction

1.1: Microbial Diversity

Biodiversity can be defined in several ways ranging from "a popular term used in scientific and media context to refer to the catalogue of living species that exist in a certain habitat or ecosystem" (Rodriguez-Valera 2002) to "an attribute of an area and specifically refers to the variety within and among living organisms, assemblages of living organisms, biotic communities, and biotic processes, whether naturally occurring or modified by humans. Biodiversity can be measured in terms of genetic diversity and the identity and number of different types of species, assemblages of species, biotic communities and biotic processes, and the amount (e.g.: abundance, biomass, cover, rate) and structure of each." (DeLong 1996). The term "BioDiversity" was first coined in 1986 and since then the concept has been the subject of much research.

In 1998 the known biodiversity on Earth consisted of 1.5 million animal species, 0.3 million plant species (Cases and Lorenzo 2002) and half a million insects (Pace 1997), compared to only 4500 prokaryotes (Torsvik *et al.* 2002). This number of prokaryotes significantly less than 1 % of the total number of species on Earth, which is an astonishingly small percentage when one considers that prokaryotes have been estimated to contain between 60-100 % of the total carbon contained in plants (Whitman *et al.* 1998).

This begs the question, why have so few microbial species been identified? There are several reasons for our lack of knowledge regarding microbial diversity. One of the most significant and well known reasons is the "Great Plate Count Anomaly", a phrase coined by Staley and Konopka (1985) referring to the discrepancy between the large number of cells which are visible by microscopy or detectable by fluorescence hybridisation compared to the numbers which can be cultured by traditional methods (Staley and Konopka 1985). For example, Torsvik *et al* achieved a viable colony count of 4.3×10^7 compared to 1.5×10^{10} bacteria $gram^{-1}$ from microscope observations of soil samples (Torsvik *et al.* 1990).

This anomaly can be explained by cells which are viable but not culturable. These may be cells which are unknown species for which no culturing method has been developed, known species which have entered a dormant state or for which the culturing conditions

are not suitable (Amann *et al.* 1995). For instance, bacteria that are dominant in a natural environment are not usually adapted to high concentrations of complex organic carbon (Connon and Giovannoni 2002). Furthermore, many microorganisms require complex nutritional requirements to grow successfully and can only then be cultured by specialised techniques (Button *et al.* 1998; Driessen *et al.* 1996; Ferris *et al.* 1996; Vancanneyt *et al.* 2001; Wirsen *et al.* 2002).

Modern microbiology relies on our ability to grow pure cultures, thus media are specifically designed to promote the growth of one particular type of microorganism, which usually means that the organisms which are capable of the fastest growth will out compete all other organisms (Rawlings *et al.* 1999). This means that it is difficult to retrieve more than a few organisms from an environmental sample using traditional methods. Even if a range of media were used to culture organisms from an environmental sample it is highly unlikely that more than a few species' of organisms would be recovered from each medium.

Conventional cultivation methods are highly biased and selective, as well as time consuming (Amann *et al.* 1995). Attempts to mimic natural conditions are often equally unsuccessful, suggestions for the lack of success in each case ranging from quorum sensing inhibition and lysogenic phages to the unknown effects of a microniche (Rodriguez-Valera 2002).

In many cases there may be complex interactions between microbes growing in a particular environment. For example, the breakdown of a compound by a particular organism may remove an inhibiting substance and thus permit growth of other organisms, as is the case for sulphate reducing bacteria. These organisms require aerobic organisms to remove acetic acid, a toxic by-product of their anaerobic sulphate reduction which would kill the cells if it was not removed by other microbial species (Kimura *et al.* 2006). Attempts to grow microbes in pure culture may never succeed for certain organisms which may rely on the presence and activities of other microorganisms in order to grow. Therefore, isolating certain microorganisms from environmental samples may not be possible unless other organisms are co-cultured, which given our limited knowledge and inability to culture most organisms may not be possible.

High throughput methods have been developed using very low nutrient media in an attempt to isolate previously unknown organisms. Although these methods are laborious a few organisms have been isolated and cultured. Some 2500 dilution cultures screened over a three year period gave up to a 1400 fold increase in numbers compared to traditional methods; however, only four unknown organisms were isolated (Connon and Giovannoni 2002).

The addition of signalling molecules such as cyclic AMP (cAMP) to media containing a low concentration of carbon substrate has been shown to increase cultivation efficiencies when using the most-probable-number (MPN) technique for enumeration. However, dot blot hybridisation revealed that the strains which had been most successfully cultured represented only a small fraction of the bacterial community, indicating limitations in the MPN method (Bruns *et al.* 2002).

With perseverance and patience there are some successes; with at least 16 of the 27 new phyla identified since 1986 having cultured representatives (Leadbetter 2003). For example, Sait and colleagues successfully cultured the first representative of the previously uncultured subdivision three of the *Acidobacteria* phylum by waiting for 12 weeks for colonies to develop (Sait *et al.* 2002). Combined with the use of very dilute nutrient broth and long incubation times the group also cultured strains representing six new genera of subdivision 1 of the *Acidobacteria* phylum.

Estimates of true prokaryotic diversity range from 13000 (Torsvik *et al.* 1990) to 36000 (Dykhuizen 1998) species in one 30 cm^3 soil sample, leading to one suggestion that there are more than a thousand million species of bacteria worldwide (Dykhuizen 1998). Furthermore, evidence suggests that there is a far greater number of organisms present in the subsurface of the Earth yet to be discovered (Pace 1997). Microbial diversity is extremely rich and for the most part unexplored. The potential for exploitation for biotechnology given the genetic and biochemical diversity which exists, and which we have little knowledge of, is enormous (Horkioshi 1995).

One of the reasons why there is such a range in estimates of microbial diversity is the controversy surrounding what is a species, and more importantly, what is defined as a species. Traditionally, taxonomists have used visible characteristics to classify higher organisms such as plants and animals, however this was not particularly suitable when

applied to microorganisms which do not exhibit a great deal of morphology and thus their limited morphologies and biochemistry were used to classify them. This undoubtedly resulted in many mistakes and it was not until the development of molecular sequencing that the true phylogenetic perspective was resolved.

By tradition, higher organisms have been defined as species if they interbred and were able to produce viable offspring. Unfortunately, given the mode of replication and genetic exchange in prokaryotes this definition does not apply. One of the most commonly used and accepted definitions of a prokaryotic species is DNA-DNA hybridisation of more than 70 % combined with $\leq$ 5 °C difference in melting temperature of DNA, where both units are considered (Wayne *et al.* 1987). Dykhuizen (1998) discusses this and concludes that defining species in terms of DNA homology will underestimate the number of species.

More recently still an operational definition of bacterial species nas been described as "a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property" (Rossello-Mora and Amann 2001). Despite the discrepancies which arise as a result of species definition there is still exists a large number of bacterial species to be discovered and characterised. But, how do we resolve this deficit in our knowledge? Recent work has focused on molecular methods in order to identify species diversity using 16S rRNA as a molecular marker in many cases.

1.2: Molecular Methods Used to Assess Diversity

Molecular markers are much more reliable for relating and classifying organisms than phenotypic information because they are more informative with regard to evolutionary relationships and they are also more readily available (Woese 1987). Molecular markers were the basis of one of the most significant discoveries in modern biology; namely that life on Earth consists of three evolutionary lines and not two as had been thought previously. Based on work with 16S rRNA Carl Woese, and colleagues, proposed that there should be a new level of taxon above the 5 kingdoms, known as a domain, and that there would be three domains, comprising archaea, bacteria and eucarya (Woese *et al.* 1990) (Figure 1.1). However, almost 20 years on from this proposal there are still those who do not accept this classification, such as Lynn Margulis and Michael Dolan (Margulis and Dolan 1999).

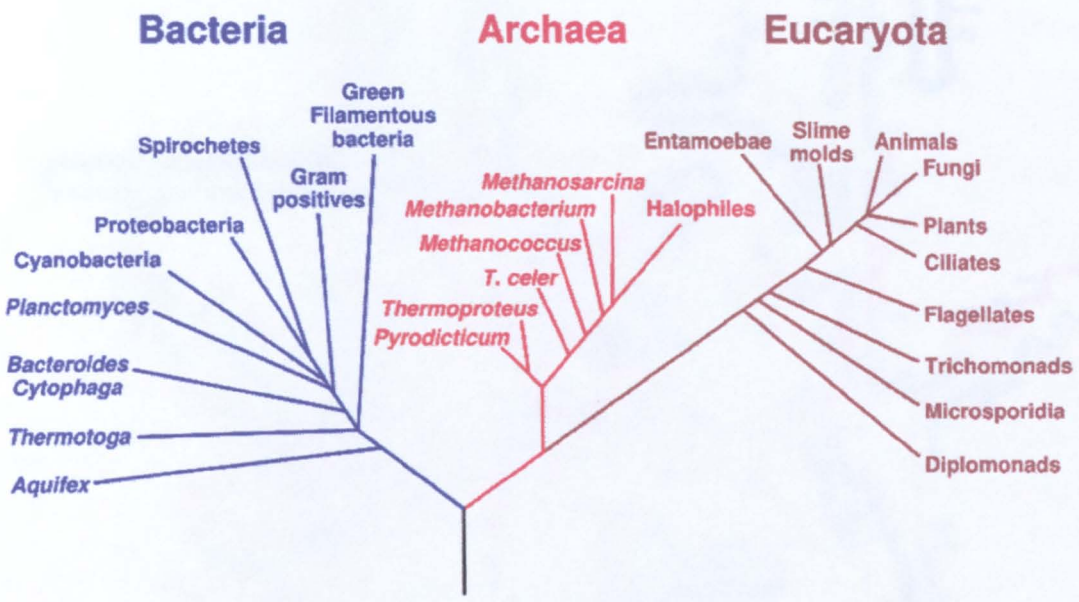


Figure 1.1: Phylogenetic tree of life. Figure from NASA Astrobiology Institute (http://nai.arc.nasa.gov/library/images/news_articles/big_274_3.jpg)

Ribosomal RNA (rRNA) (Figure 1.2) is the most commonly used molecular marker for several reasons. Firstly and most importantly, it is present in all organisms and has been for a very long time (Olsen *et al.* 1986). The overall structure of rRNA is well conserved between all organisms which means that it is easy to identify rRNA by size alone (Olsen *et al.* 1986). rRNA molecules are a necessary element of one of the most basic cellular functions, protein synthesis, and thus, form a significant proportion of cellular mass, therefore rRNA is readily recoverable from all cell types (Olsen *et al.* 1986). In addition, it is possible to sequence rRNA directly and rapidly using reverse transcriptase (Hugenholtz and Pace 1996; Woese 1987).

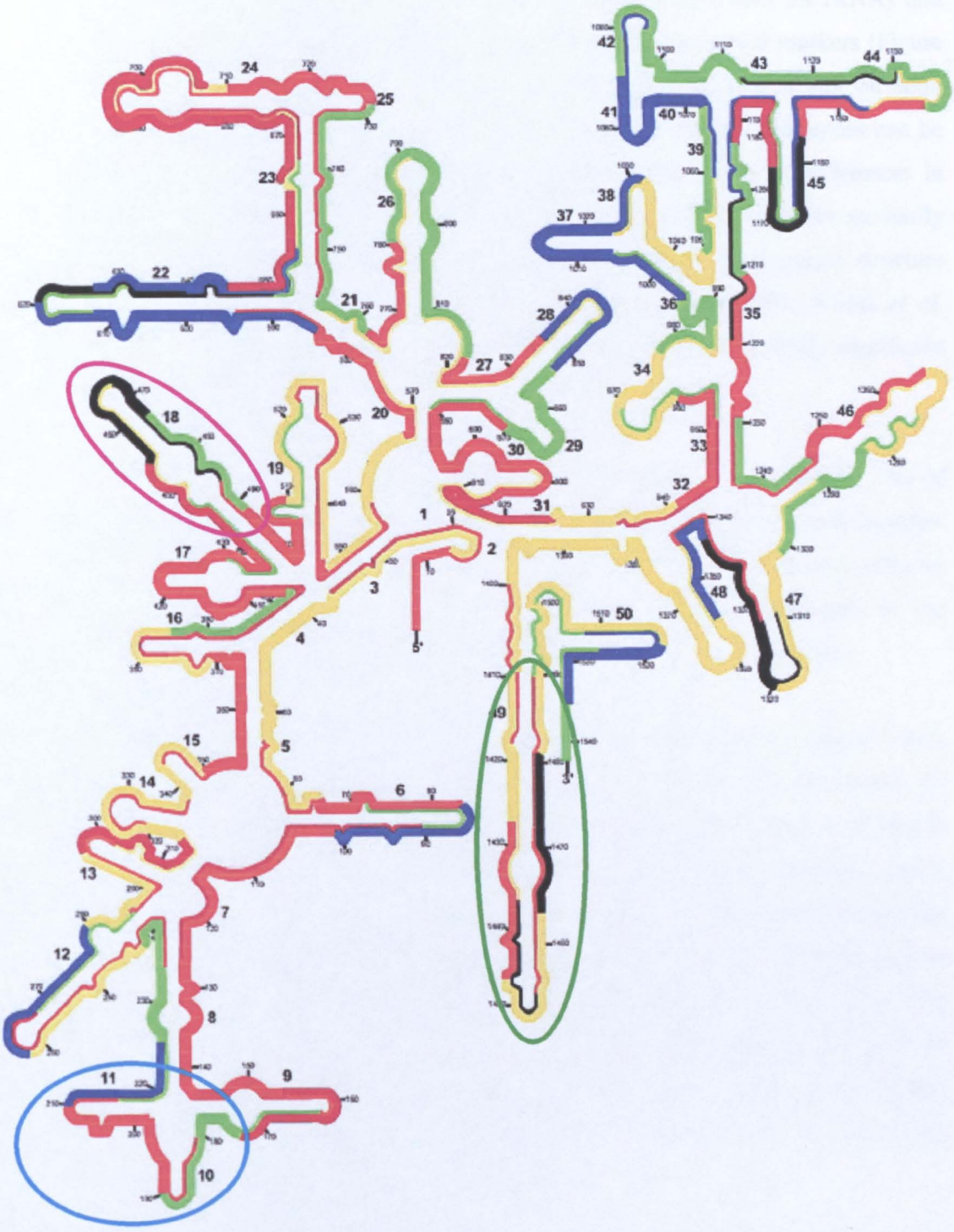


Figure 1.2: Two dimensional secondary structure of a 16S rRNA molecule from *E. coli*. Figure taken from Behrens *et al.* (2003). The magenta oval highlights the V3 region; the green oval highlights the V9 region whilst the blue circle highlights bases 180-197, the V2.2 hypervariable region.

16S rRNA molecules are large (approximately 13 times larger than 5S rRNA) and consist of many domains which also facilitates their use as molecular markers (Figure 1.2). Certain stretches of rRNA are highly conserved, showing little if any variation between species, whereas other stretches vary greatly, meaning that sequences can be aligned by matching invariable stretches and analysed on the basis of differences in highly variable loop regions. For example, archaeal 16S rRNA molecules are easily identified from bacterial 16S rRNA or eukaryal 18S rRNA by their unique structure between positions 180 and 197 (Johnson *et al.* 2001; Ma *et al.* 2004; Woese *et al.* 1990). Furthermore, the length of rRNA is sufficient to permit statistically significant comparisons (Olsen *et al.* 1986).

In 1987 over 500 species had been characterised in terms of their rRNA. As of November 8th 2007 there were 451545 16S rRNA sequences in the Ribosomal Database Project (Cole *et al.* 2005; Olsen *et al.* 1992). Improvements in molecular methods, sequencing and the genomics revolution have resulted in a vast expansion in our phylogenetic knowledge of both culturable and non-culturable microorganisms.

Furthermore, as a result of 16S rRNA sequencing from environmental samples, there has been an increase in the number of phyla from 12 to 52. Only 26 of the putative 52 phyla have cultured representatives (Rappe and Giovanoni 2003), thus we must bear in mind the limitations of this resource. For instance, whilst 16S rRNA is a good molecular marker it comprises on average only 0.05 % of the total genome and therefore is not completely representative (Rodriguez-Valera 2002). Additionally, if an organism is less than 95 % similar to a cultured organism as assessed by rRNA, very little can be predicted about its characteristics (Rodriguez-Valera 2002). The methods used to correlate the data gathered from sequencing are variable, each with advantages and disadvantages, with the most commonly used method being cluster analysis (Olsen *et al.* 1986), resulting in dendrograms such as Figure 1.3.

Several of the techniques described below rely on the extraction of total genomic DNA from environmental samples. As a starting point for all the molecular techniques described below it is essential that this process is efficient and results not only in suitable yields of DNA but also suitably sized DNA. Shearing and damage of DNA often occurs which can result in DNA which is too small to be used in the techniques described.

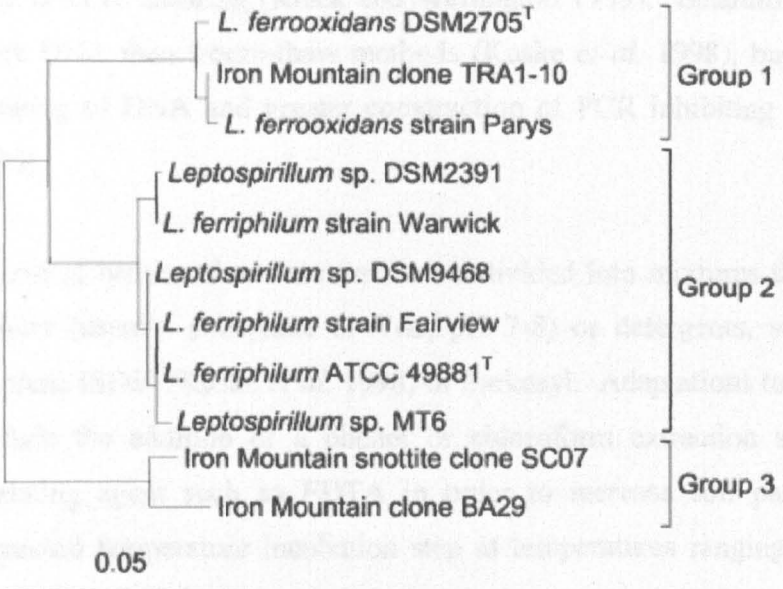


Figure 1.3: A typical dendrogram showing three phylogenetic groups of *Leptospirillum.* Figure taken from Johnson *et al* (2003).

DNA purity is also a concern, especially when dealing with soil samples where levels of PCR inhibitors, such as humic acids, are high. Furthermore, certain extraction procedures can result in a bias towards recovery of DNA from specific microorganisms and are not always truly representative. Therefore several extraction and purification methods have been developed and optimised in order to achieve suitably sized DNA.

There are two main methods of DNA extraction from sediments and soils; direct and indirect. Direct extraction encompasses all methods which lyse cells within the sediment or soil, whereas indirect methods are those methods which remove cells from the soil or sediment before lysis. Direct lysis techniques yield more DNA and are thought to yield a less biased sample of the microbial community diversity than indirect cell extraction techniques, where certain cells are much more difficult to remove from soil colloids for example (Leff *et al.* 1995).

Direct methods can be further subdivided into three basic elements, including physical disruption, enzymatic lysis and chemical lysis, of which one, two or all three components may be involved. Physical disruption methods include ultrasonication, grinding under liquid nitrogen, freeze-thawing and beadmill homogenisation with the latter two methods being the most common. Sonication is used infrequently due to a

high level of shearing (Krsek and Wellington 1999). Beadmill homogenisation yields more DNA than freeze-thaw methods (Kuske *et al.* 1998), but it also results in more shearing of DNA and greater coextraction of PCR inhibiting compounds (Leff *et al.* 1995).

Chemical lysis methods are further subdivided into mixtures that contain either NaCl, buffers (usually phosphate or Tris, pH 7-8) or detergents, such as sodium dodecyl sulphate (SDS) (Kuske *et al.* 1998) or Sarkosyl. Adaptations to chemical lysis methods include the addition of a phenol or chloroform extraction step, incorporation of a chelating agent such as EDTA in order to increase soil particle dispersal, and an increased temperature incubation step at temperatures ranging from 60 °C to 100 °C (Kuske *et al.* 1998). Enzymatic lysis using enzymes such as lysozyme, proteinase K and proteinase E often forms the final step of extraction procedures. Enzymatic lysis is insufficient alone but does improve the purity of extracted DNA, either by breaking glycosidic bonds (Krsek and Wellington 1999) within humic components or precipitating humic acids (Maarit Niemi *et al.* 2001).

Indirect lysis, also known as the cell extraction method, has been shown to yield the purest DNA and thus may be the preferred method if the DNA extracted is to be used for PCR where yield is less important (Leff *et al.* 1995). Indirect lysis is subdivided into two methods; blending methods, a method in which cells are mechanically released from the sample matrix, and cation-exchange methods, a method in which cells are dispersed by chemical disruption usually using Chelex 100 (Gabor *et al.* 2003). Indirect lysis methods are rarely used because it is difficult to process a large number of samples at one time and it is more biased, often only acquiring yields of 25 to 35 % of the total bacterial population (Frostegard *et al.* 1999).

1.2.1: Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

T-RFLP is a very useful community fingerprinting technique. The technique involves PCR amplification with the exception that one or both of the primers are fluorescently labelled. Following PCR, multiple, single restriction digests of the PCR product are carried out to generate fluorescently labelled terminal restriction fragments (T-RFs). The T-RFs generated will be of a specific length for each bacterium present in the sample and thus separation of the fragments by high resolution electrophoresis combined with automated DNA sequencing allows characterisation of a community or

environment. 16S rRNA is the most commonly used marker however any genetic marker with conserved sequence domains which would allow appropriate primer design could be utilised.

A wide range of restriction enzymes can be selected, however studies have been undertaken which revealed that *Alu*I, *Hha*I and *Tha*I are preferential since they are most discriminatory in terms of the number and size of different T-RFs generated (Osborn *et al.* 2000). Osborn *et al* (2000) assessed a range of variables which contribute to reproducibility including the initial template concentration, number of PCR cycles, choice of *Taq* polymerase, annealing temperature, restriction enzyme concentration, injection time and 16S rRNA primer sequences. The outcome of which is a highly optimised procedure which should provide reliable, reproducible results.

Using sequence information it is also possible to design a multitude of primers for specific phyla, genera or species and to analyse communities using genes other than 16S rRNA, such as those involved with degradation or a particular kind of metabolism. For example, Bruce (1997) used *mer* genes in order to analyse community diversity in terms of mercury resistance, whilst more recently Castro *et al* (2005) used *dsrA* genes to study the distribution of sulphate-reducing organisms in nutrient-impacted regions.

The procedure has several advantages over other molecular community analysis techniques such as denaturing gradient gel electrophoresis (DGGE) and other electrophoretic separation methods in that it is highly sensitive, reliable and rapid (Marsh 1999). A greater resolution can be obtained using T-RFLP than DGGE, temperature gradient gel electrophoreis (TGGE) or single strand conformational polymorphism (SSCP) with the added benefit that the output is digital. Combining this technique with the large amount of sequence information available in sequence databases it is possible to infer phylogeny of T-RFs since the position of the restriction site is not random but has a phylogenetic component; however unlike DGGE it is not possible to identify the organism responsible for each T-RF.

T-RFLP analysis has been successfully employed on a variety of samples including fungal communities in soils (Edel-Hermann *et al.* 2004), monitoring nutrient impact on contaminated soils (Mills *et al.* 2003), community structure in marine sediments (Braker *et al.* 2001), solar salterns (Cassamayor *et al.* 2002) and bioreactor sludge (Liu *et al.*

1997). Of particular relevance to this study is the analysis of microbial diversity in a macroscopic streamer growth from acidic, metal-rich mine waters in North Wales which revealed a very simple community of microorganisms (Hallberg *et al.* 2006). The study found that the community composition was remarkably different to the microbial communities found in similar acidic environments at Iron Mountain, California and the Rio Tinto, a river, in Spain.

T-RFLP could be used to monitor changes in microbial community diversity at different time points or under different conditions easily, since the process is very rapid and relatively inexpensive. However, it would not be possible to identify changes in any particular species of microorganism present. It is easily possible to analyse multiple samples concurrently, regardless of when the samples were acquired as well as to infer the abundance of the microorganisms responsible for each T-RF based on the fluorescence intensity.

Recently, web based analysis programmes such as PAT (Kent *et al.* 2003) and TAP (Marsh *et al.* 2000) have been developed which generate phylogenetic assignments based on predicted T-RFs from 16S rRNA sequences in various databases, for example T-RFLP APLAUS+ which creates a specific database of *in silico* analyses to cross reference against based on the users selection of restriction enzymes and primer sequences (Shyu *et al.* 2007). PAT is a particularly useful tool since the T-RFs generated from multiple, single restriction digests can be analysed concurrently which serves to minimise the potential possible phylogenetic affiliation of each T-RF. T-align software is also available which allows comparison of replicate data to be collated and analysed in preparation for further statistical analyses (Smith *et al.* 2005).

As with all PCR-based approaches an inherent bias is introduced as a result of several factors including the preferential binding of primers to certain species and varying copy number of 16S rRNA genes between species (Suzuki and Giovannoni 1996). However, this bias applies to all PCR-based approaches including T-RFLP, DGGE/TGGE and amplified rDNA restriction analysis (ARDRA) and has been discussed in more detail with respect to 16S rRNA and microbial diversity analysis studies by Wintzingerode *et al.* (1997).

1.2.2: Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is an increasingly popular and commonly used technique for resolving microbial populations. The technique exploits the differences in melting temperature of different DNA sequences (Muyzer *et al.* 1993). Usually, genomic DNA is extracted from a sample and short, hypervariable fragments of the 16S rRNA gene are amplified by polymerase chain reaction (PCR) using universal primers. The sample is then loaded on a vertical polyacrylamide gel and run at a constant temperature, along an increasing gradient of denaturant such as formamide or urea. At a position specific to the sequence of the DNA migrating, the DNA duplex begins to uncoil and is then trapped in the gel.

TGGE is an almost identical procedure to DGGE with the exception that instead of applying a denaturing gradient to the vertical polyacrylamide gel, there is instead a linear temperature gradient which separates the two strands of the DNA. It is usually possible to detect 50 % of sequence variants in DNA fragments up to 500 bp long, however if a GC clamp is added to the 5' end of one of the primers this can be increased to almost 100 %. The GC clamp is a GC-rich sequence, usually between 30 and 50 nucleotides in length, forming a high melting point domain which prevents complete separation of the two strands of DNA. Chemical clamps are sometimes used as an alternative to GC clamps which can be expensive to produce, however chemical clamps are covalently attached, thus bands extracted from the DGGE gel cannot be re-amplified directly.

DGGE and TGGE have been used successfully to study community complexity, changes in microbial communities and to monitor enrichment and isolation of bacteria. Muyzer *et al* (1993) applied DGGE to PCR-amplified 16S rRNA gene fragments from a biofilm in order to profile community complexity. PCR was carried out on genomic DNA preparations using primers designed to amplify the V3 region of 16S rRNA gene from sulphate-reducing bacteria and the resulting PCR products analysed by DGGE. This study showed the presence of up to 10 different 16S rRNA gene fragments, as well as identifying the presence of microorganisms which constituted less than 1 % of the total microbial population. Hybridisation analysis using group specific radioactively-labelled probes yielded further information about the species present in the biofilm, with further information obtained when DNA fragments were excised from the gel and sequenced (Muyzer *et al.* 1993).

DGGE has become an increasingly popular tool to study community diversity, and has been applied to a variety of ecosystems and environments including microbial mat communities in hot springs (Ferris *et al.* 1996a), hydrothermal vents (Muyzer *et al.* 1995), seawater (Diez *et al.* 2001; Schauer *et al.* 2000), contaminated soils (Andreoni *et al.* 2004), industrial sites (Zocca *et al.* 2004) and the Tinto River (Gonzalez-Toril *et al.* 2003). The surveys carried out on contaminated soils and on the Tinto River are particularly relevant to the present study. The Tinto River has a mean pH of 2.4 and thus bears relevance to the samples from Hoole Bank, which has an average pH of approximately 2.6. In total, 57 of the 80 bands excised from the DGGE gel yielded identifiable sequences from the Tinto river samples, with 30 of these sequences having greater than 98 % similarity to the closest relative microorganism (Gonzalez-Toril *et al.* 2003).

DGGE is an ideal tool for studying changes in a variety of communities and environments (Schafer *et al.* 2001; Van Der Gucht *et al.* 2001) with the same advantages of T-RFLP in that samples can be analysed simultaneously, regardless of when or where they were acquired. Furthermore, an estimate of the number of species present can be made simply by looking at the banding pattern, and thus the presence or absence of bands on two related samples can be compared easily. The intensity of the bands can also be used as an estimate of abundance of a specific organism (Nubel *et al.* 1999).

1.2.3: Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA)

Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA) involves digestion of a PCR amplified gene, usually 16S rRNA, with one or more restriction enzymes. This produces fragments of varying lengths which can be separated on polyacrylamide gels, producing a unique banding pattern which can be compared visually in a similar way to the results of DGGE. In contrast to T-RFLP, no fluorescent primers are used in ARDRA and the banding patterns are simply compared visually as opposed to measuring the restriction fragments using a sequencing gel. If the gene sequence is known it is possible to predict the number and size of fragments obtained from a particular restriction enzyme digest, or combination of enzymes and thus the ARDRA pattern of unknown organisms can be compared to known standards. In a similar way to DGGE, ARDRA can be carried out and the banding pattern of different environments

compared, thus it is useful in detecting changes in a population or differences in communities in different environments (Smit *et al.* 1997).

1.2.4: Gene libraries and metagenomics

Gene libraries such as fosmid and Bacterial Artificial Chromosome (BAC) libraries allow large genome fragments recovered directly from the environment to be analysed and allow characterisation of unknown species more thoroughly than by 16S rRNA analysis. Genomic DNA is usually prepared by direct lysis and partially digested with restriction enzymes to create smaller fragments of DNA which can be ligated into BACs or other vectors. Recombinant strains are then screened for a particular feature, such as ability to utilise a specific substrate (Henne *et al.* 1999) or to assess diversity (Beja *et al.* 2000).

Following screening, colonies of interest are sequenced to analyse the insert in more detail. One study, concerned with diversity, was able to sequence an insert from an uncultivated organism and identify novel open reading frames (ORFs), which could indicate a novel function (Beja *et al.* 2000). Quaiser *et al* (2002) utilised a gene library approach to isolate DNA from archaea present in soil, by carrying out genomic DNA extraction and PCR using archaeal specific 16S rRNA primers. One particular isolate found contained a complete 16S/23S operon as well as 17 genes thought to encode proteins. Analysis of the sequence showed the insert to be affiliated with the crenarchaeota, but also showed significant differences between archaeal DNA isolated from marine environments (Quaiser *et al.* 2002).

Voget *et al* (2003) utilised this technique to produce a cosmid library for which to screen for novel biocatalysts. Functional screens along with cosmid sequencing identified 12 putative agarase genes from 4 clones, and a further 7 other biocatalyst encoding genes. Thus, creation and analysis of gene libraries can provide further insight into the genomic potential residing in the environment, particularly regarding uncultured species.

Metagenomics, the study of the collective genomes from a particular environmental sample (Handelsman *et al.* 1998), uses cloning vectors to create large gene libraries of environmental DNA. Metagenomics has been carried out in a number of environments

of increasing community complexity ranging from acid mine drainage communities at Iron Mountain, California (Tyson *et al.* 2004) to the Sargasso Sea (Venter *et al.* 2004).

Metagenomic libraries are usually screened using functional-driven analysis or sequence-driven analysis (Handelsman 2004). Functional analysis identifies clones expressing a particular function and has led to the identification of novel antibiotics, antibiotic resistance genes, degradative genes and catalytic genes (Voget *et al.* 2003). The main disadvantage of function-driven analysis is that it relies upon heterologous expression. Whilst DNA from several organisms has been successfully expressed in *E. coli*, one of the most commonly used host species, it is unlikely that most genes will be successfully expressed. This is especially likely given that most screens are trying to identify novel genes or motifs, most probably from unusual microorganisms, and yet the host used is a very common bacterium. Development of high-throughput methods and appropriate screening techniques is necessary in order to further optimise the identification of novel genes, especially when the frequency of active clones is so low (Rondon *et al.* 2000). New methods are continually being developed in order to screen metagenomic libraries (Uchiyama *et al.* 2005) as the potential for biotechnological application is significant.

Sequence-based analysis utilises phylogenetic anchors which indicate phylogenetic groups in order to identify the most likely source of the inserted fragment. Alternatively, random sequencing is conducted and the phylogenetic anchor identified in the flanking sequence of any interesting genes identified. A promising application of sequence-based analysis is that in simple communities it is possible to assemble whole genomes from both cultured and uncultured organisms, something which has been applied to Iron Mountain samples and has led to two near-complete genome sequences and three partial genome sequences (Tyson *et al.* 2004). Large scale sequencing projects using metagenomic libraries have begun in recent years beginning with samples from the Sargasso Sea (Venter *et al.* 2004) which individually generated more than 1 million base pairs of sequence data. Further shotgun sequencing has been carried out in other environments resulting in an overwhelming amount of data generated already. The capacity of metagenomics to yield vast amounts of data about many aspects of microorganisms is clearly huge and is an extremely promising area of research which can only expand our understanding of microbial life.

1.2.5: Fluorescence *In Situ* Hybridisation (FISH)

Whole cell hybridisation is a method which can be used to identify the presence, or absence, of a specific organism or phylum. Fluorescently labelled probes bind to specific target sequences within the cell and can then be visualised by microscopic techniques. The most commonly used probe for both whole cell and *in situ* hybridisation is a fluorescently labelled sequence targeted to 16S rRNA. Probes can be designed to detect a specific species, genus or domain. A major advantage of FISH is that it can be carried out *in situ*, therefore no bias is introduced as a result of DNA preparation method or PCR. FISH can also be semi-quantitative, if the number of cells which fluoresce from a specific probe is compared to the total number of cells stained by DAPI (4',6-diamidino-2-phenylindole). It is also possible to detect organisms which comprise a very low percentage of a microbial population if the probe is well designed or chosen carefully. FISH has been applied to many environmental samples including acidic environments such as the Rio Tinto (Garcia-Moyano *et al.* 2007) and acid mine drainage in North Wales (Hallberg *et al.* 2006) and Iron Mountain, California (Baker *et al.* 2004).

1.2.6: Future prospects

Improvements in culturing methods have increased the number of species which have been isolated in pure culture, and will continue to do so in the future. At present molecular methods allow us to identify the closest related genetic ancestor to a particular microorganism, but little more. Perhaps with a greater understanding of genomics it may be possible to deduce what conditions we could provide which may allow us to culture the organism and thus learn more directly. This has already proven at least partially successful since Teske *et al* (1996) used DGGE analysis to identify the nature of two organisms present in co-culture and went on to improve the culturing conditions in order to isolate both organisms in pure culture (Teske *et al.* 1996). In the future it may be possible to successfully predict characteristics such as substrate utilisation, biochemical pathways or other cellular features such as biodegradative enzymes. The vast amounts of genetic information generated by large scale sequencing projects and the increasing number of environments subject to metagenomic analysis will provide a greater understanding of many facets of microbial life.

1.3: Acidic Environments

In the past many environments were considered far too harsh to harbour any life, however in recent years we have realised that "where there is liquid water on Earth, virtually no matter what the physical conditions, there is life." (Rothschild and Manicinelli 2001). Extremes of temperature, salinity, pH and pressure are commonly regarded as extreme environments, but other less typical environments such as those contaminated by heavy metals or other pollutants are also extreme. Most extreme environments are dominated by prokaryotes, both bacteria and archaea, but some extremophilic eukaryotes are known. Extreme environments of particular relevance to this research include acid mine drainage (AMD) and of most relevance, other acid tar lagoons found in various locations around the world.

1.3.1: Acid mine drainage (AMD)

Acid mine drainage refers to the waters which are discharged from abandoned metal and coal mines and is an increasing problem in many areas of the world. It is likely to continue to be a problem for many years to come as water continues to leach from abandoned mines. These waters are usually high in soluble iron and sulphate as a result of oxidative dissolution of sulphidic minerals, most prevalently pyrite (FeS_2) (Hallberg and Johnson 2003). AMD has several major effects on the environment including sedimentation of ferric iron (Figure 1.4), metal toxicity, salinisation and acidity; all of which lead to the death of fish and plants sensitive to the acid produced (Leduc *et al.* 2002).

Figure 1.4: An acidic iron-rich stream within an abandoned mine. Figure from Johnson (1998)

The overall equation for the chemical processes occurring as a result of water leaching from coal or metal mines is as follows (Johnson and Hallberg 2005):

$4FeS_2 + 15O_2 + 14H_2O \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+$

However, the four step process which results in the above overall equation begins with oxidation of pyrite by ferric iron (Equation 1), and not molecular oxygen as the above equation might suggest. The second reaction in the series (Equation 2) also occurs in the absence of oxygen. Meanwhile, both reactions 3 and 4 (Equations 3 and 4) require molecular oxygen (Hallberg and Johnson 2003).

(1) $FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+$

(2) $Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+$

(3) $2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$

(4) $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$

A key step in this cycle is the regeneration of ferric iron, which reacts with pyrite and is reduced to ferrous, which allows propagation and continuation of oxidation of the sulphide mineral. This reaction can be chemically or biologically catalysed at pH > 4, however, it is primarily biological at pH levels < 4 (Johnson and Hallberg 2003; Savage and Tyrrel 2005).

Acidity is generated by the oxidation of reduced inorganic sulphur compounds (Equations 1 and 4) and from hydrolysis of ferric iron (Equation 2), thus the presence of molecular oxygen effects the proton acidity of the water leached from mines (Hallberg and Johnson 2003). Net acidity is comprised of both proton acidity and mineral acidity, where mineral acidity is the concentration of soluble metals including manganese and aluminium. Biological processes such as ammonification, denitrification and methanogenesis along with the formation of bicarbonate from the dissolution of basic minerals offsets net acidity and can increase alkalinity in drainage streams (Johnson and Hallberg 2005). Initially it was thought that the microorganisms involved in oxidation were limited to *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, however it has been shown that a collection of organisms are involved (Leduc *et al.* 2002).

1.3.2: Acid tar lagoons

Acid tar is a waste residue of coal and petrochemical processing technologies originating from the end of the 19th Century (Milne *et al.* 1986), which have now been abandoned. Acid tar production is a result of three chemical processes; benzole refining, white oil production and oil re-refining (Nancarrow *et al.* 2001). All of the processes use concentrated sulphuric acid as a washing liquid to purify organic material, resulting in a residual tar which contains a high proportion of sulphuric acid. Historically, this waste was simply disposed to landfill, existing holes or lined lagoons, usually in close proximity to chemical plants.

Generally, acid tars are "dark-coloured liquid with a strong acrid odour" (Milne *et al.* 1986) with a varying odour between each type of acid tar production. However, all tars produce a persistent, penetrating and noxious odour; initially it is a very acrid smell due to the release of large amounts of sulphur dioxide. Sulphur dioxide release decreases after time resulting in different odours according to the method of tar production with benzole refinery acid tars possessing a strongly aromatic smell. In 1972, the "Disposal of Poisonous Wastes Act" (DPWA) was introduced which led to more regulated treatment and control of acid tar waste, however the scale of acid tar treatment and disposal prior to this legislation is unclear. Furthermore, whilst the introduction of DPWA greatly improved the waste disposal situation, the treatment and disposal of acid tar waste still followed a disorganised route, with a number of unsuccessful methods used for technical and economical reasons.

Attempts to treat acid tar waste varied according to the method of production; with the benzole refinery industry preferring to mix the tar with water or steam in an attempt to remove or dilute sulphuric acid resulting in a less acidic, tarry substance which was usually tipped on-site or in close proximity. Several other methods were used in an attempt to treat the acid tar waste however there are strong indications that none of these methods were successful, ultimately resulting in the disposal of potentially dangerous waste to an unknown number of contaminated sites. In some cases in South Yorkshire the contaminated area was covered in order to mask the disposal site; however this caused further problems in the future. Ground movements, heat and the weight of the overlying material caused the waste to begin to appear at the surface of the site, causing a danger to local residents and animals who may come into contact with the waste (Milne *et al.* 1986).

Since legislation was introduced there have been three main types of treatments; stabilisation, "pseudo-landfill" and incineration. Stabilisation methods include the use of organic and inorganic materials whilst "pseudo-landfill" methods are based on older methods which attempt to remove components of the waste before the residue is tipped into landfill sites. Consequently, almost all of the UKs acid tar waste is being handled by a waste disposal company in the West Midlands which removes the sulphuric acid from the waste and disposes of the residue in landfill. Disposal of aid tar waste via incineration using fluidised bed incineration and cement kilns is common in European countries, with cement kilns proving to be of economical advantage to both the cement industry and the producers of the acid tar waste.

The condition of disposal sites for acid tar waste and nature of the waste material itself varied greatly; therefore the environmental impact of acid tar lagoons is highly variable. There are more than 150 registered sites within UK and many unregistered ones (Smith *et al.* 2004) which are of concern due to the physical mobility of the tar and the hazardous nature of certain tar components such as polycyclic aromatic hydrocarbons (PAHs), BTEX (Benzene, Toluene, Ethylbenzene and Xylene) and sulphuric acid. Table 1.1 lists some of the acid tar lagoons in the public domain across the UK, Europe and the United States of America.

Name	**Location**	**Status**	**Volume (Tonnes)**	**Depth (m)**
Hoole Bank	Near Chester, Cheshsire	Unremediated	62000	11-14
Llwyneinion	Near Wrexham, Wales	Unremediated	94000 + 7500	~ 10
Cinderhills	Near Belper, Derbyshire	Unremediated	63600 (in 7 pits)	9-10
Rieme	Belgium	Undergoing remediation	200000	n/a
Mittelbach	Neukirchen, Mittelbach, Germany	Remediated/Undergoing remediation	> 100000	n/a
Sand Springs	Oklahoma State, USA	Remediated	135000 cubic yards	n/a

Table 1.1: A brief description of some acid tar lagoons in the public domain. Data taken from http://www.acidtarlagoons.org.uk/.

There is a great deal of heterogeneity within each acid tar lagoon and between each acid tar lagoon. The acid tar is not homogeneous, with each particular acid tar characteristics highly dependent on its production process, age, disposal environment and the presence of any co-disposed material. Most acid tars are quite viscous, and are able to migrate on the surface, through fissures and in the sub-surface. This allows acid tar to seep and leak from the lagoon and further contaminate the immediate environment. Due to the relatively high density of acid tar, water will always pool on top of tars which can cause further problems since attempts to cap several lagoons have been unsuccessful due to instability problems. The heavier capping layer pressurises the lighter tar causing it to migrate through fissures wherever possible and in acid tar lagoons which have been capped this has been shown to be one of the biggest factors for tar migration.

Acid tar lagoons which are in the public domain in the UK include Cinderhills (Derbyshire), Llwyneinion (North Wales) and Hoole Bank (Cheshire), the site at the centre of this research. Llwyneinion Lagoon (Figure 1.5) is considered the most serious tar waste disposal problem in North Wales, with estimates of remediation costs at more than £ 100 million. This acid tar lagoon is the sister site to Hoole Bank acid tar lagoon, with acid tar waste from the same chemical plant disposed at both sites, and as such is probably the most similar acid tar lagoon to Hoole Bank acid tar lagoon.

The site is divided into three tipping locations, containing a total of 94000 tonnes of liquid acid tar, covered by ponded rainwater. Within the tar body is distributed an unknown number of steel drums containing unidentified chemical waste material. The Llwyneinion site also has a long history of industrial use, including mining of a coal seam known to outcrop beneath the present location of the waste lagoon. Records indicate the presence of potentially uncapped, unfilled disused mine shafts beneath the lagoon floor.

In 1980 planning for site remediation began, following a major fire at the lagoon which burned on the surface of the lagoon for two days. It is thought that the fire began when a drum containing a sodium product decayed, causing the sodium to ignite when it came into contact with water. This caused the 75 mm layer of volatile hydrocarbons which floated on the 0.5 m of rainwater to ignite and burn (Reynolds 2002). The fire burnt off the volatile hydrocarbon, which also resulted in the evaporation of the acid water due to the heat of the fire eventually leading to the acid tar beneath the rainwater setting on

fire. Since the fire the Llwyneinion Lagoon has been covered by approximately 0.5 m of rainwater except in unusually hot dry weather when this evaporated allowing hydrocarbon fumes to be produced, causing a health risk to local residents and an odour nuisance. The seepage of volatile hydrocarbons up through the acid tar is shown in Figure 1.6 where the hydrocarbons have caused snow-covered ice to melt in certain areas of the lagoon.

Figure 1.5: Llwyneinion Lagoon, Wrexham. Figure from Reynolds (2002).

***Figure* 1.6:** Areas of snow-covered ice melt out due to uprising gas bubbles and hydrocarbons at Llwyneinion *Lagoon. Figure from* Reynolds (2002)

1.3.3: Hoole Bank acid tar lagoon, Cheshire, UK

Hoole Bank acid tar lagoon (Figures 1.7 and 1.8) is a 3.5 ha area located at National Grid Reference SJ 433 692, approximately 0.5 km east of the M53 motorway and 4 km northeast of Chester. The site is a former brickworks factory set in a rural area with residential, agricultural and commercial properties in the vicinity and surroundings.

The lagoon arose as a result of over 62000 tonnes of liquid acid tar waste from benzole refining being poured into an excavated clay pit until 1967. The waste was produced by a company called Lobitos, which was taken over by Burmah-Castrol Company, now part of BP Anaco. The sludge deposited into the pit consisted of a mixture of tar-like hydrocarbons, which included spent bentonite and absorbed heavy oil, sulphuric acid and other oily substances. The acid tar waste arrived warm and in a semi-fluid state, and therefore was simply poured into the pit. In addition to the acid tar waste, several chemical drums were also fly tipped into the sludge. However, nothing is known about the origins, contents or amounts contained within these drums.

Most of the tar waste remains viscous and mobile, however, some semi-solid tar layers have developed. The tar is described as "black, acrid-smelling, corrosive and toxic." (Nichol 2000). It is difficult to define the chemical properties of the lagoon, given the heterogeneity of the tar both in terms of its distribution and mixture, however an average breakdown reveals its composition to be 44 % sulphuric acid, 42 % oil residues, 8 % sulphated oil residues and 6 % water (Nichol 2000).

The 1.1 ha unlined lagoon is up to 9 m deep in certain places with most of the surface covered by less than 1 m of rain water, with the western side of the lagoon filled with water seasonally and after long periods of wet weather, whereas the eastern part of the lagoon is water filled continuously. The site is surrounded by farmland, a disused domestic waste disposal site and commercial business. However there are some residential properties in close proximity to the site. A report commissioned by the UK Environment Agency in 1999 (Unpublished) concluded that the site must be secured against unauthorised access and as such the site was fenced off (Figure 1.9). The report also noted that run-off water needed to be treated and that some outcrops of tar should be moved to within the lagoon.

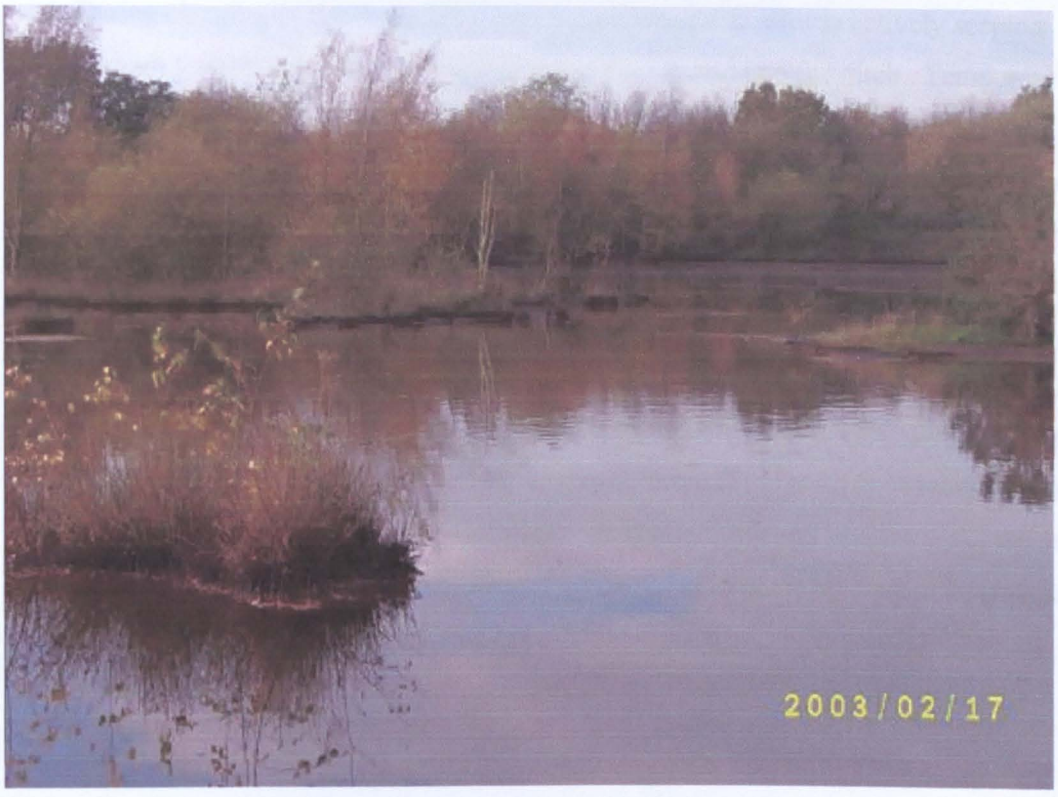


Figure 1.7: Image of Hoole Bank acid tar lagoon, Cheshire, UK.

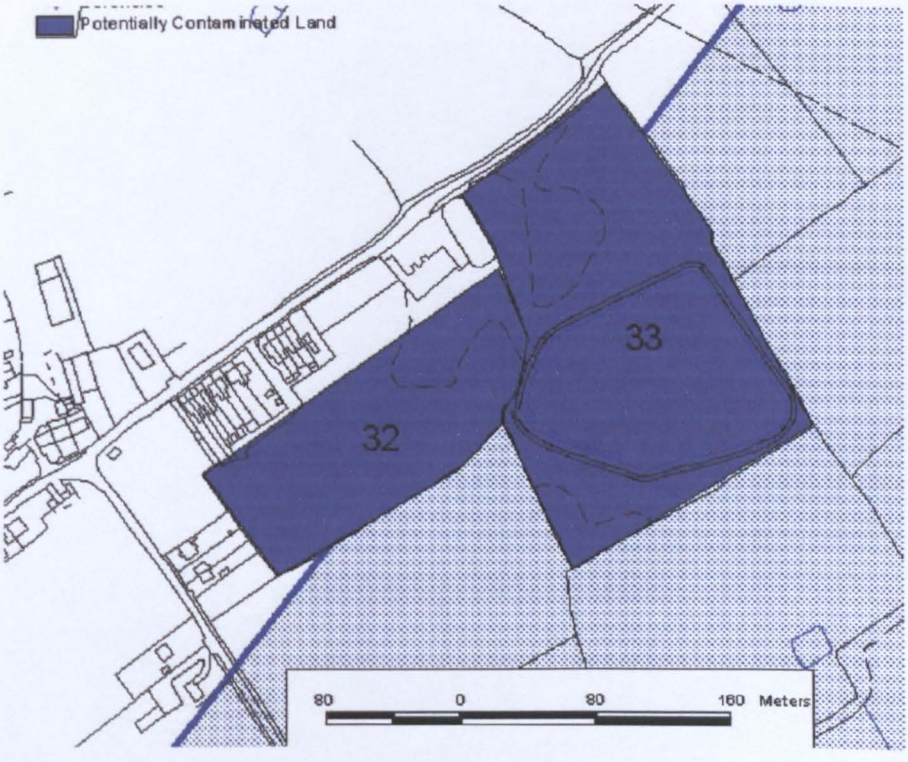


Figure 1.8: Aerial schematic of the Hoole Bank acid tar lagoon site, Cheshire. Diagram taken from Cheshire City Council Contaminated Land Strategy report (2006).

During cleaning of the drain ditch area it was noted that oil was actively seeping through the retaining bund surrounding the lagoon and into the ditch. Temporary measures were put in place but as yet no lasting solution to this problem has been found. It is expected that the bund surrounding the lagoon will need strengthening and the oil seeping from the bund will need to be contained (Chester City Council, 2006). An oil interceptor had been put in place at the head of the drainage ditch to collect and retain oil after it has seeped through the bund to prevent further pollution of the ditch. However, it is clearly visible upon inspection of the site and the interceptor that it is no longer functioning properly and has reached its capacity as it is clearly overflowing, causing the ditch to become contaminated once more (Figure 1.10). Figure 1.9 also highlights other areas of the lagoon where tar is migrating and seeping up to the surface, in this case outside of the lagoon fence boundary put in place by the Environmental Protection Team.

Figure 1.9: Fencing around the edge of Hoole Bank acid tar lagoon. Tar seepage is clearly visible outside the fence boundary as a result of tar migration.

Figure 1.10: An image of the overflow of acid tar from the oil interceptor and into the ditch at Hoole Bank acid tar lagoon.

1.3.4: Remediation of acid tar lagoons

In recent years attempts to remediate acid tar lagoons have been carried out in Belgium and the U.S.A. The acid tar lagoons in Belgium contain more than 200 000 tonnes of acid tar material in total, which will be remediated by treating the material with various additives after it has been excavated (Pensaert 2005). Full scale remediation work began in March 2005 and was expected to finish in early 2007 and should result in a neutralised, solidified product. As yet there has been no update as to the success of this work however preliminary small scale experiments were encouraging.

Acid tar sludge at a Superfund site in the U.S.A was successfully remediated ahead of schedule and below the expected costs in the mid-1990s using a process known as Dispersion by Chemical Reaction (DCR) (Grajczak 1995). DCR is a patented stabilisation process for waste treatment originally developed by Professor Friedrich R Boelsing in Germany. Hydrophobised CaO is used in the DCR process to remediate organic-contaminated materials. Calcium oxide, in its pure state, is hydrophilic therefore hydrophobic and oleophilic lime is prepared by treating CaO with natural fatty acids. This process delays the hydration step and allows the fatty acid-coated CaO reagent to preferentially adsorb oils during a mixing step. The delayed hydration then produces calcium hydroxide $Ca(OH)_2$, which is fractured into submicron sized particles (Equation 1). This hydration reaction is highly exothermic. Hydrate particles are homogeneously charged throughout their internal and external cavities with the oil phase. The finely dispersed $Ca(OH)_2$ then slowly reacts with natural CO_2 to generate relatively insoluble $CaCO_3$ (Equation 2) (Boelsing 1995):

1. $CaO + H_2O \rightarrow Ca(OH)_2 + \text{energy}$
2. $Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O$

Thus, the oil components are immobilised in a $CaCO_3$ matrix which is safe enough to handle and can be treated like ordinary soil.

These examples of remediation of acid tar lagoons and acid tar contaminated soils prove that it is possible to clean acid tar contaminated sites if the appropriate technology is applied and used correctly. The choice of remediation method used is very important and will need to be chosen carefully for each acid tar lagoon following detailed analysis of the lagoon and the properties of the acid tar at each site in order to achieve the best

possible results. The remediation of such large, contaminated sites is time consuming and may not be possible in all cases, given the differences and complexities of each acid tar lagoon. Therefore, further remediation methods such as bioremediation or the production of activated charcoal from acid tar waste provide alternative avenues for further research. Research into bioremediation of acidic environments has been carried out for several years, with the main focus of research on acid mine drainage (Johnson and Hallberg 2005) however more recently some research on bioremediation of acidic, hydrocarbon polluted environments has been carried out (Gemmell and Knowles 2000; Hallberg *et al.* 1999)

1.4: Survival of Microorganisms in Acidic Environments

The survival of microorganisms in acidic environments is a highly complex process which varies from species to species. Intracellular pH homeostasis is one of the most important factors to the survival of any cell, regardless of the external pH, therefore growth of acidophilic microorganisms in external pH as low as – 0.7 requires stringent control and regulation of internal pH in order for the cell to function. In most cases, acidophiles must maintain a near-neutral intracellular pH, which can be several pH units higher than the pH of the external environment (Matin 1990).

Proton motive force (PMF) is a key factor in energy generation and pH homeostasis for all microorganisms. The PMF describes the energised state of the cell membrane, and is composed of membrane potential, $\Delta\psi$, which is the difference in charge separation between the membrane and the external medium, and Δ pH, the difference in pH between the internal (cytoplasm) and external environment (Δ pH = $pH_{in} - pH_{out}$). PMF is calculated as follows: PMF (mV) = $\Delta\psi - 60\Delta$ pH (at 25 °C)

In most neutralophiles, PMF is approximately - 200 mV (inside negative), which results from a slightly negative Δ pH, in the approximate range -0.1 to -1.5 for most microorganisms, and a $\Delta\psi$ around 100 mV. However, acidophiles have a much greater Δ pH. This could be seen as advantageous as it allows the formation of a larger PMF and therefore for a greater proton influx though F_0F_1-ATPases and consequently increased ATP generation. However, uncontrolled influx of protons into a cell can be extremely harmful, potentially leading to cell death. An increased proton concentration within the cell inhibits protein function and stability of nucleic acids, impedes enzymatic activity, protein synthesis and transcription of DNA, ultimately resulting in

the complete dissipation of Δ pH which would result in a much decreased PMF incapable of meeting the cells energy requirements (Baker-Austin and Dopson 2007).

There are several known mechanisms by which acidophiles can minimise or prevent proton influx into the cell, as well as other mechanisms which allow the cell to tolerate and survive a small influx of protons into the cell. Mechanisms which inhibit or prevent proton influx into the cell include reversing the membrane potential ($\Delta\psi$), possessing a highly impermeable cell membrane and proton pumping. Meanwhile, cytoplasmic buffering, along with the presence of chemicals and enzymes which sequester protons within the cell and an increased number of secondary transporters help the cell to cope with proton influx (Matin 1990).

One of the most commonly observed adaptations in acidophiles is a reversal of membrane potential. All neutralophilic microorganisms have an inside negative $\Delta\psi$ whereas most acidophiles possess an inside positive $\Delta\psi$, a reversal of the normal membrane potential (Figure 1.11). A reversed membrane potential is a mechanism for decreasing proton influx into the cell as it forms a chemiosmotic barrier which deflects positively charged protons, preventing them from entering the cell. It is thought that the reversed $\Delta\psi$ is generated by a Donnan potential (Donnan 1924) of positively charged molecules, particularly accumulation of K^+ ions. The creation of a reversed $\Delta\psi$ by an accumulation of K^+ ions is supported by evidence that there is an increased number of potassium-transporting ATPases found in acidophile genomes (Futterer *et al.* 2004; Tyson *et al.* 2004). Reversal of $\Delta\psi$ results in a decrease in the overall proton motive force; however the reversed $\Delta\psi$ detracts from such a large Δ pH that the final PMF is large enough to meet the cells energy demands.

Further difficulties are encountered as a result of the reversed membrane potential. When the cell is in a reenergised state, such as in acidic media, the positive inside membrane potential results in the exclusion of permeant cations and the accumulation of permeant anions, which can begin to poison the cell. Whilst this is true there is one exception, SO_4^{2-}, the most common anion in acidic environments. Acidophiles are able to tolerate higher levels of SO_4^{2-} than other anions for two reasons. The first reason is that acidophiles have adapted systems to remove SO_4^{2-} from inside the cell, whilst the

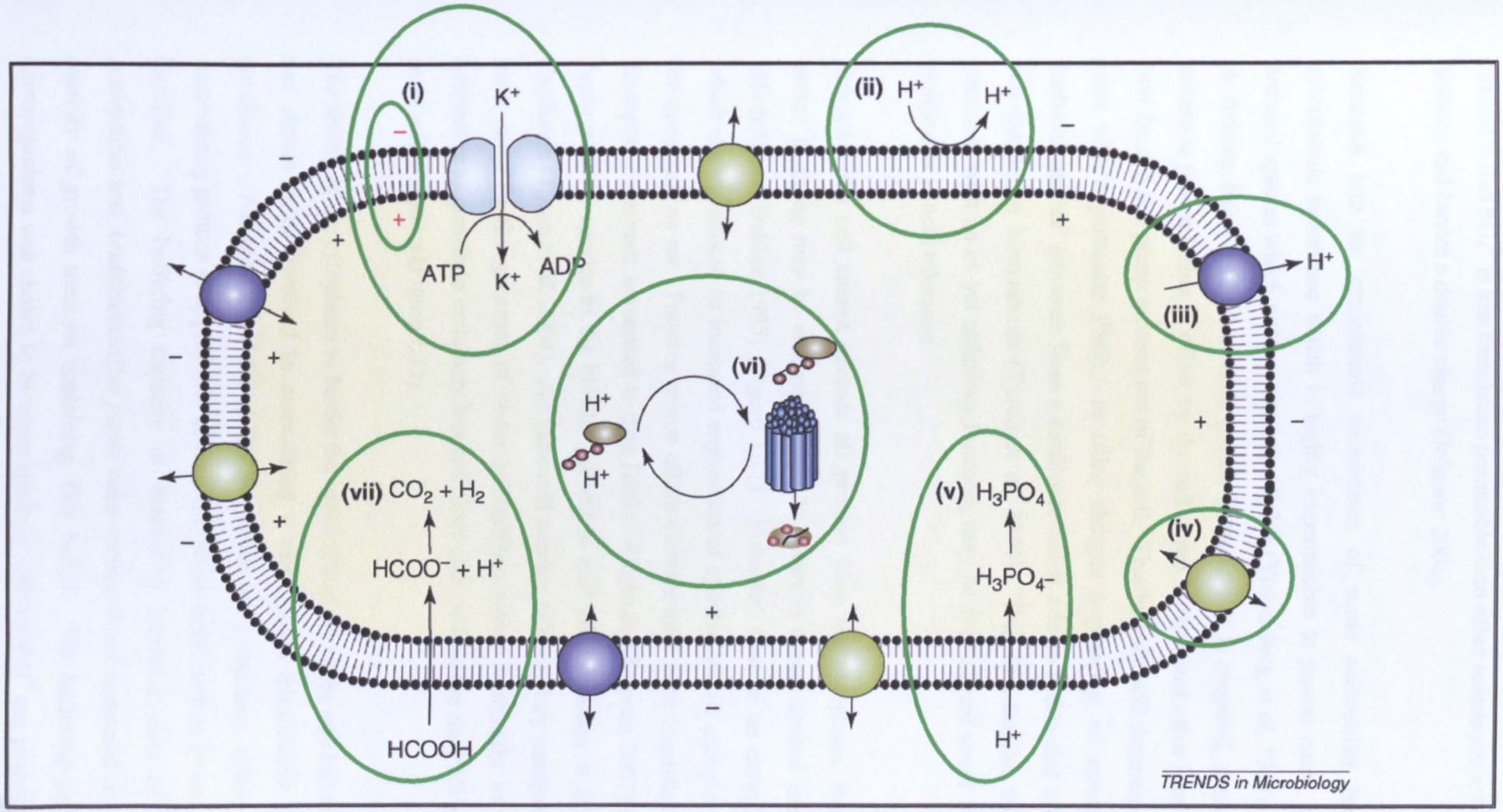


Figure 1.11: Diagrammatic representation of commonly occurring adaptations to low pH observed in acidophilic microorganisms. The green circles highlight several adaptations including (From top left, clockwise) reversed membrane potential, increased K^+ ion accumulation, impermeable cytoplasmic membrane, proton pumping, increased number of secondary transporters, cytoplasmic buffering, DNA and protein repair mechanisms and weak acid degradation pathways. Diagram adapted from Baker-Austin and Dopson (2007).

second is that SO_4^{2-} is less membrane permeable than other anions since it has additional polarity and carries a double charge (Johnson 2006).

Research into the cytoplasmic membranes of some acidophiles has revealed a cytoplasmic membrane which is highly impermeable to proton entry, particularly in archaeal species which possess tetraether lipids (Vossenberg *et al.* 1998) (Figure 1.11). A delicate balance of membrane proton permeability is required, since the need to minimise proton influx is offset by the cells need to transport other ions and nutrients into the cell and pump protons out of the cell. This balance will determine if a cell can cope with a particular PMF. *In silico* shotgun sequencing of several acidophilic bacterial species' genomes from a biofilm revealed a large and varied set of genes for cell membrane biosynthesis (Tyson *et al.* 2004). This data implies that a complex structure, with an as yet unknown function, may be formed and could be an intrinsic component in acid tolerance.

Inevitably the cell cannot exclude all protons from the cytoplasm, therefore active proton pumping may be utilised in order to maintain a near-neutral intracellular pH (Michels and Bakker 1985) (Figure 1.11). However, there is no definitive evidence which shows induced or increased expression of systems which exclude protons from the cytoplasm as yet. Putative proton efflux systems have been identified in all of the acidophilic genomes sequenced to date (Baker-Austin and Dopson 2007) including the *Leptospirillum* species in the biofilm present in acid mine drainage at Iron Mountain, California (Tyson *et al.* 2004). An increased number of secondary transporters has also been noted in the genomes of some acidophiles, which is thought to be a further adaptation to growth in extremely low pH, since cells will utilise the PMF generated for metabolic purposes (Figure 1.11).

The ability of the cytoplasm to buffer the intracellular pH of the cell has been observed and shown to be involved in maintaining a near-neutral intracellular pH in acidic conditions. All cells have the ability to buffer the cytoplasm, either releasing or sequestering protons as appropriate using basic amino acids such as lysine, arginine and histidine. The buffering capacity of respiration inhibited cells of *Thiobacillus acidophilus* and *Acidithiobacillus facile* were measured and compared to the buffering capacity of growth medium containing Tris buffer. The buffering capacity of the microorganisms was shown to be approximately 100 nmol H^+ mg protein $^{-1}$ pH unit $^{-1}$

(Matin 1990), showing a much smaller decrease in cytoplasmic acidification than would be expected given the net H^+ influx into the cells compared to the buffered medium. Comparisons of the buffering capacity between *E. coli* and *Acidithiobacillus acidophilum* were also carried out, with buffering capacities of 85 nmol H^+ mg protein $^{-1}$ and 97 nmol H^+ mg protein $^{-1}$ respectively (Zychlinsky and Matin 1983) (Figure 1.11).

Of particular concern to acidophiles is the uptake of weak acids (HA) as they function as uncouplers of the respiratory chain, which shuttle the net uniport of protons and 'uncouple' proton transport from cellular processes (Ciaramella *et al.* 2005). These compounds can cross the cell wall in their undissociated form as they are uncharged at acidic pH (the external pH) but once they enter the cell, near-neutral pH, they become undissociated (H^+ and A^-) and can no longer leave the cell in their charged form. Accumulation of weak acids by this process is problematic for cells, as protonation of the cytoplasm can occur quickly, therefore some acidophiles have developed active mechanisms of weak acid degradation in order to overcome this problem (Ciaramella *et al.* 2005) (Figure 1.11).

Other mechanisms involved in pH homeostasis include the use of DNA and protein repair chaperones and the use of iron to stabilise enzymes and enzyme complexes (Baker-Austin and Dopson 2007). However, after comparisons of the genomes of several acidophilic microorganisms there appears to be no definitive individual adaptation or set of adaptations present in every acidophile which enables growth in low pH environments.

1.5: Acidophiles

Acidophiles are most commonly defined as organisms with an optimum growth at pH 3 or below, however organisms which can grow sub-optimally at pH 4 or below may be considered moderate acidophiles. Acidic environments capable of sustaining life are usually dominated by sulphate anions, and there is usually only a low concentration of dissolved organic matter, with as little as 20 mg l^{-1} dissolved carbon in some environments (Johnson 1998). Thus, the most commonly found organisms are chemolithotrophic bacteria and archaea, however some heterotrophic acidophiles can be supported from organic matter fixed by chemolithotrophic organisms. Acidophiles are most commonly grouped according to their carbon source and method of energy generation and further subdivided based on their optimum temperature for growth

(Table 1.2). The following discussion includes several subdivisions of acidophiles since it is likely that any microorganisms isolated from Hoole Bank acid tar lagoon samples will be acidophiles.

1.5.1: Iron oxidising prokaryotes

One of the best studied and well characterised acidophilic microorganisms is the chemolithotroph *Acidithiobacillus ferrooxidans* (formerly known as *Thiobacillus ferrooxidans*). *Acidithiobacillus ferrooxidans* is a mesophilic non-motile, iron oxidising rod which attacks pyrite by oxidising Fe^{2+} to $Fe^{3+}$and has an optimum growth pH between 1.8 and 2.5 (Rawlings *et al.* 1999). *Acidithiobacillus ferrooxidans,* affiliated to the *β/γ-proteobacteria,* was one of the first acidophiles to be discovered and thus has been the subject of much study, however data suggests that it is not actually the most dominant organism in some acidic environments (Rawlings *et al.* 1999).

Mesophilic organisms belonging to the phyla *Nitrospira* and *Thermoplasmales* compose a significant majority of the iron oxidising bacteria. *Leptospirillum* species are prominent within this group of acidophiles, notably including *L. ferrooxidans,* a particularly acid tolerant organism capable of growth at pH 1.2 which uses Fe^{2+} as its sole electron donor (Johnson 1998; Rawlings *et al.* 1999). *Leptospirillum ferrooxidans* has a higher affinity for Fe^{2+} than other bacteria, which may explain why it is more dominant in environments with lower iron concentrations.

Ferroplasma isolates are also part of this group and include an organism of particular interest, *"Ferroplasma acidarmanus". Ferroplasma acidarmanus* is a facultatively anaerobic acidophilic archaeon which is able to grow between pH 0 and 1.5, but most interestingly it grows chemoorganotrophically, combining oxidation of yeast extract to iron (III) reduction (Dopson *et al.* 2004). There are also some extremely thermophilic iron oxidising prokaryotes including the obligate aerobe *Sulfolobacillus yellowstonii* and the facultative anaerobe *Acidianus brierleyi* (Table 1.2).

1.5.2: Sulphur oxidisers

Several sulphur oxidising prokaryotes are moderately or extremely thermophilic, with optimum growth temperatures above 40 °C and 60 °C respectively. *Acidithiobacillus caldus* is an aerobic moderate thermophile capable of growth up to 55 °C, known to be found in hot springs in Iceland, New Zealand and the United States and has recently

been isolated from hot springs in Monsterrat (Burton and Norris 2000). Extreme thermophiles utilising sulphur oxidation include the autotroph *Sulfolobus metallicus* and the mixotrophic *S. hakonesis*, both of which belong to the order *Sulfolobales* within the crenarchaeotal branch of the domain archaea.

Acidithiobacillus ferrooxidans is also capable of oxidising sulphur, along with other mesophilic sulphur oxidisers such as the mixotroph *Thiobacillus acidophilus*, also a member of the β/γ-*proteobacteria*. However, with a maximum growth temperature of 70 °C, *Hydrogenobacter acidophlius*, a sulphur oxidising bacterium, has the highest known growth temperature of any sulphur oxidising acidophile.

1.5.3: Heterotrophic organisms

Heterotrophic organisms are important to the ecology of acidic environments, as some species contribute to mineral dissolution both directly and indirectly. *Acidiphilium* spp. is an Fe^{3+} reducer which increases the rate of iron dissolution in geothite and jarosite. Heterotrophs such as *Thermoplasma* and *Acidocella* may utilise the natural organic compounds produced by chemolithotrophic iron- and sulphur- oxidising organisms thereby detoxifying the environment of inhibitory substances such as organic acids which would otherwise prevent growth of organisms sensitive to such compounds (Johnson and Hallberg 2003).

Many acidophilic heterotrophs are archaea, including the moderate thermophiles *Picrophilus torridus and P. oshimae*, both of which are strict anaerobes but most importantly, they have the lowest recorded pH optimum of any acidophile at pH 0.7. (Schleper *et al.* 1995). As of January 2003, there was only one fully documented strictly heterotrophic thermophilic acidophile, *Alicylclobacillus* sp (Johnson *et al.* 2003).

Acidophilic prokaryotic microorganisms

Mineral-degrading acidophiles	Thermal classification*	Phylogenetic affiliation
1a. Iron-oxidizers		
Leptospirillum ferrooxidans	Meso	*Nitrospira*
L. ferriphilum	Meso	*Nitrospira*
L. thermoferrooxidans	Mod Thermo	*Nitrospira*
"*Thiobacillus ferrooxidans*" m-1	Meso	β-*Proteobacteria*
"*Ferrimicrobium acidiphilum*"	Meso	*Actinobacteria*
Ferroplasma acidiphilum	Meso	*Thermoplasmales*
"*Fp. acidarmanus*"	Meso	*Thermoplasmales*
1b. Sulfur-oxidizers		
Acidithiobacillus thiooxidans	Meso	β/γ-*Proteobacteria*
At. caldus	Mod Thermo	β/γ-*Proteobacteria*
Thiomonas cuprina	Meso	β-*Proteobacteria*
Hydrogenobacter acidophilus	Mod Thermo	*Aquifacales***
Metallosphaera spp.	Ext Thermo	*Sulfolobales*
Sulfolobus spp.	Ext Thermo	*Sulfolobales*
1c. Iron- and sulfur-oxidizers		
Acidithiobacillus ferrooxidans	Meso	β/γ-*Proteobacteria*
Acidianus spp.	Ext Thermo	*Sulfolobales*
Sulfolobus metallicus	Ext Thermo	*Sulfolobales*
1d. Iron-reducers		
Acidiphilium spp.	Meso	α-*Proteobacteria*
1e. Iron-oxidizers/reducers		
Acidimicrobium ferrooxidans	Meso	*Actinobacteria*
1f. Iron-oxidizers/reducers and sulfur-oxidizers		
Sulfobacillus spp.	Meso and Mod Thermo	*Firmicutes*
2. Heterotrophic acidophiles (non mineral-degrading)		
Acidocella spp.	Meso	α-*Proteobacteria*
Acidisphaera rubrifaciens	Meso	α-*Proteobacteria*
Acidobacterium capsulatum	Meso	*Acidobacterium*
Acidomonas methanolica	Meso	α-*Proteobacteria*
Alicyclobacillus spp.	Meso	*Firmicutes*
Picrophilus spp.	Mod Thermo	*Thermoplasmales*
Thermoplasma spp.	Mod Thermo	*Thermoplasmales*
3. Obligate anaerobes		
Stygiolobus azoricus	Ext Thermo	*Sulfolobales*
Acidilobus aceticus	Ext Thermo	*Sulfolobales*

* Meso—mesophiles ($T_{optimum}$ < 40 °C); Mod Thermo—moderate thermophiles ($T_{optimum}$ 40–60 °C); Ext Thermo—extreme thermophiles ($T_{optimum}$ > 60 °C).

** Inferred ability to oxidize minerals (via production of sulfuric acid).

Table 1.2: Acidophilic prokaryotic microorganisms

Taken from Johnson *et al.* (2003)

1.5.4: Acidophilic eukaryotes

Surviving at low pH is arguably more difficult for eukaryotic cells than prokaryotic cells since the problems encountered by prokaryotic acidophiles (as discussed in Section 1.4) are equally applicable to eukaryotes, yet the cellular processes of a eukaryote are considerably more complex. For example, eukaryotes must cope with acid conditions on the surface of the plasma membrane. Ion channels and transporters are in contact with the low pH of the external environment, which would minimally require molecular modifications compared to the equivalent proteins of neutralophilic organisms (Messerli *et al.* 2005). Nevertheless, several algal, yeast and fungal species thrive in acidic environments, often playing key roles in the microbial communities present in these environments, particularly when biofilms are present.

A high degree of eukaryotic diversity has been reported in the Rio Tinto, Spain, with eukaryotic organisms forming up to 60 % of the biomass in some cases (Lopez-Archilla *et al.* 1993). Lineages previously undetected in the Rio Tinto such as ciliates, cercomonads, vahlkampfiid, amoebae, stramenopiles and fungi were identified by Zettler *et al* (2002), alongside the discovery of taxa never previously found in extreme habitats before. Eukaryotic organisms have also been implicated in the development and structure of biolfims present in the Rio Tinto, with 14 taxa identified from Rio Tinto biofilms in total. Algal species belonging to the genera *Dunaliella* and *Cyanidium* were found to dominate during biofilm formation (Aguilera *et al.* 2007) whilst other algal genera present in the biofilms throughout their development include *Zygnemopsis*, *Klebsormidium* (filamentous algae), *Chlamydomonas*, *Euglena* (flagellated algae) and *Chlorella* (sessile algae). Other eukaryotic organisms detected in the biofilms also included amoebae, diatoms, and several fungi (Aguilera *et al.* 2007).

Nixdorf *et al* (2001) report at least six classes of algae with acidophilic representatives found in acidic mining lakes, and their closest neutralophilic relatives (Table 1.3). The diversity of diatoms in acidic environments has also been reviewed (DeNicola 2000). At least 124 different taxa detected in acidic environments, of which 19 have been found in more than one environment and are considered abundant. However, this includes taxa detected between pH 4.5 and 5.0, whilst reports suggest that diatom diversity is significantly decreased at pH $\leq$ 3.5 (DeNicola 2000). Some of the abundant diatom taxa identified in the DeNicola (2000) review include *Achnanthes minutissima*, *Eunotia pectinalis*, *Frustulia rhomboides*, *Nitzschia communis* and *Pinnularia*.

Class	Species/taxon in acidic mining lakes	Typical genera in wetlands
Chlorophyceae	*Chlamydomonas* sp.	Chlamydomonas
	Scourfieldia cordiformis	Chlorella
	Ulotrichales	Stigeoclonium
	Chlorogonium sp.	Oedogonium
	Choricystis sp.	Spirogyra
	Schroederia setigera	Hydrodictyon
	Stichococcus sp.	
Chrysophyceae	*Ochromonas* sp.	Ochromonas
	Chromulina sp.	Chromulina
	Synura sp.	Dinobryon, Mallomonas
Dinophyceae	*Gymnodinium* sp.	Gymnodinium
	Peridinium umbonatum	Peridinium
	Amphidinium elenkinii	
Euglenophyceae	*Lepocinclis ovum*	Euglena, Trichomonas
	Trachelomonas volvocina	Phacus, Strombomonas
Bacillariophyceae	*E. exigua*	Navicula, Fragilaria
	Eunotia sp.	Pinnularia, Melosira
	Navicula sp.	Cyclotella
	Nitzschia sp.	
Cryptophyceae	*Cryptomonas marssonii*	Cryptomonas
	Cryptomonas erosa	Rhodomonas, Chilomonas
	Cryptomonas ovata	

Table 1.3: Typical algal colonisers of acidic mining lakes and their neutralophilic wetland colonising counterparts. Table from Nixdorf *et al* (2001)

Baker *et al* (2004) studied the eukaryotic diversity present at the Iron Mountain acid mine drainage site in California, revealing a lineage of the red algae *Rhodophyta*, members of the family *Vahlkampfiidae* and three fungal isolates putatively named "*Acidomyces richmondensis*" which are most closely related to the fungal class *Dothideomycetes* based on 18S rRNA sequences.

1.6: Aims of the Project

A number of samples were collected from Hoole Bank acid tar lagoon in February 2003. A range of techniques, both classical microbiology and molecular biology techniques, were used to characterise the microbial diversity found in Hoole Bank acid tar lagoon (Chapter 3).

Two specific microorganisms isolated from the Hoole Bank acid tar lagoon were chosen for further study. In Chapter 4 the eukaryotic alga, *Euglena gracilis* G46, is discussed in detail and in the final results chapter (Chapter 5) the prokaryotic bacterium, *Acidocella*, is further characterised. The results in Chapter 4 and 5 help to explain the mechanisms used by microorganisms to survive and grow in Hoole Bank acid tar lagoon.

Chapter Two: Materials and Methods

All chemicals were purchased from Sigma (Appendix B) unless otherwise stated.

2.1: Isolation of Microorganisms

2.1.1: Sample collection

Various samples were collected from around Hoole Bank acid tar lagoon into sterile 500 ml Nalgene bottles or sterile 50 ml Falcon tubes. Each sample was labelled at the time and a photograph taken at each sampling location. The pH of samples was measured using a Mettler Toledo MP225 pH meter. Samples were stored at 4 °C until required.

2.1.2: Growth media

½ Buffered Luria Broth (BLB) plates were prepared by adding 5 g yeast extract (Oxoid L21), 2.5 g tryptone (Oxoid, LP0042) and 1.5 g NaCl to 500 ml of distilled water, adjusting the pH to 3.0 with 1 M H_2SO_4 and autoclaving. A separate gelling solution was prepared by adding 10 g of agar no.1 (Oxoid, LP0011) to 500 ml of distilled water and autoclaving at 121 °C for 20 minutes. After autoclaving, both solutions were allowed to cool to approximately 50 °C, combined aseptically and gently inverted to mix before being poured into sterile plastic Petri dishes and left to set.

Acidiphilium medium (AC) plates were prepared by adding the following chemicals to 500 ml of distilled water: 0.5 g $MgSO_4.7H_2O$, 0.1 g $(NH_4)_2SO_4$, 50 mg KH_2PO_4, 50 mg KCl, 14.4 mg $Ca(NO_3)_2.2H_2O$, 1.0 g mannitol, 0.1 g tryptone soy broth. The pH was adjusted to pH 3.0 with 1 M H_2SO_4 and the solution autoclaved at 121 °C for 20 minutes. A separate gelling solution was prepared by adding 10 g of agar no.1 to 500 ml distilled water and autoclaving at 121 °C for 20 minutes. After autoclaving, solutions were cooled to approximately 50 °C, combined aseptically and inverted gently to mix, poured into sterile plastic Petri dishes and left to set. Liquid media was prepared simply by dissolving all the reagents except agar in 1 litre of distilled water and autoclaving at 121 °C for 20 minutes. The pH value of liquid media was checked after autoclaving and shown not to alter by more than 0.05 pH units.

During initial isolation of microorganisms from environmental samples, nystatin was added to all AC and ½ BLB media at 100 $\mu g\ ml^{-1}$ in order to inhibit fungal growth.

M6 medium consisted of 1 g $CH_3COONa.2H_20$, 2 g yeast extract, 2 g of tryptone (Oxoid, UK) and 10 ml of $CaCl_2$ stock solution (1g $CaCl_2$ in 1 litre of distilled water) in 1 litre of distilled water. The pH was adjusted as necessary with 1 M H_2SO_4 or 1 M NaOH prior to autoclaving at 121 °C for 20 minutes. Solid medium was produced by adding 10 g agar no 1 directly to the pH 7 medium, whilst pH 3 plates were made by making a separate gelling solution and a 2X concentrate of the medium and mixing after autoclaving as described above for AC and ½ BLB solid media.

Luria-Bertani (LB) medium consisted of 5 g yeast extract, 10 g tryptone, 5 g NaCl and 10 g Oxoid Agar No 1 in 1 litre of distilled water to produce solid medium. Liquid medium consisted of 5 g yeast extract, 10 g tryptone and 5 g NaCl per litre. Ampicillin was also added to LB in some cases, either at 100 $\mu g\ ml^{-1}$ in solid medium or 50 $\mu g\ ml^{-1}$ in liquid medium.

M9 minimal media were prepared by dissolving 10 g M9 minimal salts in 1 litre distilled water and adjusting the pH as necessary with 1 M H_2SO_4 or 1 M NaOH. Phosphate-free minimal medium was prepared as follows: 5 g NaCl, 5 g $(NH_4)_2SO_4$, 5 g KCl and 6 g Trizma base in 1 litre of water. The pH was adjusted to pH 3 or pH 7 with 1 M H_2SO_4 or 1 M NaOH as necessary.

2.1.3: Initial isolation of microorganisms using spread plating

Approximately 200 µl of raw environmental sample was spread plated onto both AC and ½ BLB plates. Each plate was labelled, sealed and incubated at 25 °C. Growth was monitored daily for 14 days and any colonies arising were streaked onto fresh plates in order to isolate pure cultures. After the third generation of plate growth, a single colony from each isolate was inoculated into approximately 10 ml of the appropriate liquid medium and sub-cultured into 250 ml flasks, containing 50 ml of medium, and shaken at 250 rpm at 26 °C.

2.1.4: Initial isolation of microorganisms using chemostat culture

A chemostat with a working volume of 1 litre was used to isolate microorganisms from environmental samples. Temperature was maintained at 25 °C with an aeration rate of 500 ml $minute^{-1}$. Samples were taken on a daily basis over the 7 – 10 day incubation period.

2.2: Growth conditions

2.2.1: *Euglena gracilis* Z and G46

Wild-type *Euglena gracilis* Z (CCAP 1224/5Z) and *E. gracilis* G46 (CCAP 1224/46) were routinely grown in M6 medium, at pH 7 and pH 3 respectively. Both organisms were grown under 8 hours light and 16 hours darkness at approximately 27 ± 2 °C.

2.2.2: *Acidocella sp.* (29)

Acidocella 29 was routinely grown in batch culture in AC medium at pH 3 (Section 2.1.1) at 26 °C and shaken at 250 rpm.

2.3: Chlorophyll Content

Chlorophyll content of algal cultures was determined by centrifuging a 5 ml sample at 3000 *g* for 10 minutes. Following centrifugation, the supernatant was removed and the pellet resuspended in 80 % acetone (Fisher Scientific) for 5 minutes. The sample was then centrifuged again for 10 minutes at 3000 *g* and the absorbance of the resulting supernatant measured against an 80 % acetone blank at 645 and 663 nm (Bruinsma 1961).

The chlorophyll concentration was then calculated as follows:

$$\frac{(\text{Absorbance at 645 nm} \times 202) + (\text{Absorbance at 663 nm} \times 80.2)}{5} = \mu g \text{ chlorophyll ml}^{-1}$$

2.4: Protein Determination

Determination of the amount of protein in a sample was measured using the Bradford Assay (Bradford 1976). 0.1 ml of the sample was placed into a test tube and 0.9 ml of 1 M NaOH added. The sample was then vortexed and heated at 90 °C for 10 minutes. The test tube was cooled on ice for 5 minutes prior to centrifugation at 3000 *g* for 10 minutes. 0.1 ml of the resulting supernatant was added to 3 ml of Bradford's reagent, vortexed and the optical density measured in the Unicam Heliosα spectrophotometer against 0.1 ml water plus 3 ml Bradford's reagent blank at 595 nm after a minimum of 5 minutes incubation.

2.5: Photosynthesis and Respiration Measurements

2.5.1: Oxygen electrode preparation

A modified Clark-type oxygen electrode (Hansatech Instruments Ltd, Norfolk, UK) was prepared for use by adding a few drops of 2.3 M KCl to the electrode disk before an approximately 1 inch square piece of cigarette paper was placed on top of the centre of the disk. The electrode Teflon membrane was placed on top of this and forced tightly onto the electrode disc using a membrane applicator and held in place by an O-ring (Figure 2.1). The disc was then placed into the chamber and connected to the control box. Oxygen was removed from the chamber by adding sodium dithionite to allow calibration of the electrode.

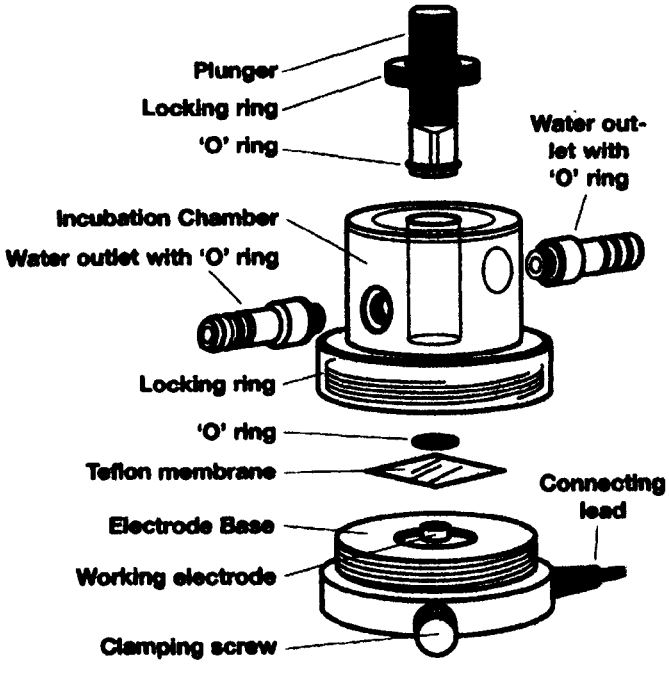


Figure 2.1: Schematic diagram illustrating the key components of an oxygen electrode. Figure from http://www.rankbrothers.co.uk/prod1exp.htm.

The reaction chamber, working volume 2 ml, was maintained at a constant 30 °C by circulating water from a temperature controlled water bath. A 12 V, 100 W tungsten-halogen lamp was used to illuminate the chamber when required, approximate output intensity 1800 μmol m^{-2} s^{-1}.

2.5.2: Algal pH shock experiments

Rates of oxygen evolution and uptake were measured in *E. gracilis* G46 cells grown in M6 medium at pH 3 and in *E. gracilis* Z cells grown in M6 medium at pH 7 using the oxygen electrode. Rates of oxygen evolution and uptake were also measured after pH shock experiments in which cells were resuspended in medium of a different pH to the growth medium. In steady state experiments cells were resuspended in fresh medium of the same pH as that of the growth medium. In shock experiments, cells were resuspended in different pH media ranging from pH 2 to pH 9 and rates of oxygen evolution and uptake were measured immediately. In all cases chlorophyll content of the cells was determined (Section 2.3) and samples adjusted to 30 μg chlorophyll ml^{-1}.

In all cases, 2 ml of the concentrated cells were pipetted into the chamber and left in the dark for 2 minutes. After the 2 minutes acclimatisation the samples were illuminated for approximately 2 minutes until oxygen evolution was linear, followed by approximately 2 minutes in the dark until oxygen uptake was linear. At the end of each experiment, the sample was carefully removed using a plastic pasteur pipette and the chamber rinsed several times with distilled water before the next sample was applied. Each sample was repeated in triplicate.

The rate of oxygen evolution or uptake was calculated as follows:

$$\text{Respiration rate} = \frac{\text{standard}}{\text{range}} \times \frac{\text{number of units}}{\text{time}} \times \frac{60}{\text{mg chlorophyll per sample}}$$

Standard: Oxygen solubility (0.406 μmoles O_2 ml^{-1} at 30 °C)

Range: Units taken from calibration

Number of units: Number of units covered in a certain period time, either with or without light

Time: The length of time in minutes for which the sample was measured

60: This converts the time from minutes to hours

mg chlorophyll present in sample: This relates to the chlorophyll content of the sample for example 30 μg chlorophyll ml^{-1} = 0.060 mg

The rate of respiration was taken as being the rate of oxygen uptake in the dark. To calculate the rate of photosynthesis it was assumed that the rate of respiration in the

light was equal to dark respiration. Therefore, the rate of photosynthesis was equal to oxygen evolution in the light plus oxygen uptake in the dark. This assumption is unlikely to be completely valid, however since the rate of respiration is generally a small proportion of the photosynthetic rate the error involved is likely to be small (Jackson and Volk 1970).

2.5.3: Bacterial pH shock experiments

5 to 10 ml of overnight *Acidocella* 29 culture were concentrated and resuspended in 2 ml M9 minimal medium at various pH values over the range pH 2 – 7. 2 ml samples were placed in the electrode chamber and the rate of respiration measured for 5 minutes after a linear decrease in oxygen was recorded. The electrode chamber was rinsed thoroughly with distilled water between samples. The rate of respiration was calculated using the equations in Section 2.5.2, with the exception that "mg chlorophyll present in the sample" is replaced by "μg of protein present in the sample", which was measured using the Bradford assay as described in Section 2.4.

2.5.4: Sodium orthovanadate inhibition of proton efflux in *E. gracilis*

Sodium orthovanadate, Na_3VO_4, was used as an inhibitor of Non-F_1F_0 ATP-ases in order to inhibit active proton efflux. Sodium orthovanadate was prepared in 100 mM stocks and used at 1 mM concentrations (20 μl of 100 mM stock added to 2 ml samples). *E. gracilis* G46 and *E. gracilis* Z were prepared by measuring chlorophyll concentration (Section 2.3) and concentrating the cells to 30 μg chlorophyll ml^{-1}.

Concentrated cells were resuspended in pH 3 and pH 7 phosphate-free minimal media (Section 2.1.2) and the rate of respiration was measured immediately. After 5 minutes of a linear respiration rate, 20 μl of 100 mM sodium vanadate was added and the respiration rate recorded for a further 5 minutes after a linear rate was achieved. This was carried out for both pH 3 and pH 7 resuspended cells. A further two sets of duplicate samples were also prepared; incubating the cells in phosphate-free minimal medium for 60 minutes with and without 1 mM sodium vanadate, both at pH 3 and pH 7, and the rates of respiration measured as previously described.

2.5.5: Sodium orthovanadate inhibition of proton efflux in *Acidocella* 29

15 – 25 ml samples of overnight *Acidocella 29* culture were centrifuged at 3000 *g* in the bench centrifuge for 10 minutes. Concentrated cells were resuspended in pH 3 and pH

7 phosphate-free minimal media (Section 2.1.2) and the rate of respiration measured after immediate resuspension. After 5 minutes of a linear respiration rate 20 µl of 100 mM sodium vanadate was added and the respiration rate recorded for a further 5 minutes after a linear rate was achieved. This was carried out for both pH 3 and pH 7 resuspended cells. A further two sets of duplicate samples were also prepared; incubating the cells in phosphate-free minimal medium for 60 minutes with and without 1 mM sodium vanadate, both at pH 3 and pH 7, and the rates of respiration measured as previously described. At least 1 ml of *Acidocella* 29 culture was retained for protein determination as described in Section 2.4. Rates of respiration were calculated as described in Section 2.5.2.

2.6: Cell separation using the silicone oil technique

Centrifugation through silicone oil was used to completely separate cells from the medium. A range of Dow Corning silicone oils (Fisher Scientific, UK) were used to obtain a range of different density oils by mixing individual oils in specific ratios. In order to determine the density of silicone oil required, cell samples of the desired chlorophyll or protein content as appropriate, were applied to the oil and centrifuged. *E. gracilis* samples were concentrated to 30 µg chlorophyll ml $^{-1}$, whilst *Acidocella* 29 samples were adjusted to an OD_{595} of 0.5. Oil of the correct density produced a pellet separated from the medium by a layer of oil with a non-inverted meniscus (Figure 2.2). Both *E. gracilis* G46 and *Acidocella* 29 required a 3:1 mix of 550 and 200 oils respectively to separate cells.

2.6.1: Determination of cell volume

Chlorophyll content of the cells was measured according to Section 2.3. Cells were then adjusted to 30 µg chlorophyll ml^{-1}. 5 µl of ^{14}C-dextran (1.85 MBq ml^{-1}) (MW = 70000 Da) was added to a 1 ml sample of concentrated cells and 10 µl of $^{3}H_2O$ (1.85 MBq ml $^{-1}$) added to a further 1 ml sample before both samples were vortexed thoroughly. After 5 minutes the microcentrifuge tubes were vortexed again. 300 µl of cell suspension were then layered onto 300 µl of 3:1 550:200 silicone oil in microcentrifuge tubes, and this process repeated twice to result in triplicate samples from each initial 1 ml sample.

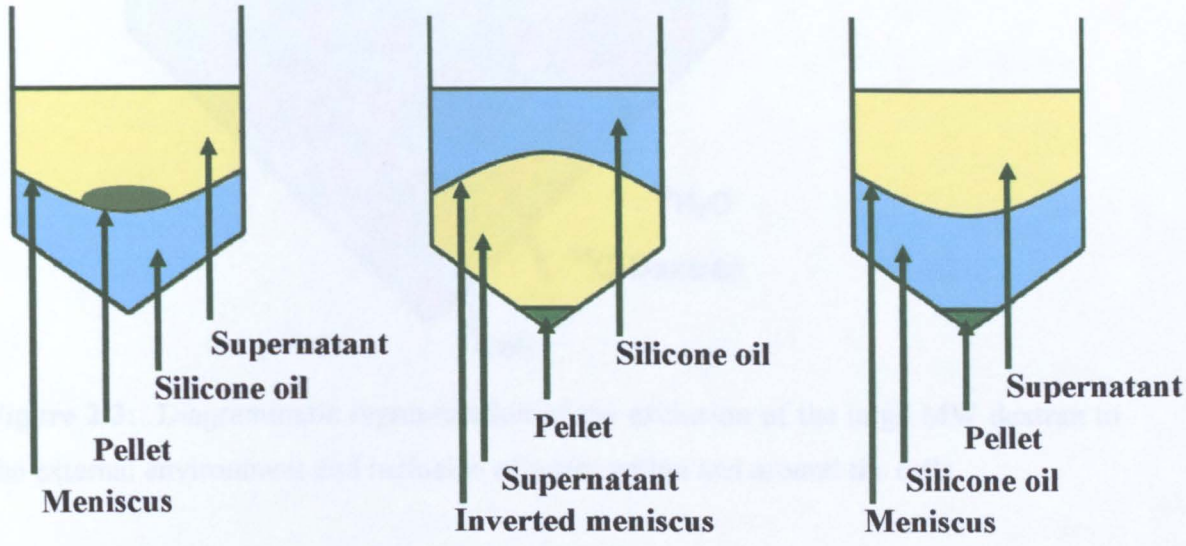


Figure 2.2: Diagram highlighting the possible and desired results when attempting to acquire the correct density silicone oil required to separate cells from their growth media

The microcentrifuge tubes were then centrifuged at 11400 *g* for 1 minute. 100 µl samples of supernatant were carefully removed from each microcentrifuge tube and placed into separate scintillation vials each containing 5 ml of FluoranSafe scintillation fluid (VWR) and labelled appropriately. The lower half of each microcentrifuge tube, containing the cell pellet, was carefully cut off using a razor blade and placed upside down into a microcentrifuge tube containing 300 µl of distilled water. The microcentrifuge tubes were centrifuged for approximately 15 seconds at 11400 *g* in order to dislodge the pellet from the microcentrifuge tube into the distilled water.

After the pellets were dislodged the microcentrifuge tip was removed and discarded before the entire pellet was resuspended in the water. All 300 µl of cell suspension was pipetted into a scintillation vial containing 5 ml of FluoranSafe scintillation fluid and labelled appropriately. All the vials were placed in racks and counted for 5 minutes per vial in a Beckman LS 1801 Liquid Scintillation Counter.

The $^{3}H_2O$ was evenly distributed throughout the pellet, whereas the ^{14}C-dextran was only found in the spaces between the cells and the pellet due to its high molecular weight (Figure 2.3).

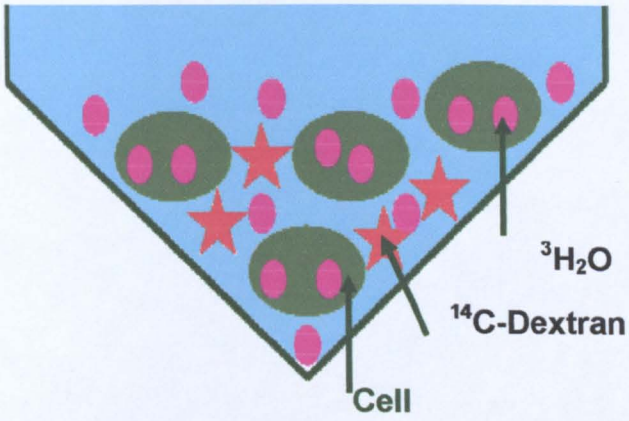


Figure 2.3: Diagrammatic representation of the exclusion of the large MW dextran to the external environment and inclusion of water within and around the cells.

The pellet volume (PV) and the extracellular volume (ECV) were calculated from the ratio of $^{3}H_2O$ and ^{14}C-dextran in the pellet and supernatant fractions respectively using the following equations (Hard and Gilmour 1996):

$$\text{Pellet volume (PV) (µl)} = \frac{^{3}H_2O \text{ DPM in pellet}}{^{3}H_2O \text{ DPM in supernatant} \times 3} \times 300$$

$$\text{Extracellular volume (ECV) (µl)} = \frac{^{14}C\text{-dextran DPM in pellet}}{^{14}C\text{-dextran DPM in supernatant} \times 3} \times 300$$

Intracellular volume (ICV) was calculated by subtracting the ECV from the total PV (Rottenberg 1979).

2.6.2: Determination of intracellular pH

The internal pH of the cells was measured using a weak acid (Rottenberg 1979). ^{14}C-benzoic acid was used when the external pH was lower than pH 7.0 in order to obtain a measurable accumulation of the isotope. The silicone oil method employed was identical to that described in Section 2.6.1, except that 10 µl of ^{14}C-benzoic acid (3.7 MBq ml^{-1}) was added to a 1 ml sample of cells.

In order to calculate the intracellular pH the following equations were used:

1. $\frac{\text{DPM } ^{14}C\text{-benzoic acid supernatant}}{100} = \text{DPM } ^{14}C\text{-benzoic acid in 1 µl} = \mathbf{A}$

2. Multiply **A** by ECV = DPM ^{14}C-benzoic acid within the pellet, outside the cells = **B**

3. Subtract **B** from the DPM ^{14}C-benzoic acid pellet and divide by ICV = DPM μl^{-1} cell volume = **C**

4. Ratio of **C/A** = $\frac{\text{Concentration of } ^{14}\text{C-benzoic acid inside the cell } (a_i)}{\text{Concentration of } ^{14}\text{C-Benzoic acid outside the cells } (a_o)}$

If the pKa of the probe (weak acid) is more than 1.5 units below the external pH then:

$$\Delta pH = -\log (a_i/a_o)$$

If the pKa of the probe (weak acid) is less than 1.5 units below the external pH then:

$$pH_i = -\log [a_i/a_o(10^{pK} - 10^{pH_o}) - 10^{-pK}]$$

2.6.3: Killed controls

Intracellular pH was also measured using killed *E. gracilis* G46 and *E. gracilis* Z as further controls. In these instances, 50 µl of Grams iodine was added per ml of sample and the procedure carried out as previously described (Section 2.6.2).

2.6.4: Measurement of isotope uptake over time

In order to determine uptake of any isotope by *E. gracilis* G46 cells, a time course experiment was carried out. Essentially the silicone oil method (Section 2.6.1) was carried out using only one isotope. An appropriate volume of isotope was added to seven 1 ml samples of cells and the silicone oil procedure carried out at 5, 15, 30, 60, 120, 180 and 240 minute intervals. The vials were placed in racks and counted for 5 minutes per vial in a Beckman LS 1801 Liquid Scintillation Counter. The average dpm for the pellet and supernatant triplicates per time point were calculated and plotted against time.

2.6.5: Measurement of membrane potential

The silicone oil procedure was carried out as described previously (Section 2.6.1), with the exception that 5 µl of ^{3}H-TPP^{+} (tetraphenylphosphonium) (1.85 MBq ml^{-1}) was added to a 1 ml aliquot of cells. Membrane potential ($\Delta\psi$) was then calculated as follows:

1. $\frac{\text{DPM } ^3\text{H-TPP}^+ \text{ Supernatant}}{100} = \text{DPM } ^3\text{H-TPP}^+ \text{ in 1 µl} = \mathbf{A}$

2. Multiply **A** by ECV (calculated from parallel samples treated with ^3H_2O and ^{14}C-dextran as in Section 2.6.1) = ^{3}H-TPP$^+$ within the pellet, but outside the cells = **B**

3. DPM ^{3}H-TPP$^+$ in the pellet minus **B** and divide by ICV (calculated from parallel samples treated with ^3H_2O and ^{14}C-dextran as in Section 2.6.1) = DPM µl^{-1} cell volume = **C**

4. Ratio of **C/A** = $\frac{\text{Concentration of TPP}^+ \text{ inside the cell } (a_i)}{\text{Concentration of TPP}^+ \text{ outside the cells } (a_o)}$

Using the Nernst equation, where:

R = 8.3143 Joules mol^{-1} K^{-1}

T = 296 K (23 °C)

F = 96.487 Joules ml^{-1} mV^{-1}

Z = 1 (charge on ionic species)

At 23 °C and converting from ln to log (x2.303):

$\Delta\psi$ (mV) = -58.8 x log (a_i/a_o)

2.6.6: Measurement of the effect of valinomycin and gramicidin on membrane potential

A 10 mg ml^{-1} stock solution of valinomycin was prepared in 95 % ethanol and diluted 1 in 10. 3 µl of this dilution was added to a 1 ml sample of *E. gracilis* G46 or *E. gracilis* Z, resulting in a final concentration of 3 µg ml^{-1} valinomycin, and incubated for 10 minutes prior to a further 5 minute incubation after 5 µl of ^{3}H-TPP$^+$ was added. The silicone oil procedure was carried out as previously described and the membrane potential calculated according to Section 2.6.5. The same procedure was used to measure the effect of gramicidin, with the exception that a 20 mg ml^{-1} stock solution was made initially resulting in a final concentration of 6 µg ml^{-1} gramicidin. Two controls were carried out; one using ^{3}H-TPP$^+$ alone, in order to measure the typical membrane potential for comparison, and the other with the addition of 3 µl of 95 % ethanol for 10 minutes prior to adding the ^{3}H-TPP$^+$ in order to ascertain if the solvent

used to dissolve both the valinomycin and gramicidin had an effect on membrane potential.

2.7: **Fluorescence Activated Cell Sorting (FACS)**

The basis of the flow cytometric method lies in the cell sample being passed through a beam of light of a defined wavelength. The sample is loaded into a flow cell which delivers the sample into a stream of mild saline solution. This mixture is then pushed through a narrow channel which forces the liquid in the centre to form a fast moving laminar flow of liquid. This process forces the particles in the centre of the flow into single file resulting in the particles passing across the beam of light which transects the laminar flow once only. This is the principle of hydrodynamic focusing (Figure 2.4).

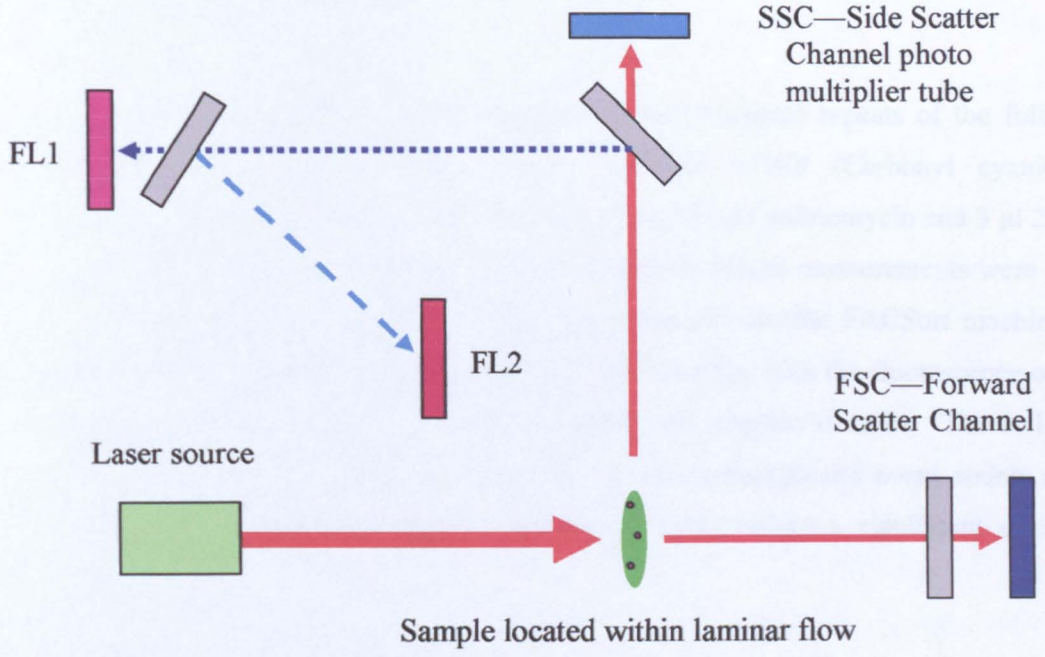


Figure 2.4: A diagrammatic representation of the photon detectors within the FACSort apparatus. The laser beam transects the laminar flow. The FSC detector picks up photons which pass directly through the sample, whereas the SSC is located at 90 ° to the source. The FL1 and FL2 (and indeed FL3) detectors are located downstream of the SSC. The grey boxes represent filters which aid the detectors located behind them.

The wavelength of the beam of light which transects the laminar flow can be tailored to obtain specific information from the sample. Detectors located on the other side of the laminar flow can receive information about the degree of side and forward scatter of the

light beam by the particles within the core of the laminar flow. The $DiOC_2$ stain component of the *Bac*Light kit exhibits green fluorescence in all bacterial cells, but the fluorescence shifts towards red emission as the dye molecules self associate at higher cytosolic concentrations caused by larger membrane potentials ("Molecular Probes Product Information Sheet MP34950").

2.7.1: Measurement of $\Delta\psi$ in *Acidocella* 29 using FACS

The *Bac*Light Bacterial Membrane potential kit (Molecular Probes, Invitrogen) was used in order to determine the effect of various reagents on $\Delta\psi$, using a FACSort machine in order to determine the ratio of red:green fluorescence. An appropriate volume of cells were centrifuged and resuspended in 18 ml of filter sterilised pH 3.0 phosphate buffered saline (PBS) (Sambrook and Russell 2001) to produce a final OD_{595} of approximately 0.5. Samples were prepared with 1 ml of cell suspension in each flow cytometry tube, to which 10 µl of 3 mM $DiOC_2$ solution was added to all tubes except for the three unstained controls.

To the remaining samples containing $DiOC_2$ stain triplicate repeats of the following treatments were also prepared: 10 µl 500 µM CCCP (Carbonyl cyanide 3-chlorophenylhydrazone); 3 µl 100 % ethanol, 3 µl 10 mM valinomycin and 3 µl 20 mM gramicidin. Samples were incubated for 15 minutes before measurements were made. A 1 ml sample of filter sterilised PBS was mounted onto the FACSort machine and compared to 1 ml samples of unstained and stained cells, with the fluorescence of cells in both the FL1-H and FL2-H axes confirming the position of cells. The cell event count region, known as "R1", was located to reduce background event counts and to contain as much of the cell population as possible before a significant error was incurred.

Instrument settings were adjusted such that the unstained samples produced approximately equivalent FL1-H and FL2-H median values. Samples were then stabilised for approximately 30 seconds before the three repeats, following which 20000 events in the R1 region were counted. The ratio of red:green fluorescence was then calculated using the median FL2-H value divided by the median FL1-H value for each sample.

2.7.2: Measuring intracellular pH in *Acidocella* 29 using FACS

The fluorescent probe LysoSensor Green DND-189 (Molecular Probes, Invitrogen) was employed as an alternative method to NMR for determination of intracellular pH in *Acidocella* 29. Cells were adjusted to an OD_{595} of 0.5 log, resuspending the pellets in AC pH 3 medium containing 1 μM LysoSensor Green DND-189 before returning cell samples to their normal growth conditions for 2 hours. After 2 hours cells were pelleted and the supernatant removed completely before resuspending in fresh filter sterilised AC pH 3 medium without any probe immediately prior to FACS.

1 ml samples of cells were mounted onto the FACSort and a gated population of cells assigned based on comparisons between filter sterilised AC pH 3 and stained cells as described in Section 2.7. *E. coli* were used as a negative control, in order to ensure that any fluorescence observed, was not due to aberrant binding of the probe. Intracellular pH was measured as a function of the median FL1-H.

2.8: Nuclear Magnetic Resonance (NMR)

2.8.1: *E. gracilis*

100 ml of 7 day old (approximately 25 – 35 μg chl ml^{-1}) *E. gracilis* G46 or *E. gracilis* Z culture were centrifuged at 3000 *g* for 10 minutes and the pellet resuspended in 2 ml of 10 mM Tris pH 3 or pH 7 for ^{31}P-NMR experiments. Cells were then added to 5 mm NMR tubes (Wilmad/Lab glass, Buena, N.J.) and deuterium oxide added to a final concentration of 10 % v/v before being pelleted further in a hand centrifuge. NMR measurements were recorded in a Bruker Avance DRX-500 (11.7 T) at 202.45 MHz with a 10 μs pulse (90 °) with an acquisition time of 0.68 s and a relaxation delay of 1 s, typically using 128 transients, giving a total time per measurement of 4 minutes. Spectra were processed by Fourier Transformation using a 5 Hz exponential broadening using FELIX (Accelrys Inc, San Diego, CA). Chemical shifts are reported relative to 1 M phosphoric acid in ppm. Calibration samples of 50 mM sodium phosphate were prepared at pH values 2.91, 3.64, 4.03, 4.54, 4.96, 5.52, 5.99, 6.53 and 8.3. The pH of test samples was determined by interpolation.

2.8.2: *Acidocella* 29

100 ml of *Acidocella* 29, OD_{595} = 0.5, were centrifuged and resuspended in 1 ml of 10 mM Tris pH 3 or pH 7. ^{31}P-NMR experiments were carried out exactly as described above (Section 2.9.1) with the exception that 4096 transients were recorded.

^{1}H NMR measurements were carried out in a Bruker Avance DRX-500 (11.7 T) at 500 MHz using a 90 ° pulse approximately 12 μs calibrated for each sample with an acquisition time of 0.7 s and a relaxation delay of 1.5 s, during which time the water signal was suppressed by saturation. Spectra were typically acquired using 200 transients. Chemical shifts are reported relative to DSS (2,2-Dimethyl-2-silapentane- 5-sulfonate sodium salt) in ppm. Calibration samples of 50 mM lactic acid in 50 mM sodium phosphate were prepared at pH values: 8.3, 6.53, 5.99, 5.52, 4.96, 4.54, 4.03, 3.64, 2.91 and 2.27. The pH of test samples was determined by interpolation.

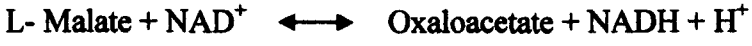
2.9: Malate Dehydrogenase Activity

2.9.1: Preparation of *E. gracilis* cell free extract

E. gracilis G46 and *E. gracilis* Z were grown in batch culture (Section 2.2.1) and the chlorophyll content of the cells determined (Section 2.3). *E. gracilis* G46 and *E. gracilis* Z cells were harvested by centrifugation at 3000 *g* for 10 minutes and resuspended in pH 7 M9 minimal medium. Cells were disrupted using a French Pressure Cell Press (Simoamico, SLM Instruments, INC). The crude extract was then centrifuged in 1 ml samples in a microcentrifuge at 11400 *g* for 1 minute and the resulting supernatant removed into a fresh microcentrifuge tube and kept on ice.

2.9.2: Enzyme activity determination

Malate dehydrogenase is an enzyme found in the tricarboxylic acid (TCA) cycle which catalyses the following reaction:



The assay mixture contained:

2.0 ml M9 (minimal salts medium)

0.3 ml 1.5 mM NADH

0.2 ml 7.5 mM oxaloacetate, pH 7.5

0.1 ml cell free extract

0.4 ml distilled water

Absorbance was measured in a Heliosα Spectrophotometer at room temperature (~20-25 °C) in 4.0 ml cuvettes with a 1 cm light path in all cases. All reagents, with the exception of oxaloacetate, were added to the cuvette and the background rate of reaction

measured at 340 nm for 1 minute. Positive rates were reduced to zero, whilst any negative background rate was recorded and subtracted from the final rate. The reaction was started by the addition of oxaloacetate and the decrease in absorbance at 340 nm measured for 1 minute against a water blank. Enzyme activity is expressed as µmoles NADH oxidised min^{-1} mg^{-1} of protein.

Enzyme assays were carried out as both shock experiments, where cell free extract was added into the cuvette and the rate of reaction measured immediately, and incubated experiments where the rate of reaction was measured after 120 minutes incubation of the cell-free extract in M9 minimal medium at the appropriate pH at room temperature. Oxaloacetate and NADH solutions were prepared fresh daily due to their instability. The extinction coefficient of NADH at 340 nm is 6.22×10^3 litre $mole^{-1}$ cm^{-1}.

2.10: Agarose Gel Electrophoresis

All DNA samples were analysed by electrophoresis using 0.8 – 1.3 % TAE gels. These comprised 0.8 – 1.3 g low melting point agarose (Roche), 2 ml 50X TAE (Appendix D) and distilled water to 100 ml. This solution was then heated in a microwave until the agarose had melted, after which it was allowed to cool whilst being stirred, and 5 µl of ethidium bromide (Biorad #161-0433) was added prior to pouring into a Biorad Subcell GT electrophoretic tank with a 14, 20 or 30 well comb. Once the gel had set, it was covered with 1X TAE buffer (Appendix D) and run at 90 – 120 V using a Biorad PowerPack 300. Gels were visualised using the Uvitec "Uvidoc" mounted camera system.

2.11: Genomic DNA Extraction

2.11.1: Environmental samples

Direct extraction of total community genomic DNA was carried out using the commercially available PowerSoil kit (Mo Bio Laboratories Inc, California) following the manufacturers guidelines with the following exceptions: 500 µl of each sample was used instead of 0.25 g and an additional 5 minute incubation at 70 °C was carried out after the addition of solution C1 and prior to the 10 minute vortex, as suggested by the protocol in order to increase cell lysis. Successful extraction of genomic DNA was verified by resolving a 2 µl sample of the eluate by gel electrophoresis (Section 2.10) against 1 µl of GeneRuler 1 kb ladder (Fermentas International Inc, Canada). Samples containing community genomic DNA were labelled and stored at – 20 °C until required.

2.11.2: *E. gracilis* G46

The method used for genomic DNA extraction from *E. gracilis* G46 was adapted from Chen *et al* (2001). Cells were grown in M6 medium, initially at pH 3, for 7 days before four 1 ml samples were centrifuged at 11400 *g* for 10 minutes. The supernatant was poured off immediately and each pellet resuspended in 250 µl of cetyltrimethylammonium bromide (CTAB) solution (2% CTAB w/v, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA).

The samples were then combined into two microcentrifuge tubes, each containing 500 µl, and incubated at 65 °C for 60 minutes. DNA was recovered using phenol-chloroform isoamylalcohol and ethanol precipitation (Sambrook and Russell 2001) before resuspending air-dried DNA pellets in 50 µl of MilliQ water.

2.11.3: Unknown microorganisms

Genomic DNA was extracted from cells using the commercially available QIAgen genomic Tip 20/G kit following the manufacturer's protocol. Genomic DNA was resuspended at 55 °C for 2 hours before a 2 µl sample was resolved by agarose gel electrophoresis (Section 2.10) against 1 µl of GeneRuler 1 kb ladder (Fermentas International Inc, Canada).

2.12: Phylogenetic Identification of Unknown Organisms

2.12.1: 16S rRNA PCR

Following extraction of genomic DNA from a particular microorganism, polymerase chain reaction (PCR) was carried out in order to amplify the 16S rRNA gene. The primers used to amplify the 16S rRNA gene were 27F and 1492R (Lane 1991) (Appendix C). The reaction mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl Distilled Water, 5 µl 10X Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1µl genomic DNA and 0.5 µl Taq polymerase (Bioline).

Amplifications were carried out in a MyCycler thermocycler (BioRad) and began with an initial denaturation step consisting of 94 °C for 3 minutes followed by 30 cycles consisting of 1 minute at 94 °C, 1 minute at 50 °C, and 2 minutes at 72 °C followed by a final extension at 72 °C for 5 minutes.

2 µl of the PCR reaction was added to 2 µl of Orange G loading dye and analysed on a 1 % agarose gel against 1 µl of 1 kb GeneRuler ladder (Fermentas) to confirm the correct sized product had been amplified. PCR reactions were then cleaned up using QIAgen PCR purification kit as per the manufacturer's protocol.

2.12.2: 18S rRNA PCR

The algal 18S rRNA gene was amplified using primers 18SF and 18SR (Sittenfeld *et al.* 2002) in a MyCycler Thermocycler (BioRad) using the following programme: 4 minutes at 94 °C, followed by 25 cycles of 30 seconds at 94 °C, 30 seconds at 50 °C and 6 minutes at 72 °C, with a single 15 minute extension at 72 °C. The reaction mixture contained the following reagents: 39 µl Distilled Water, 5 µl 10X Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1 µl genomic DNA and 0.5 µl Taq polymerase (Bioline).

The yeast 18S rRNA gene was amplified using NS1 (White 1990) and 18L (Hamby and Zimmer 1991) primers. The reaction mixture contained 39 µl Distilled Water, 5 µl 10X Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1 µl genomic DNA and 0.5 µl Taq polymerase (Bioline). Amplification was carried out using an initial denaturation at 94 °C for 3 minutes followed by 30 cycles of 94 °C for 1 minute, 50 °C for 1 minute and 72 °C for 2 minutes, with a final extension at 72 °C for 5 minutes.

Amplification products were purified with a QIAquick-spin kit (Qiagen, Germany) and their expected size verified by agarose gel electrophoresis (Section 2.10).

2.13: Ribulose-1,5-bisphosphate Carboxylase/oxygenase PCR

Amplification of exon 5 of the large subunit of the Ribulose-1,5-bisphosphate carboxylase/oxygenase gene (363 bp) from *E. gracilis* G46 was carried out using RbcL5F and RbcL5R primers (Appendix C). The reaction mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl Distilled Water, 5 µl 10X Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1µl genomic DNA and 0.5 µl Taq polymerase (Bioline).

Amplifications were carried out in a MyCycler thermocycler (BioRad) and began with an initial denaturation step consisting of 94 °C for 3 minutes followed by 30 cycles

consisting of 1 minute at 94 °C, 1 minute at 58 °C, and 2 minute at 72 °C followed by a final extension at 72 °C for 5 minutes. Successful amplification was verified by agarose gel electrohoresis (Section 2.10) and sequenced externally by Cogenics (Appendix B).

2.14: Denaturing Gradient Gel Electrophoresis (DGGE)

2.14.1: PCR

Following direct extraction of community DNA from environmental samples PCR was carried out to amplify short, highly variable fragments of the 16S rRNA gene in order to assess the microbial diversity present at the lagoon using Denaturing Gradient Gel Electrophoresis (DGGE). Two different primer sets were used; primers P2 and P3 amplify the 202 bp hypervariable V3 region of the bacterial 16S rRNA gene from position 341 to 543 on the *E. coli* 16S rRNA gene (Muyzer *et al.* 1993) and a second pair of primers, 1055F and 1406R, amplify a 323 bp region of the bacterial 16S rRNA gene which incorporates the hypervariable V9 region from position 1070 to 1392 of the *E. coli* 16S rRNA gene (Ferris *et al.* 1996a).

PCR conditions for P2 and P3 primers were as follows: 37.5 µl of distilled water, 5 µl 10X reaction buffer, 2.5 µl 50 mM $MgCl_2$, 1 µl 25 mM dNTPs, 1 µl forward primer, 1 µl reverse primer, 0.5 µl 10 ng μl^{-1} BSA, 1 µl community DNA and 0.5 µl Taq polymerase (Bioline). Touchdown PCR began with an initial denaturation step of 94 °C for 2 minutes followed by cycles of denaturation at 94 °C for 1 minute, an annealing temperature of 65 °C for 1 minute, which was decreased by 0.5 °C per cycle until touchdown at 55 °C was reached, and extension at 72 °C for 30 seconds. This cycle was then repeated a further 5 times with a constant annealing temperature of 55 °C. A final extension at 72 °C for 3 minutes completed the reaction.

The temperature cycle for V9 PCR was 5 minutes of denaturation at 94 °C, followed by 10 cycles of denaturation at 94 °C for 1 minute, 1 minute of annealing at 53 °C, with a decrease in annealing temperature of 1 °C per cycle, and extension at 72 °C for 3 minutes. This was followed by 20 cycles of 94 °C for 1 minute, 43 °C for 1 minute, 72 °C for 3 minutes and a final extension at 72 °C for 10 minutes. Agarose gel electrophoresis against 1 µl of Fermentas 100 bp GeneRuler ladder was carried out in order to verify the correct size product was amplified in each instance.

2.14.2: Preparation of solutions

For a 10 % polyacrylamide gel, denaturing solutions were prepared in a volumetric flask as follows:

% Denaturant	30	40	60	70
40 % Acrylamide	25 ml	25 ml	25 ml	25 ml
50X TAE buffer	2 ml	2 ml	2 ml	2 ml
Formamide	12 ml	16 ml	24 ml	28 ml
Urea	12.6 g	16.8 g	25.2 g	29.4 g
Distilled Water	Up to 100ml	Up to 100 ml	Up to 100 ml	Up to 100 ml

Solutions were decanted into a 100 ml Duran bottle, covered with aluminium foil and kept at 4 °C.

2.14.3: Casting a gel

Glass plates were cleaned with acetone and any tissue from the cleaning process removed prior to use to ensure that an even gradient was formed. The plastic spacers and comb were also cleaned with acetone and any excess tissue also removed. The spacers were placed onto the clean side of the large glass plate and the clean side of the small glass plate placed faced down onto the spacers. The clamps were placed on each side of the glass plates and loosely tightened. The cardboard spacer was inserted and the clamped plates were gently tapped downwards to ensure that both plates were flush at the bottom and the clamps tightened further. The grey foam strip was placed along the bottom of the casting stand and the plates inserted onto the casting stand with the small plate at the back. This was then clamped in place by turning the small levers at the side of the casting stand.

15 ml of 30 % and 60 % denaturant solution were pipetted into separate universal tubes and 120 µl of 10 % ammonium persulphate (APS) (made freshly) and 6 µl of TEMED added to both universal tubes. The tubes were gently inverted to mix. Each solution was drawn up into a syringe, which had been rinsed thoroughly with water prior to use, and pressure applied to the syringe until the solution was approximately half way along the tube. The syringes were attached to the gradient forming wheel, with the low denaturing solution on the left hand side for bottom pouring gels and the high denaturing solution on the right hand side. The T-piece and needle were then carefully

attached to both syringe tubes and the needle placed between the two glass plates in the centre. The gradient wheel was then very gently pushed to pour the gel. Once the gel was full the comb was eased in between the two plates and left to set for approximately 60 minutes.

Whilst the gel was setting 7 litres of 1X TAE was prepared and poured into the gel tank. The core was added to the tank and set to heat to 65 °C. Once the gel was set the comb was carefully removed and the wells very gently rinsed with 1X TAE to remove any excess material. The gel plates were then clamped into the core, inserted into the tank, the temperature decreased to 60 °C and TAE added to the top buffer reservoir. Samples were prepared by adding 2 µl of loading buffer II (Appendix D) to 8 µl of PCR product and all 10 µl of each sample loaded onto the gel. Electrophoresis was carried out at 80 V for 16 hours at 60 °C. After electrophoresis the gel was removed from the tank and stained with a solution containing 2 µl of SYBR Gold (Invitrogen) in 20 ml of 1X TAE for 20 minutes under aluminium foil. After 20 minutes the stain was gently rinsed from the gel using distilled water and the gel placed into the UV transilluminator for visualisation using LabWorks Image Analysis Software (UVP Inc, California).

2.14.4: Identification of bands

Small fragments of individual bands were excised by pressing a cut-off 200 µl pipette tip onto the band and placing this into a microcentrifuge tube. 10 µl of ultrapure-DNAse, RNAse-free water was added and the sample incubated at 4 °C overnight to allow DNA to diffuse from the gel fragment. 5 µl of this solution was then used as a template for PCR using the appropriate primers and PCR programme for the sample (Section 2.14.1). Successful amplification products were gel excised using a QIAgen gel extraction kit followed by a further PCR step using only dATP to add a Poly-A tail to the fragment and finally purified using a QIAgen kit as per the manufacturer's protocol. Excised fragments were then ligated into the cloning vector pCR2.1 (Figure 2.5) (Invitrogen) using the following conditions: 4.5 µl insert, 1 µl pCR2.1 (0.025 ng µl $^{-1}$), 1 µl T4 DNA ligase (Roche) , 1µl ligase buffer and 2.5 µl ultrapure water. The ligation reaction was incubated at 4 °C overnight and used to transform competent *E. coli* strain DH5α cells.

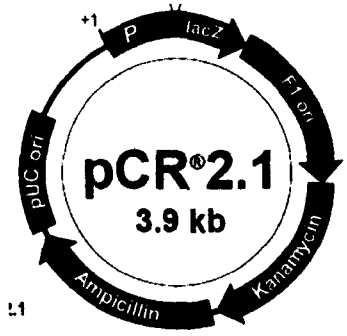


Figure 2.5: Cloning vector pCR2.1 (Invitrogen)

100 µl aliquots of competent *E. coli* DH5α cells (Appendix A) were defrosted on ice for 5 minutes before all 10 µl of the ligation reaction was carefully pipetted on top of the cells and very gently mixed. The cells were then incubated on ice for 30 minutes prior to a 90 second heat shock at 42 °C followed by a 5 minute recovery period on ice. 100 µl of SOC (Appendix D) was added to the cells prior to incubation at 37 °C for 30 – 60 minutes. 80 µl of 100 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 40 µl of 100 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) were added to the cells before plating onto LB ampicillin plates (100 µg ml^{-1}) and incubating at 37 °C overnight.

Individual white colonies were stabbed with sterile pipette tips and used to inoculate 4 ml of LB ampicillin (50 µg ml^{-1}). Cultures were shaken at 250 rpm at 37 °C overnight and plasmids extracted using QIAgen spinprep kit as per the manufacturer's protocol. Restriction digests were carried out for 2 hours at 37 °C in order to verify the correct sized insert using: 2 µl of spinprep, 1 µl of *Eco*R1 (Promega, 12 U $µl^{-1}$), 1 µl of 10X reaction buffer B (Promega) and 6 µl of ultrapure water. All 10 µl of the reaction were analysed by gel electrophoresis against 1 µl of both 1 kb and 100 bp Gene Ruler ladder (Fermentas). Plasmid preparations containing the correct sized insert were sequenced using M13 forward and reverse primers by Cogenics (UK).

2.15: T-RFLP

2.15.1: PCR

Forward primer 63F and reverse primer 1389R (*E.coli* numbering) were labelled with the fluorescent phosophoramidite dyes, 6-FAM and HEX respectively (Figure 2.6), in order to amplify the near-full length 16S rRNA gene with fluorescent labels (Osborn *et al.* 2000). Amplification was carried out using an initial denaturation of 2 minutes at 94

°C, followed by 30 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minute followed by a 10 minute final extension at 72 °C. Each reaction contained 38.5µl of distilled water, 5 µl 10X buffer, 2.5 µl 50 mM $MgCl_2$, 1 µl 25 mM dNTPs, 1 µl forward primer, 1 µl reverse primer (MWG Biotech, Germany), 0.5 µl 10 ng μl^{-1} BSA, 1 µl community DNA and 1 µl of Taq polymerase. Amplification products were verified by agarose gel electrophoresis (Section 2.10) and purified using QIAquick columns (QIAgen), eluting in a final volume of 50 µl of MilliQ ultrapure water.

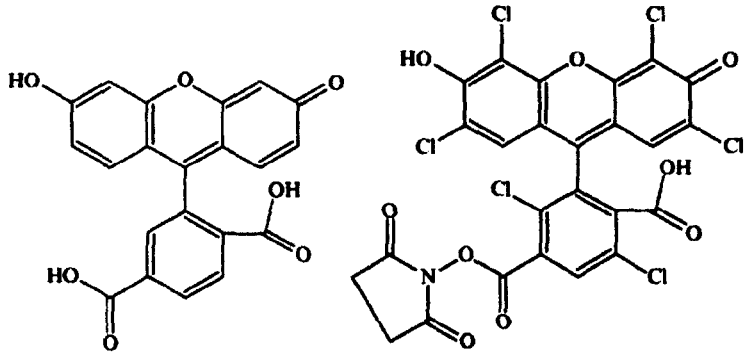


Figure 2.6: Structure of phosophoramadite dyes, 6-Fam and 6-Hex.

2.15.2: Restriction digest

10 µl of PCR product was digested with 20 units of either *Hha*I or *Alu*I (Promega) at 37 °C for 3 hours. A mastermix of enzyme, 10X reaction buffer (Promega buffer C or B respectively) and distilled water was prepared and added to each 10 µl PCR product separately to give a total volume of 15 µl per sample. Samples were frozen immediately after 3 hours incubation to stop any further digestion prior to the next stage of the procedure.

2.15.3: Fragment analysis

5 µl of PRISM GeneScan-500 ROX size standard (Applied Biosystems) was added to 1 ml of Hi-Di formamide and 9.5 µl of this mix added to 48 wells of a 96-well ABI-compatible plate, using only the odd lines. The GeneScan-500 ROX size standard contains labelled markers of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bp which allow T-RF size to be determined during analysis in much the same way as a molecular weight ladder in an agarose gel. The 35, 50, 250 and

340 bp markers are excluded when assigning the size markers due to their inconsistent migration.

0.5 µl of sample was added into each well, with unused wells made up to 10 µl with ultrapure water. The plate was then covered with a grey septa and checked for air bubbles. If any air bubbles were present the plate was spun to remove these. The samples were then denatured for 3 minutes at 94 °C using a thermocycler and placed into the black and white clips before being put into the input stack of the ABI3730 48-well capillary sequencer. A 10 second injection time was used. The laboratory work was assisted by protocols provided by the Sheffield Molecular Genetics Facility which is funded by the Natural Environment Research Council, UK.

Samples were analysed using GeneMapper v 4.0 software (Applied Biosystems) using a standard protocol prepared by the Sheffield Molecular Genetics Facility. This involves importing sample data into the GeneMapper programme and choosing the "AFLP" analysis protocol. The raw data from several samples was viewed in order to determine the position of the primer flare; for Hoole Bank acid tar lagoon samples the average position was approximately 1650 bp therefore this was input into the analysis parameters. Green, Red and Blue dyes were selected in order to analyse forward and reverse terminal restriction fragments (T-RFs) and in order to include the ROX size markers. The analysis range was set from 50-500 bp; although some samples contained T-RFs larger than 500 bp the largest ROX size standard is 500 bp therefore this sets the cut off point. Once the analysis parameters were set, the analysis method was applied to the raw sample data and both blue (Forward) and green (Reverse) T-RF profiles generated for each sample.

The GeneMapper programme then generated a bin set based on the analysis parameters. However, the generated bin set was deleted and replaced by manually scanning each sample and adding bins appropriately. This was carried out for each dye with each digest, generating four separate panel and bin sets per sample. The panel and bin sets were saved and added into the initial anlaysis settings in order to generate a table of T-RFs, including size (in bp), peak height and peak intensity. This data was then exported into an Excel workbook and analysed using T-align (Smith *et al.* 2005) for further statistical analysis using the software package MVSP. SigmaPlot was used to draw graphs.

2.16: Phylogenetic Tree Construction

Phylogenetic trees were constructed in several stages. Initially the query sequence was subjected to BlastN comparison provided by NCBI (Altschul *et al.* 1990) and the sequences from the highest identity matches obtained from the NCBI database for further comparison. The sequences were then aligned using ClustalW (1.83) (Chenna *et al.* 2003) with all parameters set at their default values.

2.17: Photosystem II Fluorescence Measurements

Chlorophyll content of *E. gracilis* G46 and *E. gracilis Z* cultures was measured according to Section 2.3 and adjusted to 15 μg chlorophyll ml^{-1}, resuspending the pellets in M6 medium at pH 3 or 7 as appropriate. A Walz Fluorometer was used to measure the fluorescence produced from photosystem II (PSII) using WPI Duo 18 software. Cells were placed into a quartz cuvette and dark adapted for 1 minute before the measuring beam was switched on (300 μmoles m^{-2} s^{-1}) which determined F_0. 30 seconds later a high intensity flash of light determined the F_m value. A further 30 seconds later the actinic light was switched on (450 μmoles m^{-2} s^{-1}) and the fluorescence trace followed for 4 minutes. During this time the high intensity pulse was switched on every 60 seconds. After switching off the actinic light the high intensity pulse was used to measure F_m for a further 3 minutes at 60 second intervals.

2.18: Characterisation of *Acidocella*

Characterisation of *Acidocella* 29 was carried out using the API 20 NE kit from bioMérieux (NC, USA) as directed in the manufacturer's protocol. Carbon source utilisation was determined by inoculating a 96-well microtitre plate containing 197.5 μl of pH 3 M9 minimal media plus 2.5 μl of 1 M carbon source in each well with 50 μl of *Acidocella* 29. Each carbon source was tested in triplicate. The 96-well plate was incubated at 26 °C for 7 days to allow any growth which may occur to be detected. The plate was read using a plate reader at 600 nm and growth in each well recorded as present (+) or absent (-).

The effect of aluminium, cadmium, copper, nickel and zinc sulphates on the growth of *Acidocella* 29 was also tested. 175 μl of M9 minimal media was pipetted into each well of a 96 well micro titre plate, to which 4 wells out of each row of eight, 2.5 μl of 1 M stock solution of metal sulphate was added, whilst 25 μl of the same 1 M stock solution was added into the remaining four wells. 50 μl of *Acidocella* 29 was added to three out

of four wells for both 10 mM and 100 mM metal concentrations, with the remaining well acting as a control for metal precipitation. An extra 22.5 µl of M9 minimal media was then added to the wells in which only 2.5 µl of metal solution was added.

Utilisation of volatile hydrocarbons was tested by preparing 1 %, 0.1 % and 0.01 % (v/v) solutions of benzene, toluene, ethylbenzene, xylene, decane, and hexadecane. 100 µl of *Acidocella* 29 was added to 1 ml samples of each solution in a microcentrifuge tube and sealed with parafilm. 100 µl of *Acidocella* 29 was also added to a 1 ml sample of M9 minimal media and a 1 ml sample of AC29 media, as negative and positive controls respectively. Sealed microcentrifuge tubes were then placed in a sealed jar to ensure no volatile hydrocarbons escaped in order to protect other cultures from harm.

2.19: LysoSensor Imaging of *E. gracilis*

The LysoSensor Yellow/Blue DND-160 probe (Molecular Probes, Invitrogen) was employed to visualise *E. gracilis* G46 cells. The probe fluoresces at different wavelengths dependent on its pH; therefore it can be used to determine intracellular pH. However, it was used in this instance simply to visualise the compartmentalisation of eukaryotic cells and to identify if different compartments of the cell had different pH values.

1 µl of the probe was added to 1 ml aliquots of M6 medium at pH 3 and pH 7 and this media incubated at 37 °C for 1 hour. 2 ml of *E. gracilis* G46 or *E. gracilis* Z were centrifuged at 11400 *g* for 10 minutes and the resulting pellets resuspended in 1 ml of the probe containing medium. Cells were then incubated under normal growth conditions for 3 hours before being centrifuged at 11400 *g* for 10 minutes. Pellets were then resuspended in 1 ml of M6 medium pH 3 or 7 as appropriate. One drop of the sample was placed onto a glass slide using a pasteur pipette and covered with a cover slip for visualisation using a CCMI Deltavision Deconvolution Microscope.

Chapter Three: Characterisation of Microbial Diversity in Hoole Bank Acid Tar Lagoon

3.1: Introduction

The aims of this work were to analyse the microbial diversity present in samples from Hoole Bank acid tar lagoon in order both to compare the results of classical and molecular techniques and to further our knowledge of the microbial community present in this environment. The work was carried out in two distinct branches; classical microbiology analysis using batch and continuous culture techniques and molecular microbial analysis using Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE), as discussed in Sections 1.2.1, 1.2.2, 2.14 and 2.15. Classical culture methods used to isolate microorganisms for further study were chosen such that microorganisms which were isolated could be grown readily.

3.2: Results

3.2.1: Sample collection

A total of 21 samples were collected from Hoole Bank acid tar lagoon. Samples were numbered according to their corresponding photograph taken during sampling at the lagoon. The pH of samples was measured upon returning to the laboratory and not on site. Samples were also categorised into 4 groups based on the sample type. The sample groups were (Figures 3.1a-d respectively):

- Group 1 - Thick Sludge external to Lagoon
- Group 2 - Lagoon edge
- Group 3 - Green Biofilm
- Group 4 – Surface water

Table 3.1 gives further details of each of the samples.

Figure 3.1a-d (From Top Left, clockwise): Group 1 - Thick Sludge external to Lagoon; Group 2 – Lagoon edge; Group 3 – Green biofilm; Group 4 - Water

Sample	Description	Group	pH
27	Entrance path near treatment plant	1	5.2
28	Lagoon edge	2	2.17
28A	Lagoon edge along from 28 – dug from bank	2	3.92
29	Green biofilm	3	3.40
30	Mud/Sludge from lagoon edge	1	2.98
33	Water near island	4	2.57
35	Fully crusted side pond along from islands	1	2.62
37	Standing water near surface tar deposits	4	4.15
38	Sludge near to sample 37	1	4.6
41	Water near surface tar deposits	4	4.4
43	Water coming up through surface tar deposits	4	1.8
44	Pond near entrance path with deposits	1	3.45
45	Green Biofilm and water	3	2.88
46	Green Biofilm at lagoon edge	3	3.07
47	Fully crusted lagoon edge	2	3.30
49	Lagoon edge, some crust	2	3.06
50	Lagoon edge, some crust	2	2.69
53	Lagoon edge	2	2.50
54	Entrance path near treatment plant	1	2.39
55	Weathered tar scrapings from surface tar deposits	N/A	4.09
58	Water at lagoon edge	4	2.79

Table 3.1: Details of the samples collected from Hoole Bank acid tar lagoon, including the corresponding photograph number, sample description, pH and grouping. Group 1 - Thick Sludge external to Lagoon; Group 2 - Lagoon edge; Group 3 - Green Biofilm; Group 4 – Surface water. N/A = Not applicable.

3.2.2: Classical microbiology analysis of microbial diversity

In order to detect and assess microbial diversity in Hoole Bank acid tar lagoon, agar plates were prepared as described in Section 2.1.2 and approximately 200 µl of each sample collected from Hoole Bank acid tar lagoon was plated onto each of the media (Section 2.1.2). Plates were incubated at 25 °C and growth monitored daily. Initially fungal growth out-competed other microbial growth therefore nystatin was added to plates and the procedure repeated.

Four organisms were isolated using this direct plating technique and identified following growth of pure cultures, extraction of genomic DNA and amplification of the 16S rRNA gene. Continuous culture methods were also employed and led to the isolation of one organism (*Burkholderia*). Several other organisms which have not been identified were cultured directly from other lagoon samples however it was either not possible to obtain pure cultures of these organisms or to subculture them for more than 1 generation. Table 3.2 gives details of the organisms isolated.

Organism	**% Identity**	**Class**	**Isolation media**	**Sample**	**Sample pH**	**Sample type**
Euglena gracilis G46	97 %	Euglenales (Order)	½ BLB	46	3.07	3 - Biofilm
Acidocella 29	97 %	α-proteobacteria	AC	29	3.40	3 - Biofilm
Burkholderia sp	97 %	β-proteobacteria	½ BLB	35	2.62	1 - Thick sludge
Gordonia sp.	99 %	Actinobacteria	½ BLB	30	2.97	1 - Thick sludge
Rhodotorula sp.	98 %	Microbotryomycetes (Subclass)	½ BLB	54	2.39	1 - Thick sludge

Table 3.2: Organisms isolated from Hoole Bank acid tar lagoon samples

A limited number of organisms were isolated from direct plating, with one organism isolated from continuous culture experiments, *Burkholderia* sp.

Although *Burkholderia* is involved in medical problems and is commonly thought of as a clinical isolate, the isolation of a *Burkholderia* species from Hoole Bank acid tar lagoon was not entirely surprising. This genus has been isolated or identified in clone libraries from several other environmental samples including several isolates from soil (Witzig *et al.* 2007), permafrost (Hansen *et al.* 2007), 300 year old volcanic deposits (NCBI accession number DQ490294), and soil exposed to phenanthrene (Bodour *et al.* 2003) amongst other environments.

The isolation of *Gordonia sp.* from the acid tar lagoon samples is slightly more unusual as *Gordonia* species are often clinical isolate. However, the *Gordonia* genus has been isolated from various environmental samples including activated sludge foam (NCBI accession number AF150493), aerobic sludge for oilfield produced water treatment (*Gordonia sp.* SCNU1; NCBI accession number DQ256752) and from a biofilter shown to degrade hexane. Figure 3.2 shows the phylogenetic position of the *Gordonia sp.* isolate from Hoole Bank acid tar lagoon, and the closest phylogenetic species, which is *Gordonia sp.* SCNU1, which was isolated from aerobic sludge.

Rhodotorula is a basidiomycetous yeast, commonly isolated from the environment, which has been found in extremely acidic environments previously (de Siloniz *et al.* 2002; Gadanho *et al.* 2006; Kawai *et al.* 2000). It is a particularly acidophilic species of yeast capable of growth at low pH (Nguyen *et al.* 2001) therefore the isolation of this species from Hoole Bank acid tar lagoon is not unexpected. *Euglena gracilis* G46 and *Acidocella* 29 were further characterised in Chapters 4 and 5 respectively.

The results from these attempts to isolate microorganisms from Hoole Bank acid tar lagoon show only a very limited number of organisms were isolated. However this was expected for many reasons, most of which are discussed in more detail in Section 1.1. In particular, the media used for isolation of organisms was deliberately chosen to ensure that any organisms successfully cultured could be grown routinely and relatively quickly in order to aid further study of these organisms. It is likely that many more organisms would have been isolated if alternative media, and alternative techniques such as the overlay method of Johnson *et al* (1987), were used to isolate organisms. The overlay method employed by Johnson *et al* involves the preparation of solid medium which has a heterotrophic organism contained within the bottom layer of agar. This functions to detoxify the upper layer of agar, which can contain organic

contaminants from agarose, such as pyruvic acid, which are inhibitory to the growth of most acidophilic organisms.

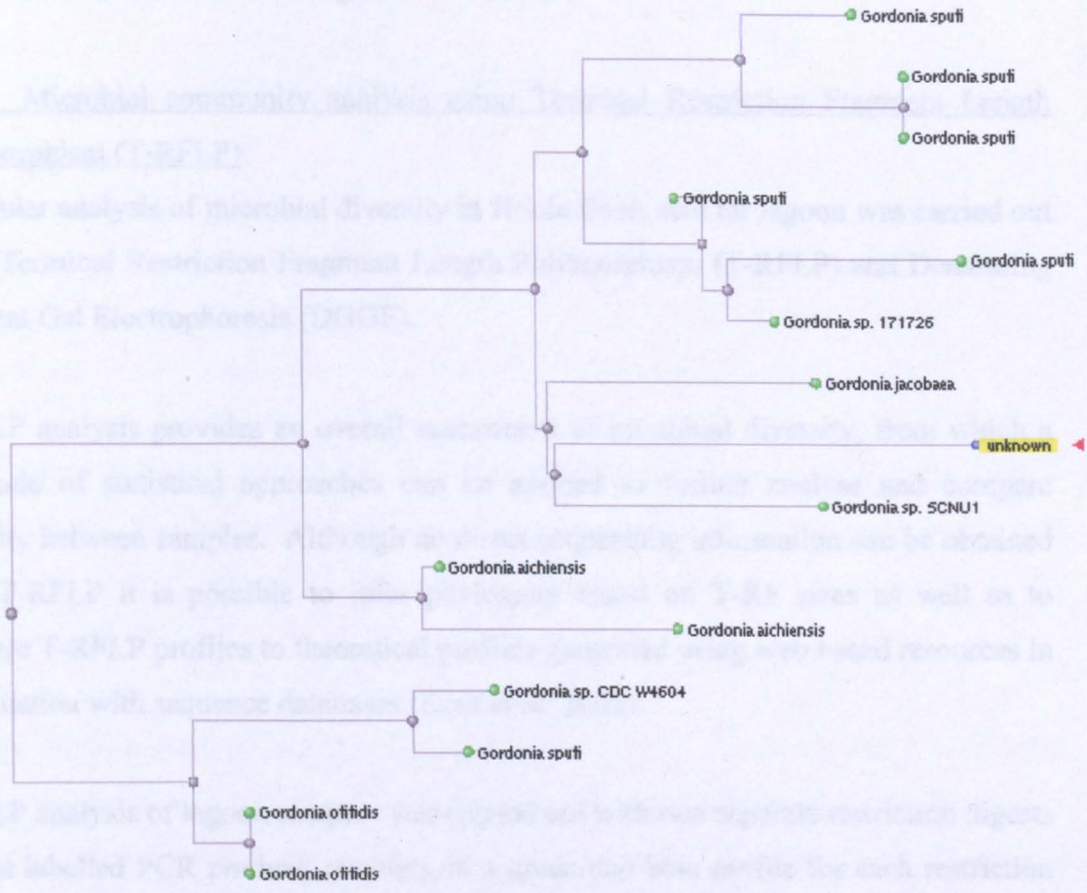


Figure 3.2: Neighbour joining phylogenetic tree of the 16S rRNA gene, with a maximum sequence difference of 0.05. "Unknown" (highlighted in yellow) represents the Hoole Bank *Gordonia* isolate, with *Gordonia sp.* SCNU1, an isolate from aerobic sludge the closest known relative.

Overall the conditions used to isolate organisms were probably far from favourable for the majority of organisms present in lagoon samples and therefore it follows that a significant percentage of the culturable proportion of organisms present in lagoon samples was not successfully cultured. There are many factors which will have affected the growth and isolation of microorganisms from Hoole Bank lagoon samples, as discussed in Section 1.1. For example, the addition of nystatin to inhibit fungal growth is likely to have altered the normal growth conditions for many organisms. The use of

aerobic conditions in all attempts to isolate organisms would immediately preclude the growth of any anaerobes and some, if not all, microaerobic organisms. Also the lack of alternate electron acceptors such as SO_4^{2-} and Fe^{3+} amongst other factors would have further reduced the number of organisms isolated.

3.2.3: Microbial community analysis using Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Molecular analysis of microbial diversity in Hoole Bank acid tar lagoon was carried out using Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE).

T-RFLP analysis provides an overall assessment of microbial diversity, from which a multitude of statistical approaches can be applied to further analyse and compare diversity between samples. Although no direct sequencing information can be obtained from T-RFLP it is possible to infer phylogeny based on T-RF sizes as well as to compare T-RFLP profiles to theoretical profiles generated using web based resources in combination with sequence databases (Kent *et al.* 2003).

T-RFLP analysis of lagoon samples was carried out with two separate restriction digests of dual labelled PCR product, resulting in a green and blue profile for each restriction digest of each sample (Figure 3.3). The HEX (blue) and FAM (green) dyes (Section 2.15.1) are covalently attached to the PCR primer such that they are incorporated at the 5' ends of both strands of amplified DNA. Thus, only the terminal fragments are labelled with either dye following restriction digestion. The presence of either the FAM or HEX dye allows the size of the fragment to be determined, with green, blue and red (size standard) channels measured on the capillary sequencer.

Selection of primers and restriction endonucleases was based on the evidence presented by Osborn *et al* (2000), resulting in the use of the enzymes *Alu*I and *Hha*I, (also known as *Cfo*I), in combination with the primers 63F and 1389R.

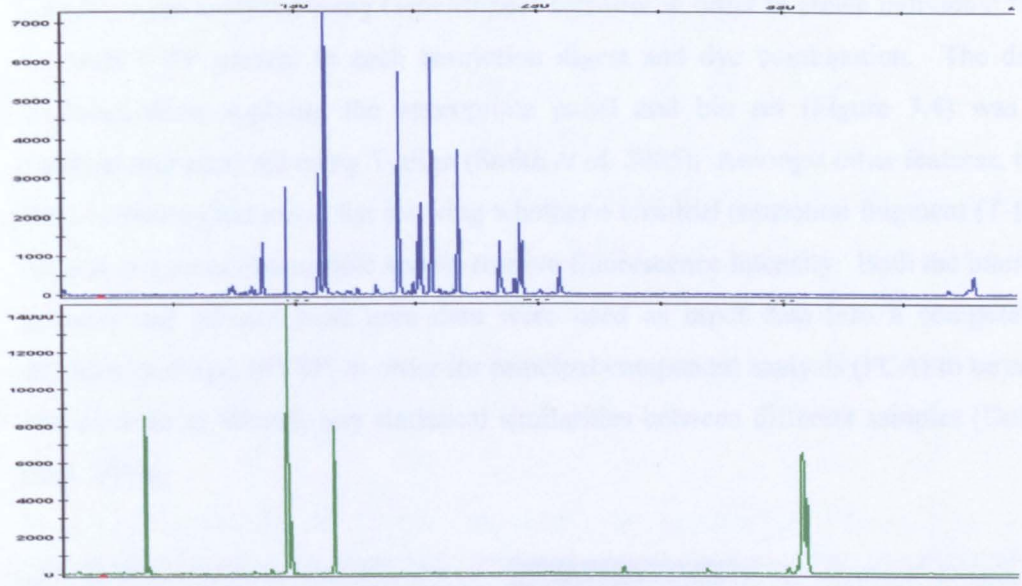


Figure 3.3: Screenshots from GeneMapper of forward (Top) and reverse (Bottom) T-RF profiles. The horizontal axis represents the size of the T-RF in bp, whilst the vertical axis represents the fluorescence intensity, which is an indicator of the abundance of a T-RF.

This primer pair was chosen for several reasons. Firstly, almost the entire 16S rRNA gene is amplified using these primers, with the exception of one hypervariable region. Secondly, these primers are internal to the most commonly used 16S rRNA gene primers, 27F and 1492R, which facilitates *in silico* prediction of T-RF sizes for most 16S rRNA genes in databases and finally because this pair has been shown to be more useful in bacterial ecological studies than other primer pairs (Marchesi *et al.* 1998).

The choice of restriction enzymes used were the same as Osborn *et al* (2000), based on the predictions that *Alu*I and *Hha*I give the most discrimination, in terms of the number of different predicted T-RFs that would be produced from different species. Osborn *et al* also report that *Tha*I gives equally good discrimination, however *Alu*I and *Hha*I were chosen for this work on the basis of cost. Based on these restriction digests the following four profiles were generated for each sample:

- *Alu* Blue (Forward)
- *Alu* Green (Reverse)
- *Hha* Blue (Forward)
- *Hha* Green (Reverse)

Samples were analysed using GeneMapper software in order to create individual "bins" for each T-RF present in each restriction digest and dye combination. The dataset produced from applying the appropriate panel and bin set (Figure 3.4) was then exported and analysed using T-align (Smith *et al.* 2005). Amongst other features, the T-align software produces a list showing whether a terminal restriction fragment (T-RF) is present in a particular sample and its relative fluorescence intensity. Both the interstitial (binary) and percent peak area data were used as input data into a computational statistics package, MVSP, in order for principal component analysis (PCA) to be carried out, in order to identify any statistical similarities between different samples (Dollhopf *et al.* 2001).

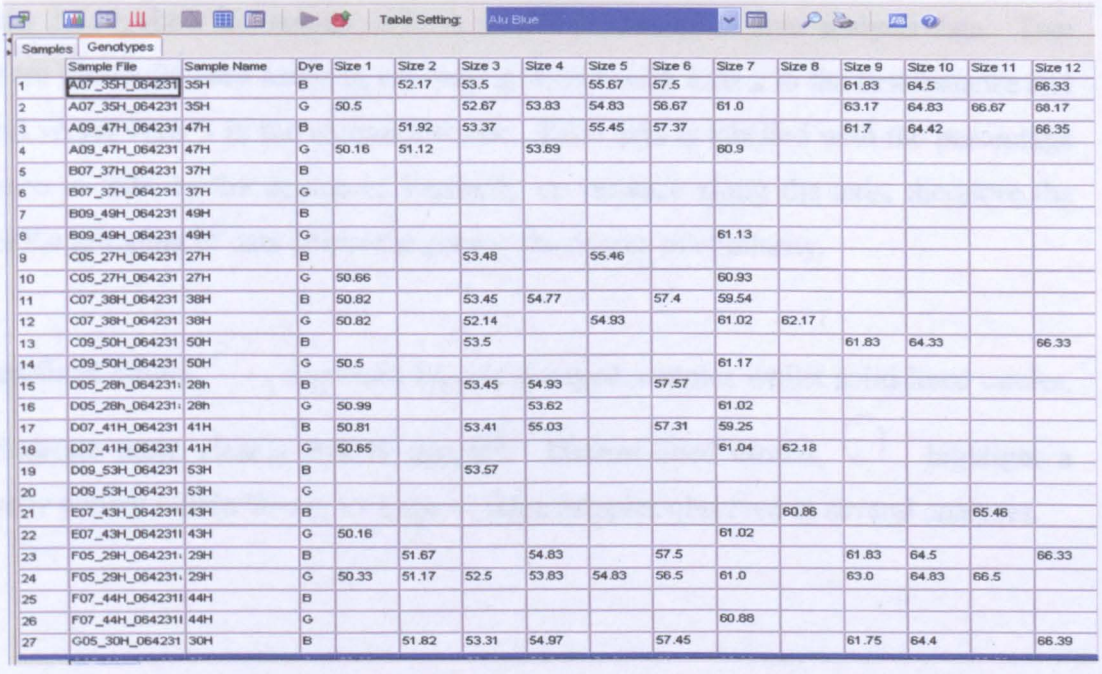


Table Setting: Alu Blue

Samples | Genotypes

	Sample File	Sample Name	Dye	Size 1	Size 2	Size 3	Size 4	Size 5	Size 6	Size 7	Size 8	Size 9	Size 10	Size 11	Size 12
1	A07_35H_064231	35H	B		52.17	53.5		55.67	57.5			61.83	64.5		66.33
2	A07_35H_064231	35H	G	50.5		52.67	53.83	54.83	56.67	61.0		63.17	64.83	66.67	68.17
3	A09_47H_064231	47H	B		51.92	53.37		55.45	57.37			61.7	64.42		66.35
4	A09_47H_064231	47H	G	50.16	51.12		53.69			60.9					
5	B07_37H_064231	37H	B												
6	B07_37H_064231	37H	G												
7	B09_49H_064231	49H	B												
8	B09_49H_064231	49H	G							61.13					
9	C05_27H_064231	27H	B			53.48		55.46							
10	C05_27H_064231	27H	G	50.66						60.93					
11	C07_38H_064231	38H	B	50.82		53.45	54.77		57.4	59.54					
12	C07_38H_064231	38H	G	50.82		52.14		54.93		61.02	62.17				
13	C09_50H_064231	50H	B			53.5						61.83	64.33		66.33
14	C09_50H_064231	50H	G	50.5						61.17					
15	D05_28h_064231	28h	B			53.45	54.93		57.57						
16	D05_28h_064231	28h	G	50.99			53.62			61.02					
17	D07_41H_064231	41H	B	50.81		53.41	55.03		57.31	59.25					
18	D07_41H_064231	41H	G	50.65						61.04	62.18				
19	D09_53H_064231	53H	B			53.57									
20	D09_53H_064231	53H	G												
21	E07_43H_064231	43H	B								60.86			65.46	
22	E07_43H_064231	43H	G	50.16						61.02					
23	F05_29H_064231	29H	B		51.67		54.83		57.5			61.83	64.5		66.33
24	F05_29H_064231	29H	G	50.33	51.17	52.5	53.83	54.83	56.5	61.0		63.0	64.83	66.5	
25	F07_44H_064231	44H	B												
26	F07_44H_064231	44H	G							60.88					
27	G05_30H_064231	30H	B		51.82	53.31	54.97		57.45			61.75	64.4		66.39

Figure 3.4: Data table from GeneMapper software. The size of T-RFs (in bp) present in each sample is shown. Also shown in the table (but not in this figure) is the peak height and peak intensity corresponding to each T-RF in each sample.

The difference between the two analyses is that the interstitial data analysis provides a simple breakdown of T-RF data, only identifying if a particular T-RF is present or absent in each sample whilst the percent peak area analysis may allow the abundance of a particular T-RF to be inferred. Samples were plotted using PCA of both interstitial data and percent peak area.

PCA is a mathematical transformation that changes the data to a new coordinate system such that the greatest variance by any projection of the data comes to lie on the first coordinate (called the first principal component), the second greatest variance on the second coordinate, and so on. Thus, the closer data points on PCA graphs cluster the less variance there is between the samples and conversely the greater the distance between data points, the greater the variance between them. Simply, PCA takes a 3D set of data points and rotates it such that the maximum variability is visible (Dollhopf *et al.* 2004; Dollhopf *et al.* 2001)

The results of PCA on the interstitial analysis data are presented in Figures 3.5a-h whilst Figures 3.6a-h show the results of PCA on the percent peak area analysis data. Two plots are shown for each analysis, comparing Axis 1 with Axis 2 in the first instance and Axis 2 versus Axis 3 in the second instance. Each axis is labelled with the percentage variance to indicate the degree of similarity or variance along the axis, therefore the closer the grouping of data points the greater the degree of similarity.

Dotted-lined circles,  , represent loosely grouped samples whilst solid-lined circles, indicate more closely linked samples. Dashed-lined circles,  , highlight a diversity pattern within Group 1 - Lagoon Edge samples observed in several analyses.

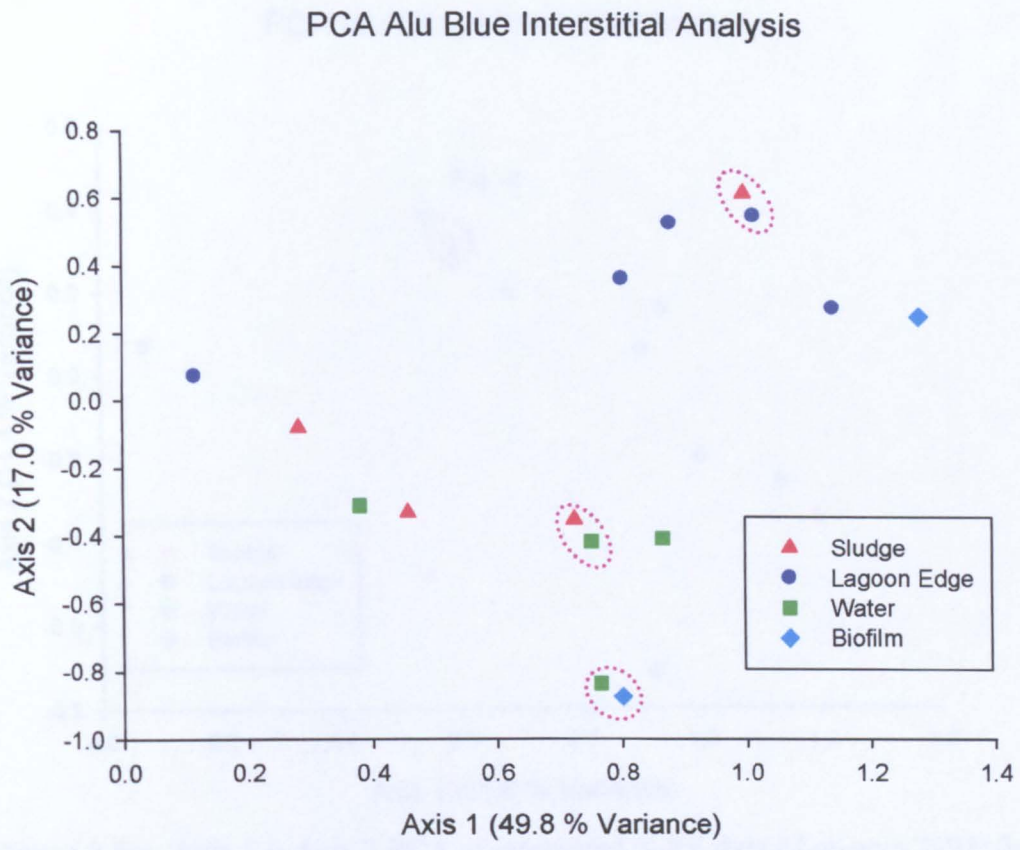


Figure 3.5a: Axis 1 v Axis 2 PCA of interstitial T-RF data of forward T-RF from *Alu*I digest

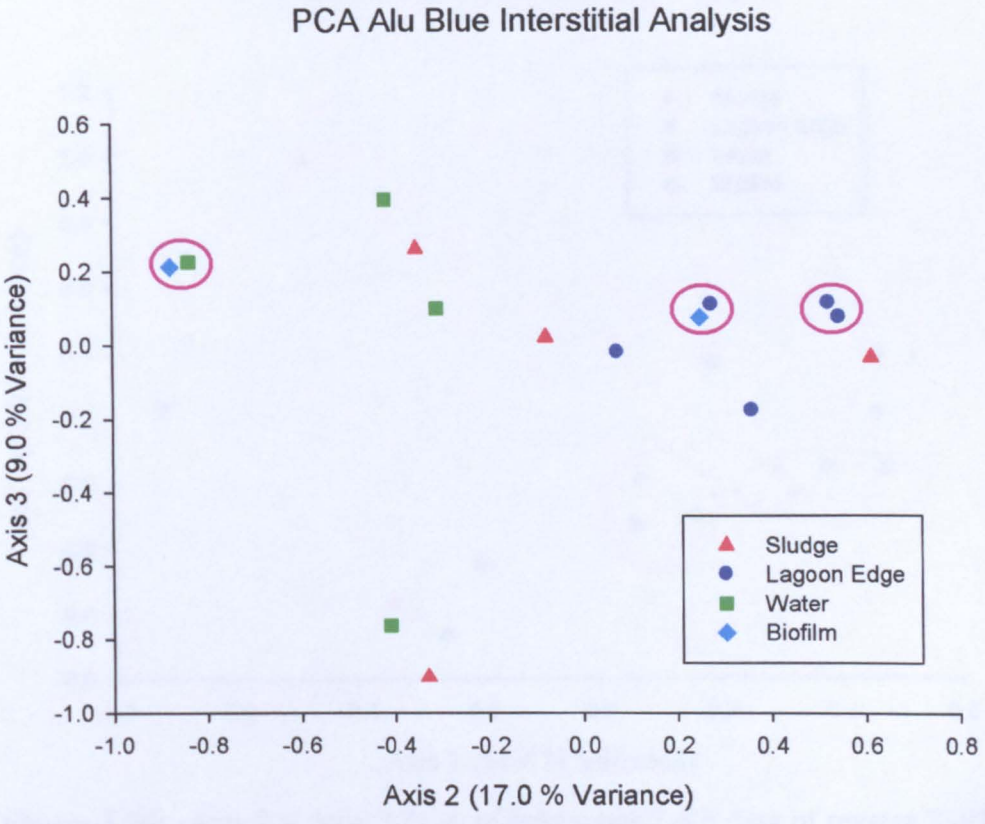


Figure 3.5b: Axis 2 v Axis 3 PCA of interstitial T-RF data of forward T-RF from *Alu*I digest

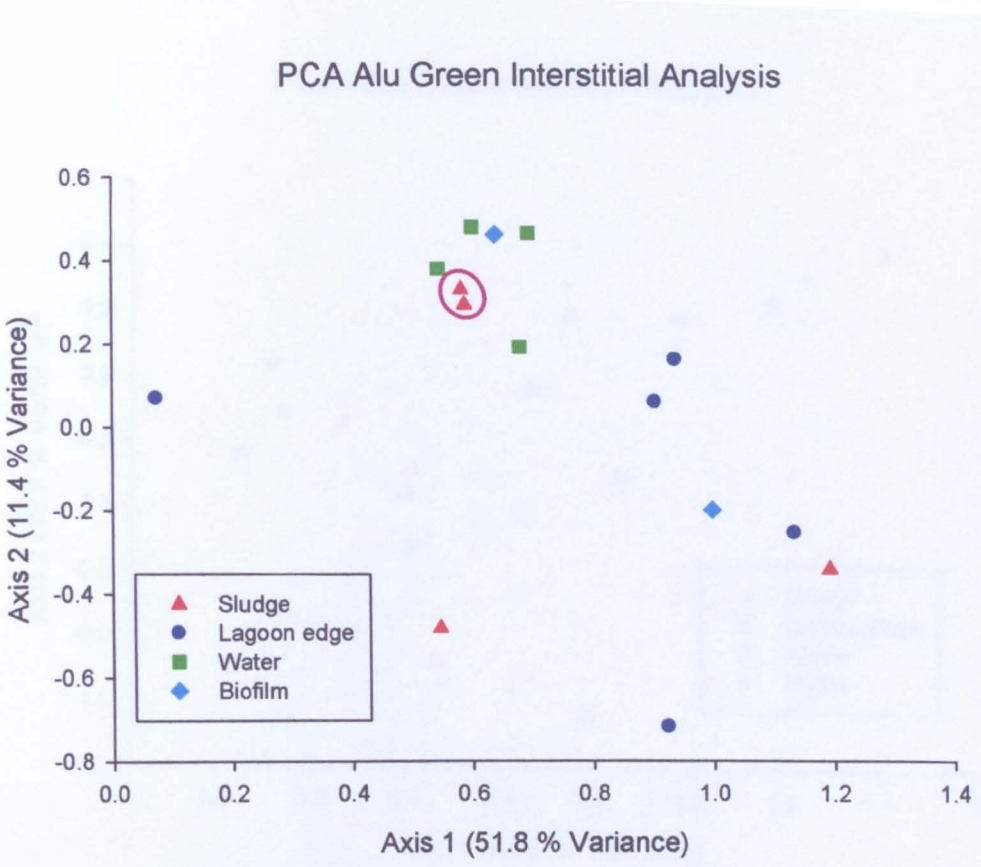


Figure 3.5c: Axis 1 v Axis 2 PCA of interstitial T-RF data of reverse T-RF from *Alu*I digest

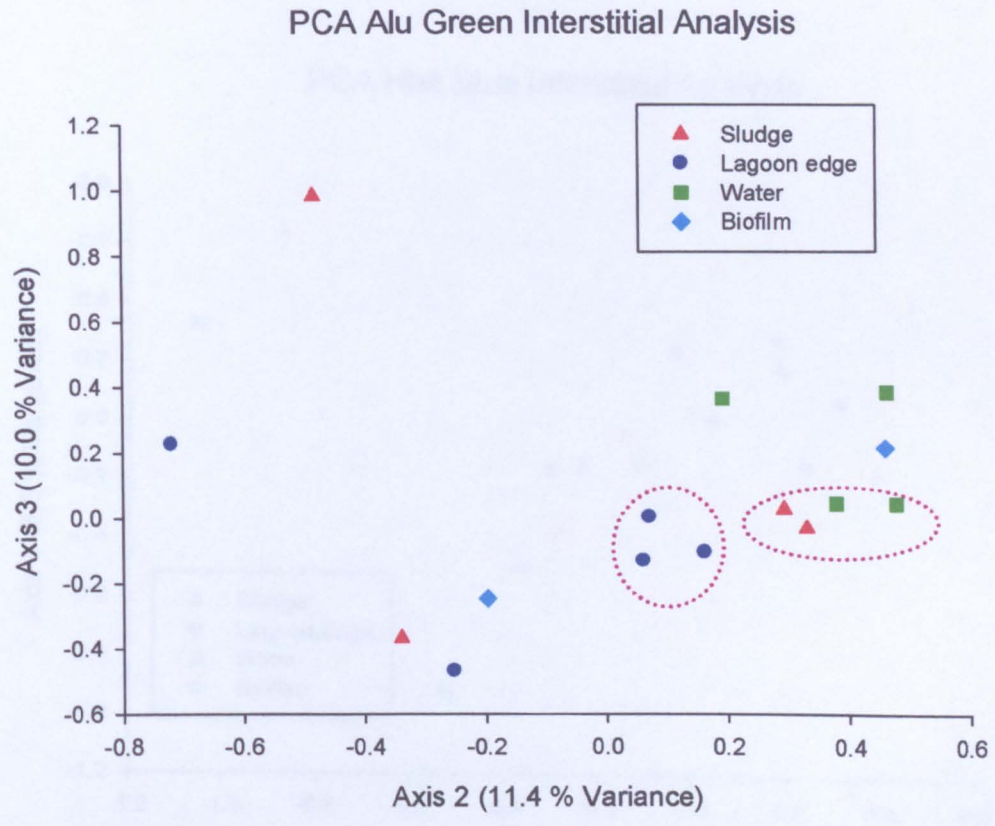


Figure 3.5d: Axis 2 v Axis 3 PCA of interstitial T-RF data of reverse T-RF from *Alu*I digest

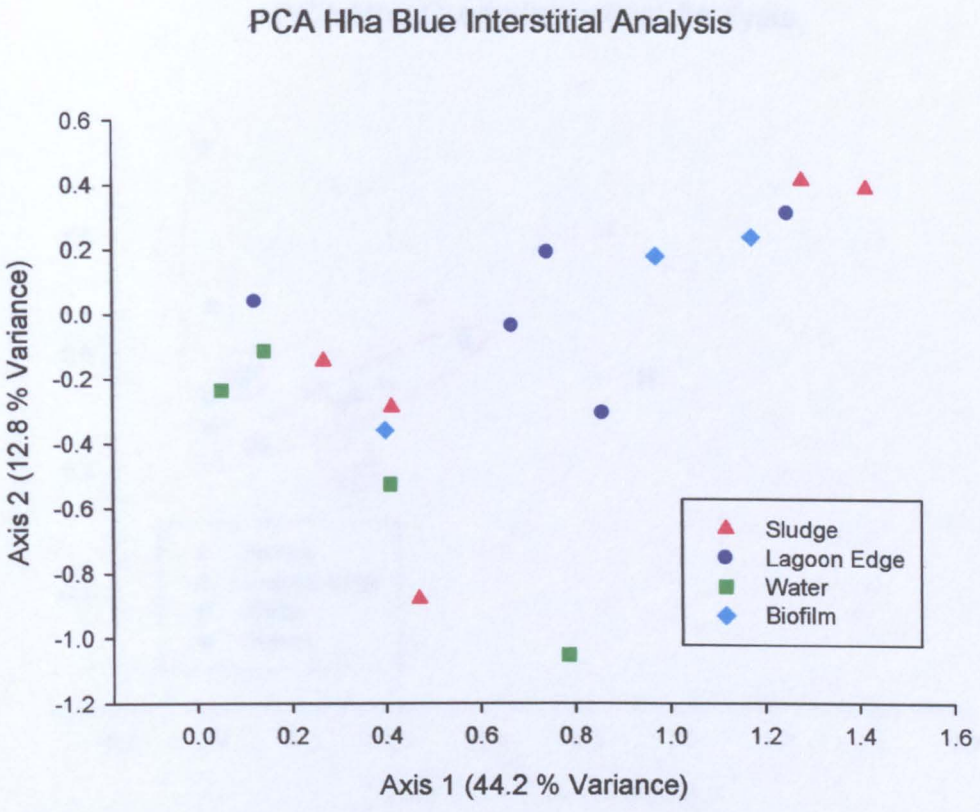


Figure 3.5e: Axis 1 v Axis 2 PCA of interstitial T-RF data of forward T-RF from *Hha*I digest

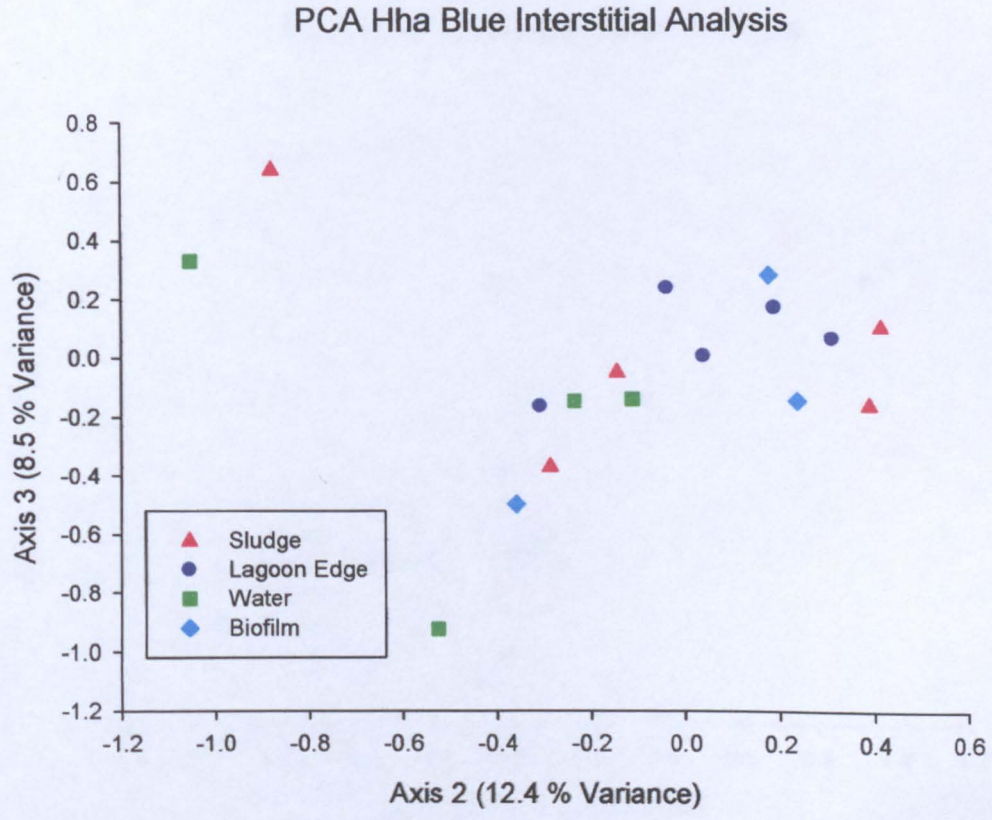


Figure 3.5f: Axis 2 v Axis 3 PCA of interstitial T-RF data of forward T-RF from *Hha*I digest

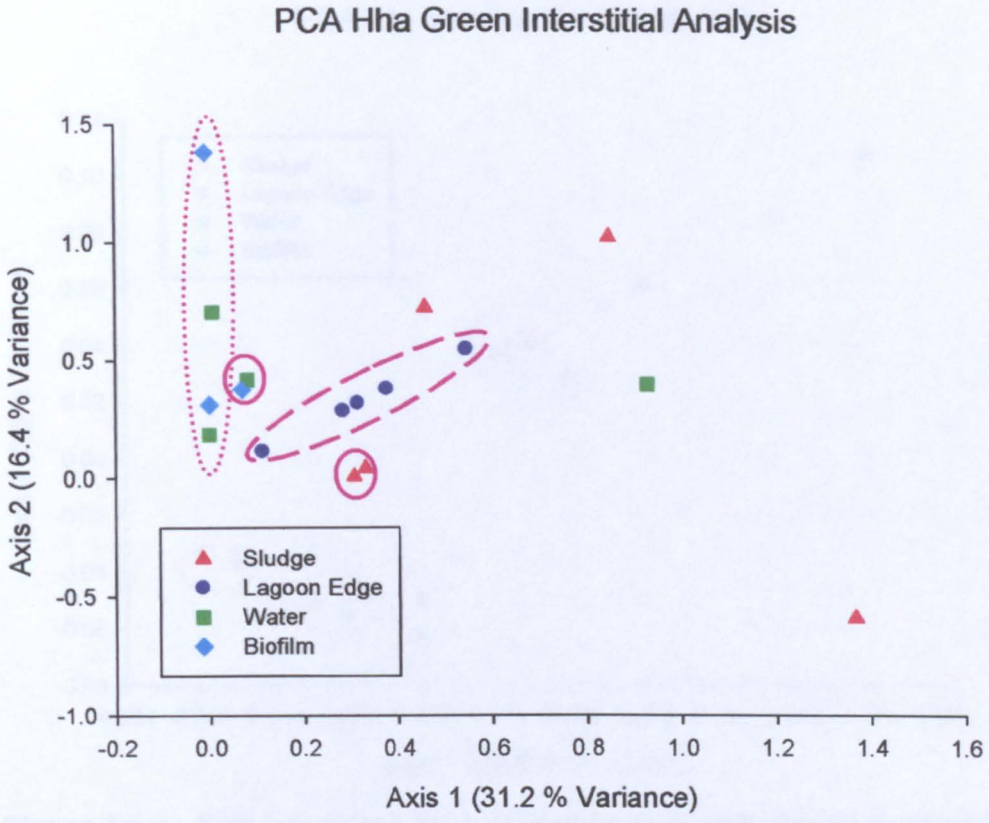


Figure 3.5g: Axis 1 v Axis 2 PCA of interstitial T-RF data of reverse T-RF from *Hha*I digest

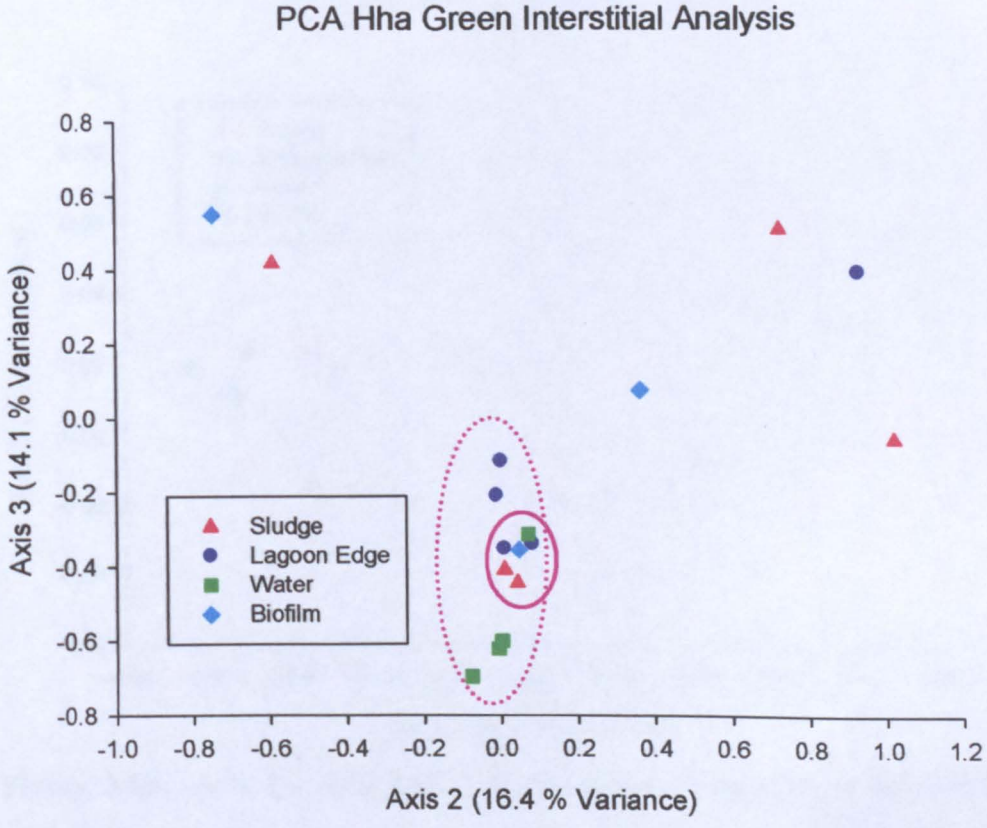


Figure 3.5h: Axis 2 v Axis 3 PCA of interstitial T-RF data of reverse T-RF from *Hha*I digest

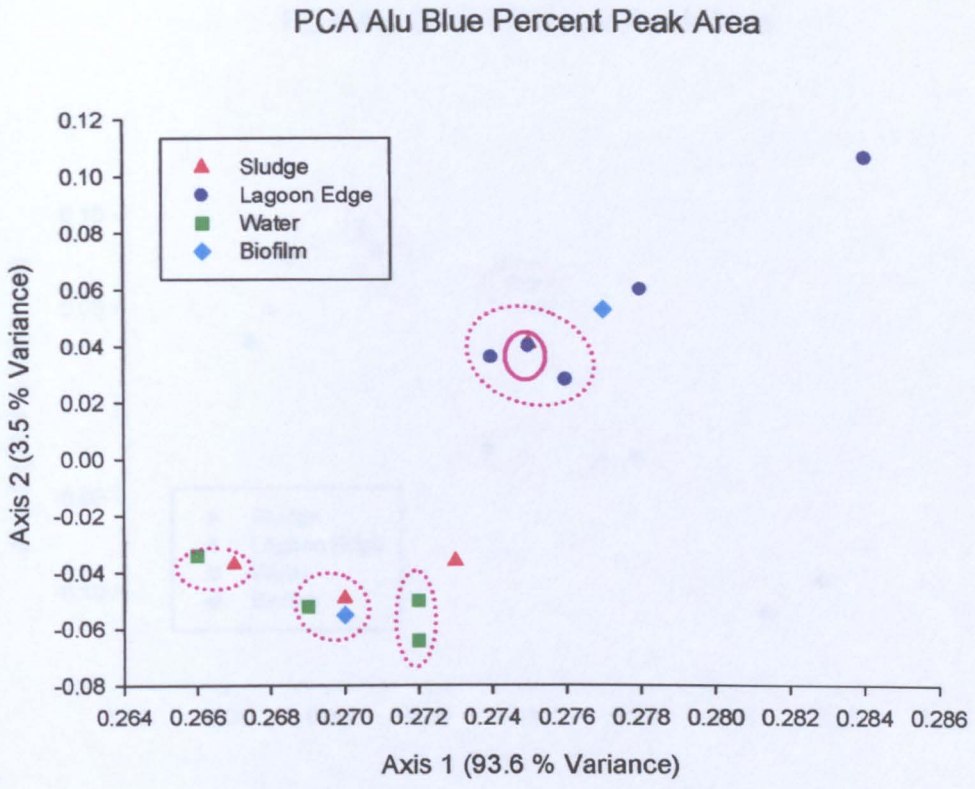


Figure 3.6a: Axis 1 v Axis 2 PCA of percent peak area data of forward T-RFs from *Alu*I digest

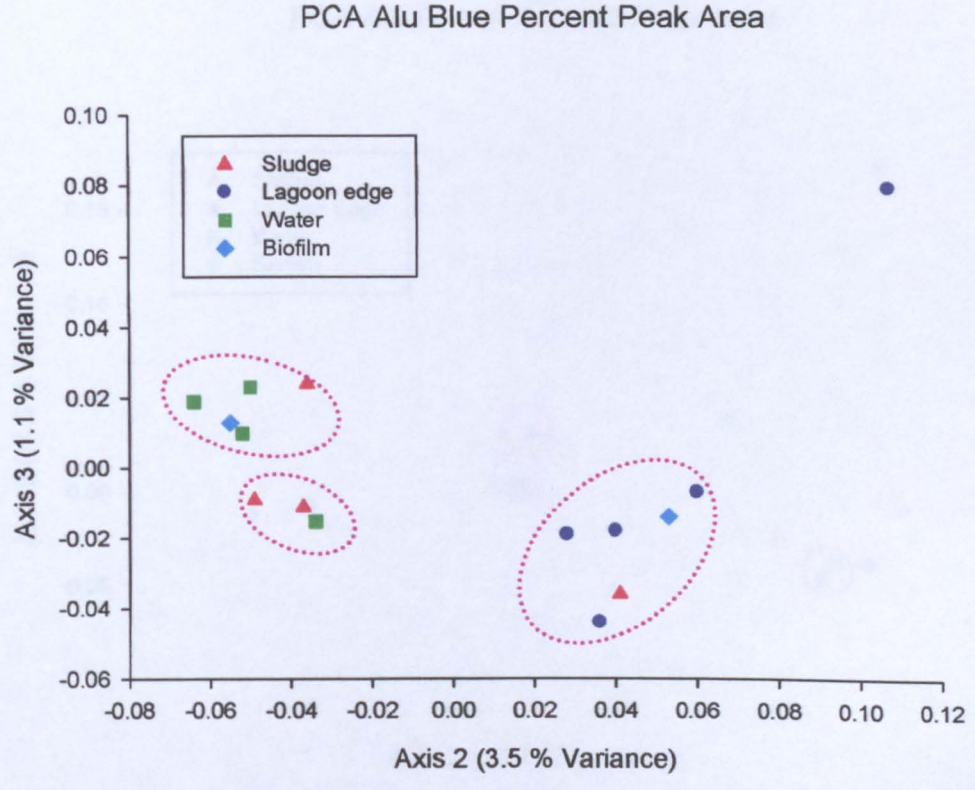


Figure 3.6b: Axis 2 v Axis 3 PCA of percent peak area data of forward T-RFs from *Alu*I digest

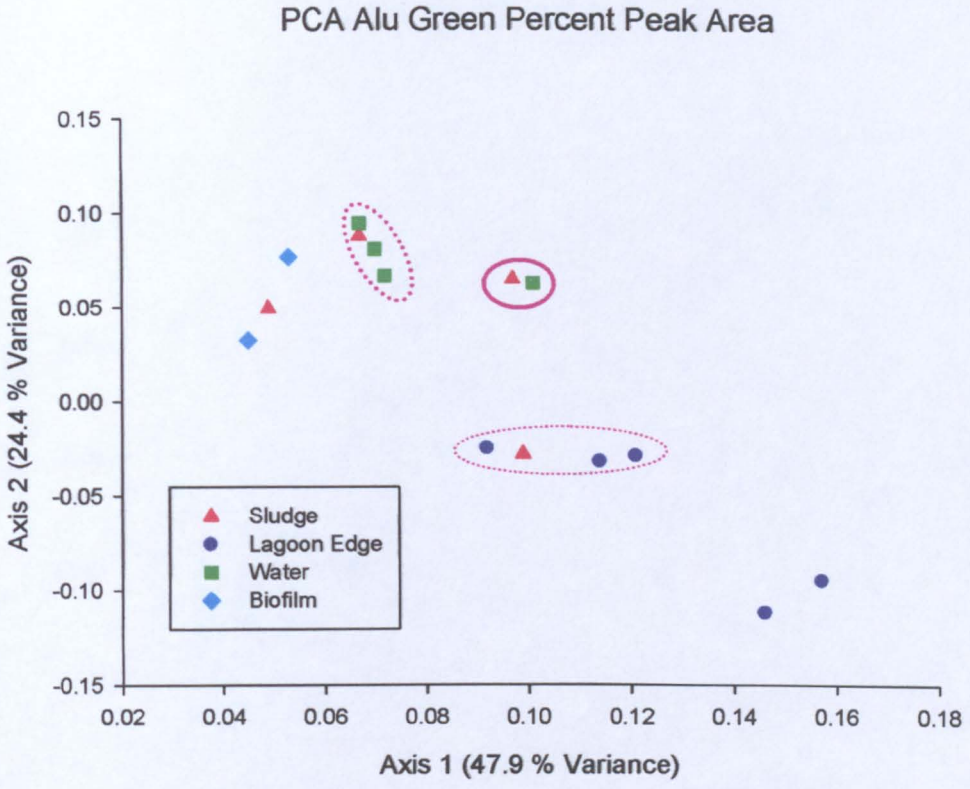


Figure 3.6c: Axis 1 v Axis 2 PCA of percent peak area data of reverse T-RFs from an *AluI* digest

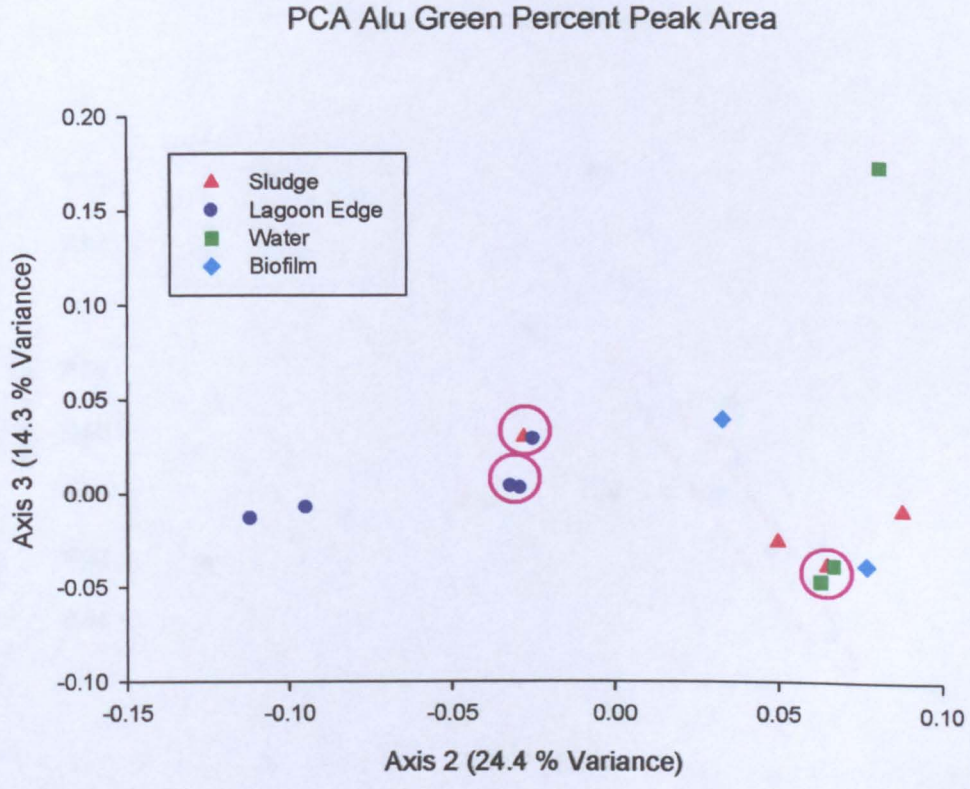


Figure 3.6d: Axis 2 v Axis 3 PCA of percent peak area data of reverse T-RFs from an *AluI* digest

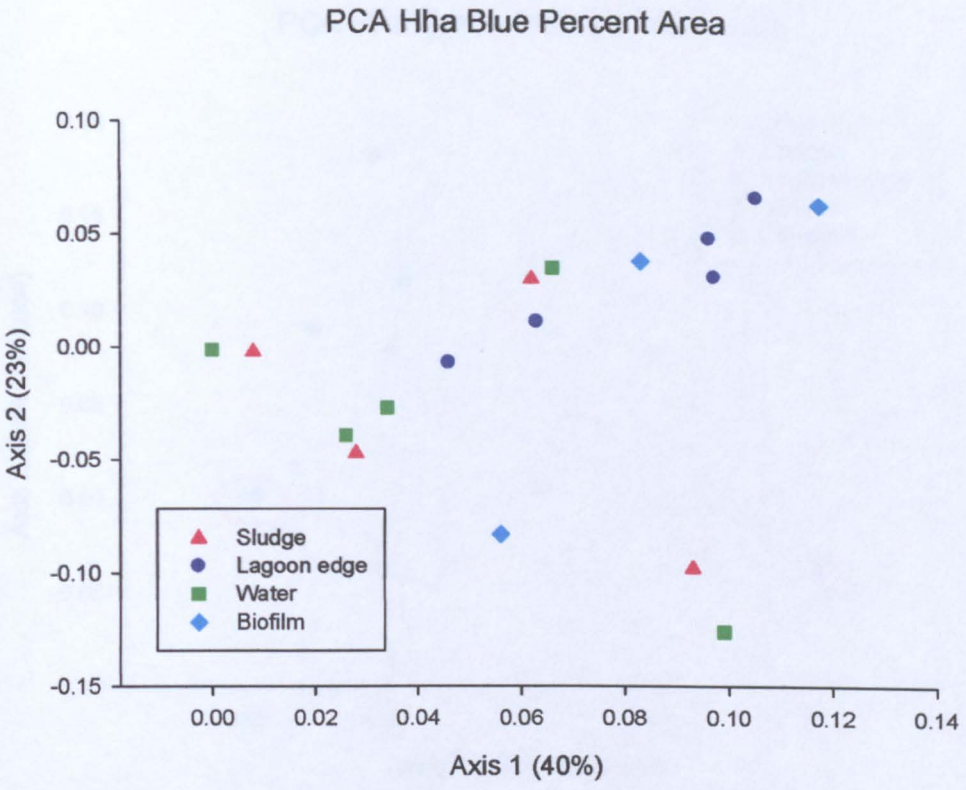


Figure 3.6e: Axis 1 v Axis 2 PCA of percent peak area data of forward T-RFs from an *HhaI* digest

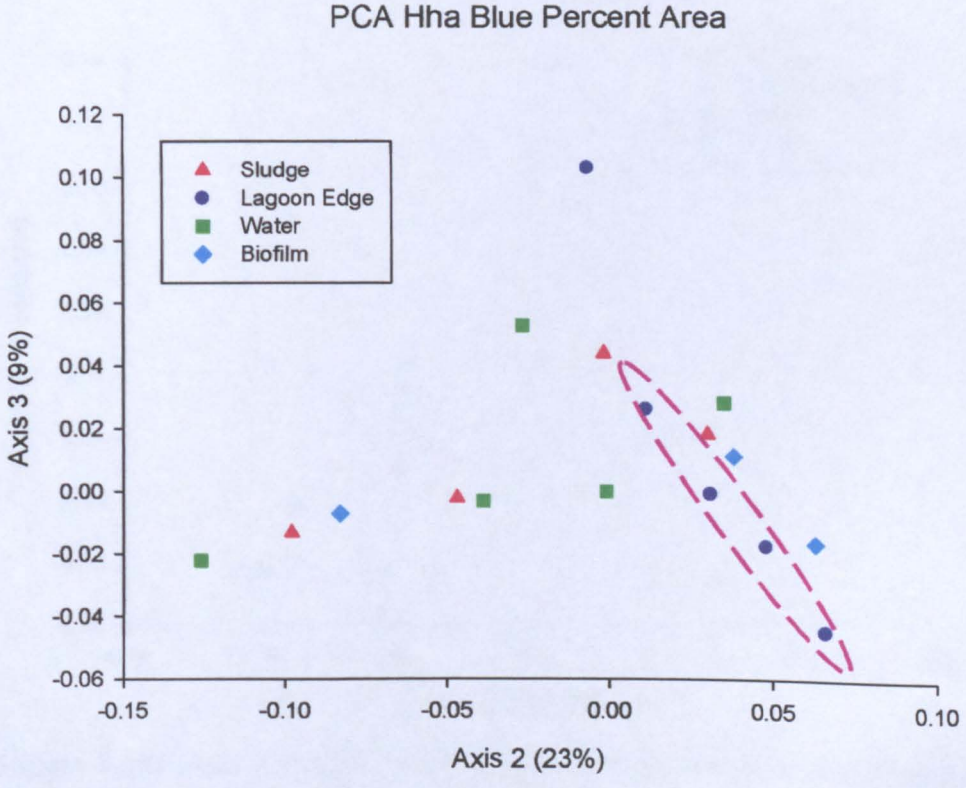


Figure 3.6f: Axis 2 v Axis 3 PCA of percent peak area data of forward T-RFs from an *HhaI* digest

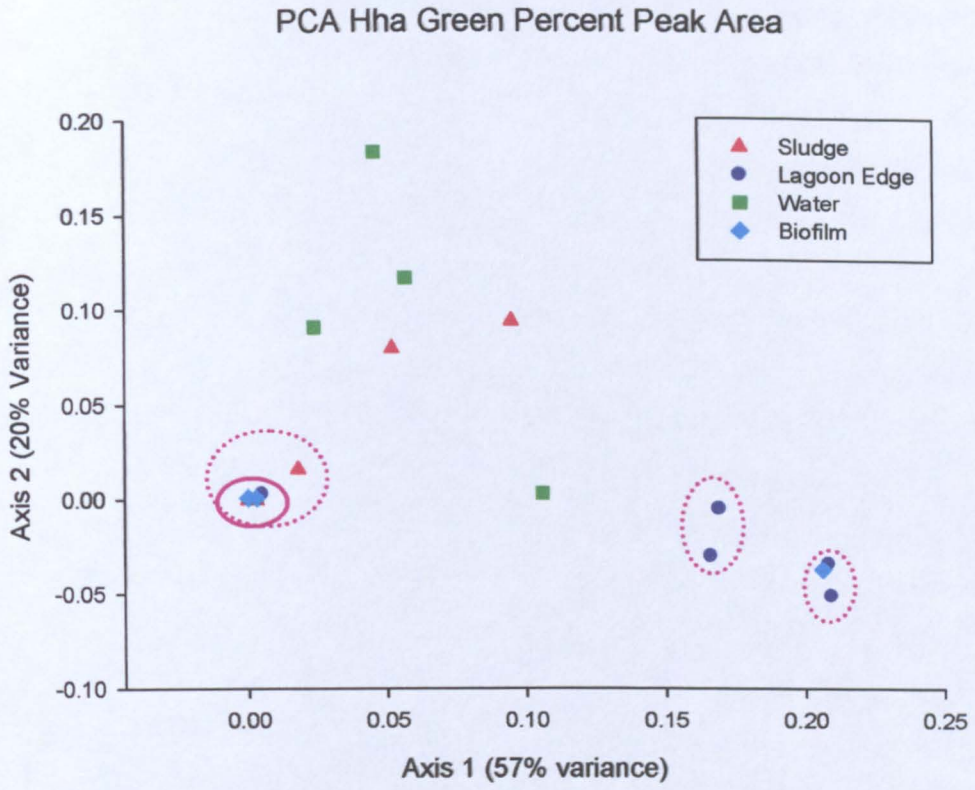


Figure 3.6g: Axis 1 v Axis 2 PCA of percent peak area data of reverse T-RFs from an *HhaI* digest

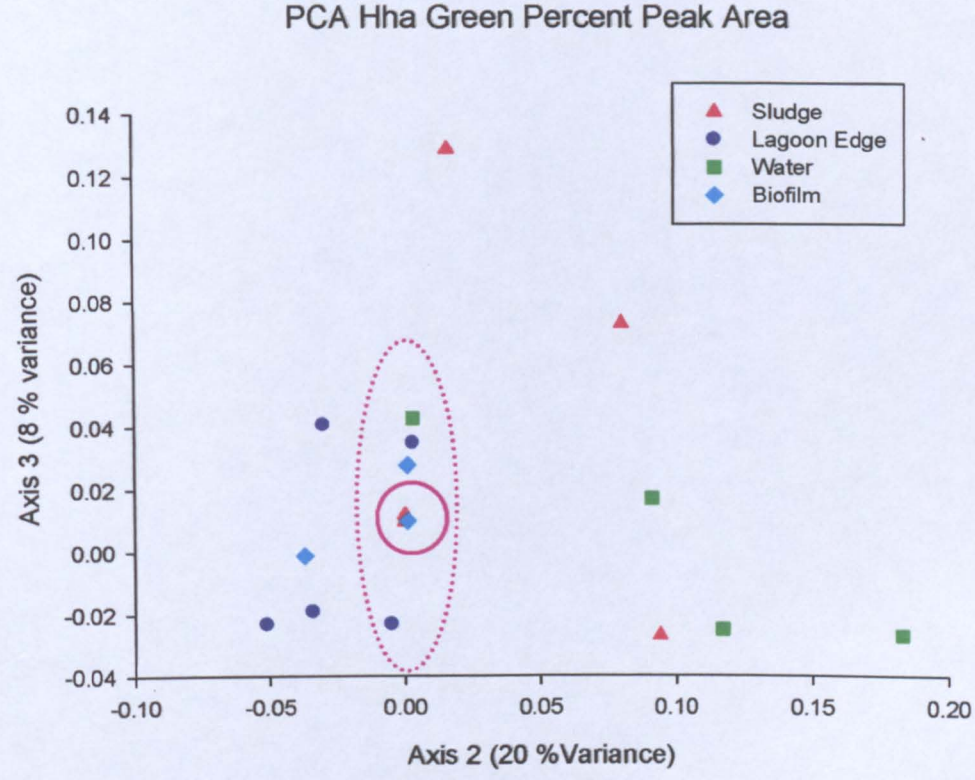


Figure 3.6h: Axis 2 v Axis 3 PCA of percent peak area data of reverse T-RFs from an *HhaI* digest

The results of the statistical analyses of T-RFLP data indicate that there are no clear diversity trends within each sampling group, with little tight clustering of samples on the basis of their sample type. Some analyses indicate that there are similarities in the diversity present between different samples, shown particularly strongly by the overlapping of data points in Figures 3.5h, 3.6d, 3.6g and 3.6h. Whilst samples could be colour coded and grouped based on sample pH, instead of by sample type, the distribution of data points would be identical and since there are very few tight clusters it is unlikely that this would highlight any further evidence linking microbial diversity to conditions within the lagoon. For example, the data points which cluster in Figure 3.6g are samples 29, 35, 38, 46 and 47 which have pH values of 3.40, 2.62, 4.60, 3.07 and 3.30 respectively. Given the wide ranging pH values of these samples it is unlikely that pH could be considered a determining factor in microbial diversity correlations. However, the clustering of certain data points such as those in Figures 3.5h, 3.6d, 3.6g and 3.6h suggests that there may be some other, as yet unknown, factor which may correlate with microbial diversity such as oxygen levels, the concentration of a particular carbon source or the availability of a terminal electron acceptor for example.

The T-RFLP data from Hoole Bank acid tar lagoon samples have also be represented using histograms in Figures 3.7a-d. This allows a more direct, visual comparison of the T-RFs within each group of sample types. In this instance the forward T-RFs from *Hha*I restriction digests have been chosen for comparison, however histograms for each restriction digest-dye combination could be created. The histograms show every T-RF in a particular sample, which allows direct visual comparison between samples to identify which T-RFs are present or absent and the relative abundance of a particular T-RF within a sample. For example, in this digest-dye combination, the T-RF at 170.00 bp is not only present in many samples, but is obviously abundant in many of the samples since it has a large percentage peak area which can been seen easily in Figures 3.7a-d. In addition to the histograms showing T-RF size and abundance for *Hha*I forward T-RF analyses, Table 3.3 lists some of the most abundant T-RF sizes for each dye-digest combination and the number of occurrences of these T-RFs.

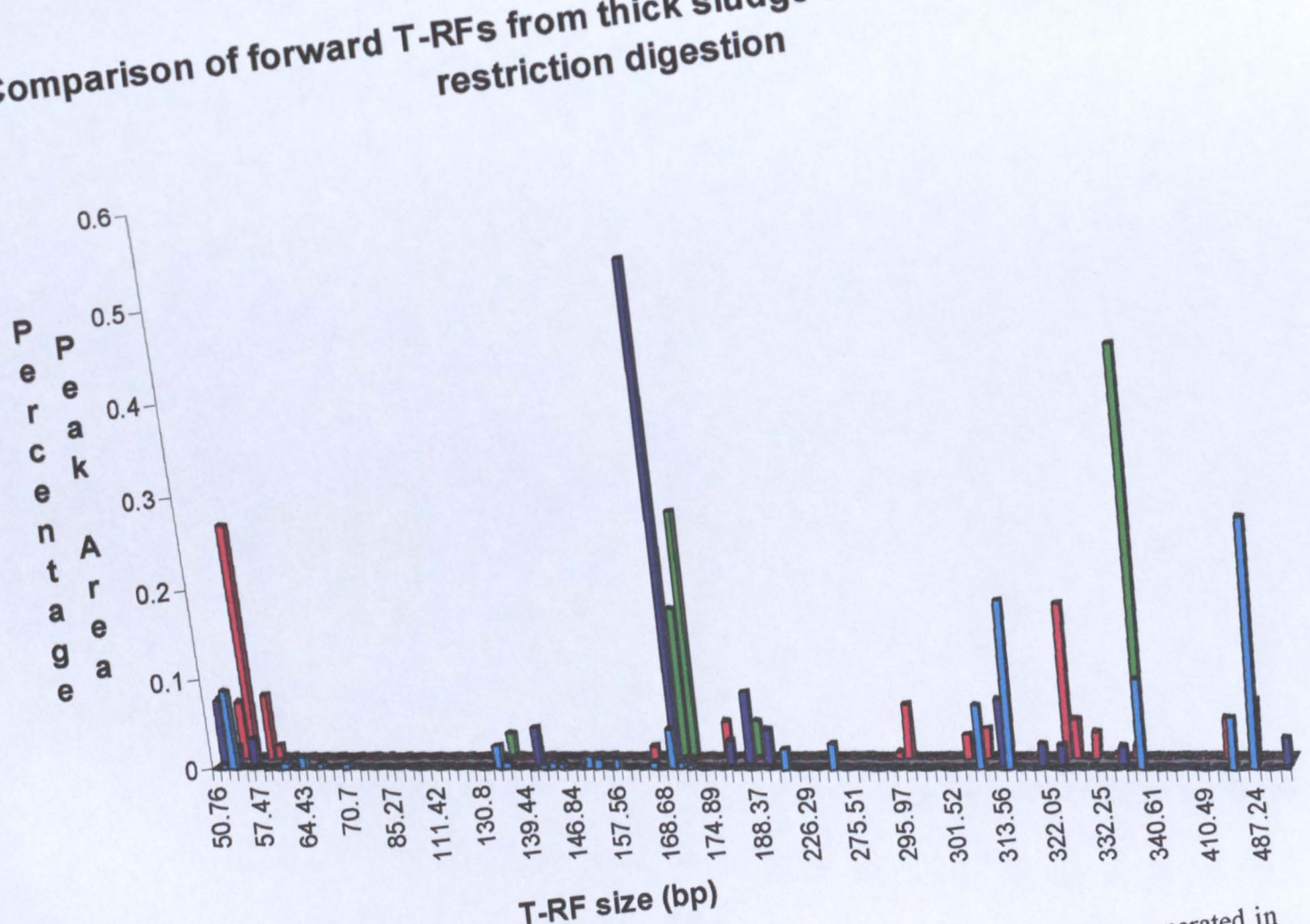


Figure 3.7a: Histogram showing the size (bp) and percentage peak area of T-RFs generated in *Hha*I forward analysis of thick sludge samples (Group 1).

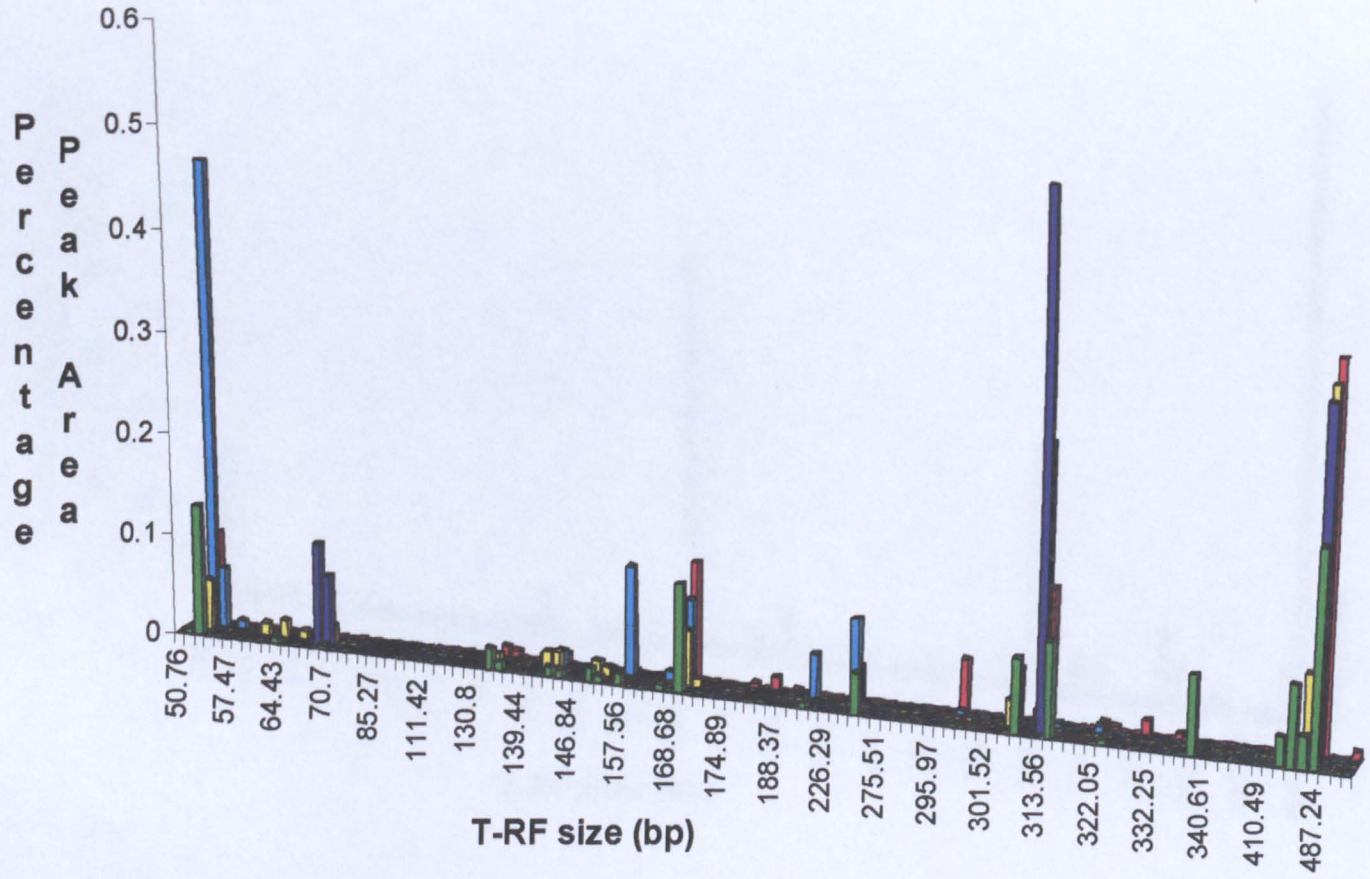


Figure 3.7b: Histogram showing the size (bp) an percentage peak area of T-RFs generated in *Hha*I forward analysis of lagoon edge samples (Group 2).

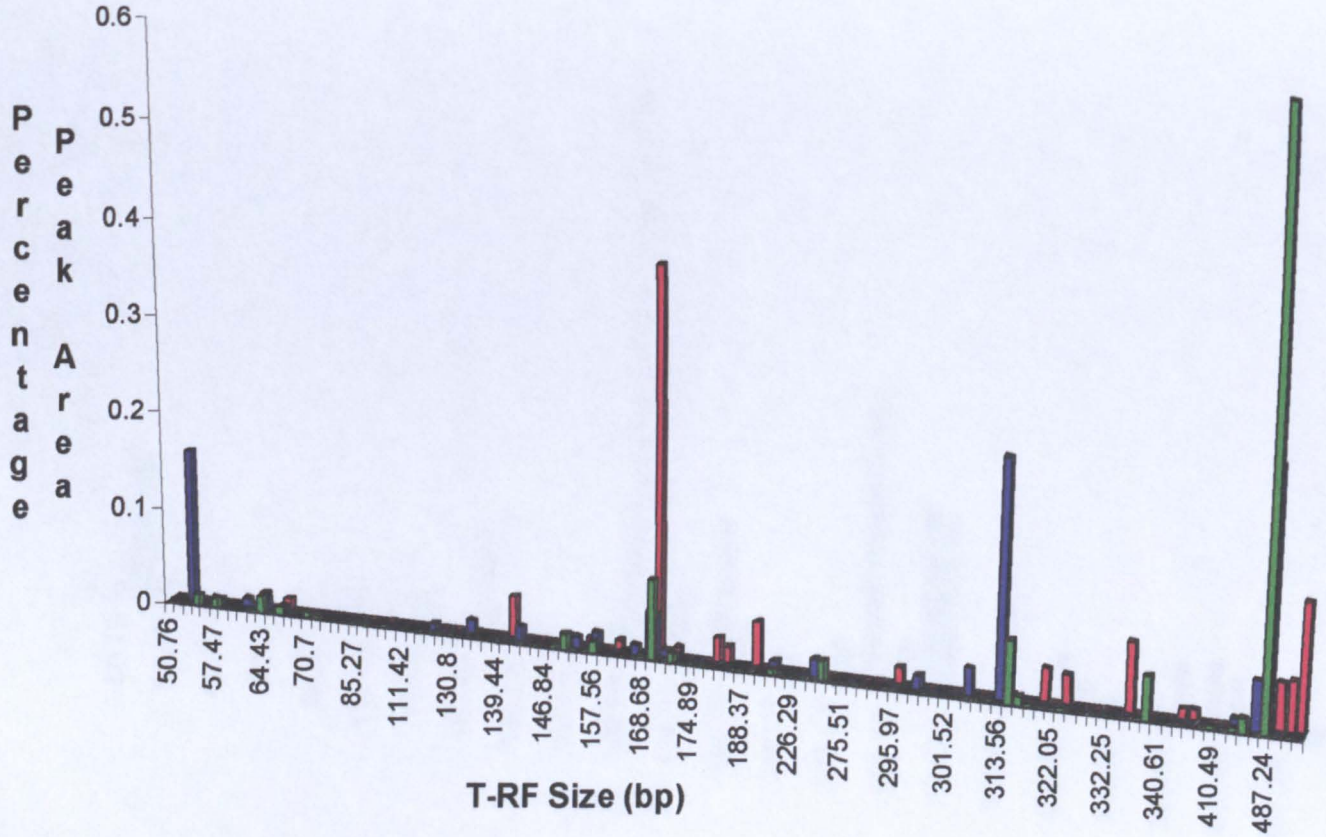


Figure 3.6c: Histogram showing the size (bp) and percentage peak area of T-RFs generated in *Hha*I forward analysis of green biofilm samples (Group 3).

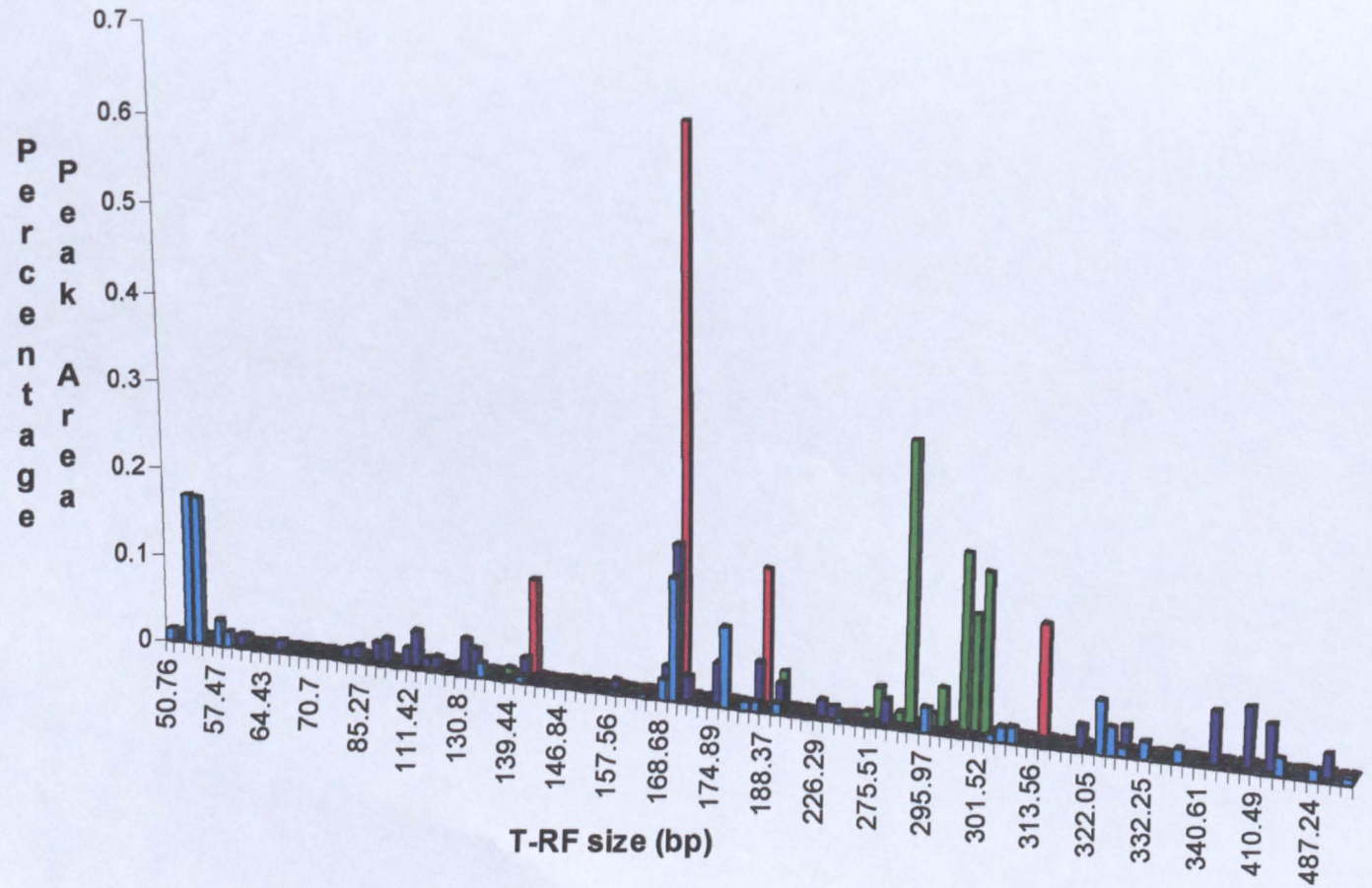


Figure 3.7d: Histogram showing the size (bp) and percentage peak area of T-RFs generated in *Hha*I forward analysis of water samples (Group 4).

*Alu*I Forward		*Alu*I Reverse		*Hha*I Forward		*Hha*I Reverse	
Size T-RF (bp)	Number of T-RFs	Size T-RF (bp)	Number of T-RFs	Size T-RF (bp)	Number of T-RFs	Size T-RF (bp)	Number of T-RFs
89.58	9	58.78	11	53.45	10	61.01	10
112.22	13	67.82	13	157.56	10	113.15	10
113.61	12	69.17	12	168.68	9	189.45	12
129.6	10	126.44	10	170	14	293.07	9
139.96	9	127.91	14	322.94	10	298.13	12
142.42	10	130.07	12	487.24	10	300.42	9
163.32	12	331.69	13				
195.66	9	335.2	9				
238.83	12						

Table 3.3: Selected T-RFs from each digest/dye combination showing some of the most commonly occurring T-RFs in each combination and the number of occurrences of each T-RF. The two T-RFs in red indicate the two most commonly occurring sized forward T-RFs, with an incidence of these T-RFs in 13 *Alu*I and 14 *Hha*I samples.

The number of T-RFs present in each sample for each digest-dye combination was calculated using data produced from the T-align analysis (Smith *et al.* 2005) and the combined total of all digest-dye combinations calculated for comparison. Table 3.4 shows the number of T-RFs present in each sample, with samples grouped into their appropriate sample group, whilst Table 3.5 shows the same data organised into increasing sample pH.

Each T-RF should represent an individual bacterial species, therefore the presumption that increased numbers of T-RFs is equal to increased microbial diversity has been made. There appears to be very little correlation between sample type and microbial diversity in thick sludge, lagoon edge, or water samples. However, two of the three biofilm samples, 45 and 46, show a greater degree of correlation between sample type and microbial diversity than most other samples. These two samples are particularly geographically close therefore it is possible that there is a connecting factor such as presence/absence of a carbon compound or terminal electron acceptor, oxygen availability or another unknown factor linking the microbial diversity in these samples.

The data in Table 3.5 also shows little correlation between microbial diversity and sample pH. Initial hypotheses would suggest that microbial diversity would increase with increasing pH yet this does not seem to be the case based on the T-RFLP data obtained in this study.

		AluI Blue	*AluI* Green	*HhaI* Blue	*HhaI* Green	Total
Thick Sludge	27	16	10	13	7	46
	30	31	29	34	25	119
	35	ND	ND	42	43	85*
	38	32	24	27	50	133
	44	9	11	7	9	36
	54	ND	ND	ND	ND	ND
Lagoon Edge	28	ND	ND	18	11	29
	28A	37	27	ND	ND	64*
	47	32	24	32	31	119
	49	3	3	4	3	13
	50	26	17	19	9	71
Green Biofilm	29	ND	ND	36	48	84*
	45	38	19	25	8	90
	46	29	15	19	21	84
Water	33	26	16	18	16	76
	37	20	28	4	6	58
	41	38	14	33	16	101
	43	12	16	30	30	88

Table 3.4: Number of T-RFs present in each sample, correlated by sample type
ND = Not determined * = Total is not from all dye-digest combinations.

pH	Sample	*AluI* Blue	*AluI* Green	*HhaI* Blue	*HhaI* Green	Total
1.8	43	12	16	30	30	88
2.17	28	ND	ND	18	11	29*
2.39	58	ND	ND	ND	ND	ND
2.57	33	26	16	18	16	76
2.62	35	ND	ND	42	43	85*
2.69	50	26	17	19	9	71
2.88	45	38	19	25	8	90
2.98	30	31	29	34	25	119
3.06	49	3	3	4	3	13
3.07	46	29	15	19	21	84
3.3	47	32	24	32	31	119
3.4	29	ND	ND	36	48	84*
3.45	44	9	11	7	9	36
3.92	28A	37	27	ND	ND	64*
4.15	37	20	28	4	6	58
4.4	41	38	14	33	16	101
4.6	38	32	24	27	50	133
5.2	27	16	10	13	7	46

Table 3.5: Total number of T-RFs in each sample, correlated by increasing sample pH
ND = Not determined. * = Total is not from all dye-digest combinations.

Further analysis of T-RFLP data was carried out using publicly available programmes on the world wide web, specifically Phylogenetic assignment tool (PAT) (Kent *et al.* 2003). PAT is an online tool which can be used to assign or infer phylogeny to a particular sized T-RF. The advantage of PAT is that the programme employs the user uploaded T-RFLP data directly to produce a results table which includes all the potential microbial species (for which there are 16S rRNA gene sequences in public databases) which would generate a particular T-RF in one or more restriction digests. Thus, both *Alu*I and *Hha*I T-RFLP data are analysed concurrently, therefore only T-RFs present with corresponding sizes in both restriction digests are found, producing much fewer possibilities than considering both restriction enzymes separately. Table 3.6 lists some of the possible species which match the 170.00 bp *Hha*I forward T-RF and the 112.22 bp *Alu*I forward T-RF (the two most commonly occurring forward T-RFs).

It was not possible to infer the phylogeny of every T-RF generated from the Hoole Bank T-RFLP data or to infer the phylogeny of a particular T-RF to less than five species in some instances using PAT. The phylogeny of T-RFs which were not identified using both *Hha*I and *Alu*I data could be inferred more loosely using PAT with a single digest or using an alternative programme such as TAP. Whilst both these programmes are extremely useful tools, they also provide further challenges since the PAT analysis of T-RFLP data from Hoole Bank acid tar lagoon produced several hundred potential phylogenetic assignments in total.

Species	Details	NCBI Accession	Reference
Acidovorax sp. OS-6	Activated sludge	AB076844	(Khan *et al.* 2002)
Uncultured bacterium SJA-23	Anaerobic, trichlorobenzene - transforming microbial consortium	AJ009457	(Wintzingerode *et al.* 1999)
Uncultured bacterium clone BANW401	Subsurface groundwater during polylactate stimulated chromate bioremediation	DQ264407	(DeSantis *et al.* 2007)
Alicycliphilus denitrificans K601	Cyclohexanol-degrading, nitrate-reducing β-proteobacterium	AJ418042	(Mechichi *et al.* 2003)
Diaphorobacter nitroreducens KSP4.	Poly(3-hydroxybutyrate)-degrading denitrifying bacterium isolated from activated sludge	AB076856	(Khan and Hiraishi 2002)
Hydrogenophaga sp. YED1-18 ATCC BAA-306	Arsenite oxidizing biofilms at Hot Creek	AY168753	(Salmassi *et al.* 2006)
Uncultured bacterium oc52	Microbial fuel cell enriched with artificial wastewater	AY491593	(Phung *et al.* 2004)
Uncultured bacterium BE24FW032601C18 W17-3.	Fracture-derived groundwater in a deep gold mine of South Africa	DQ088741	(Lin *et al.* 2006)

Table 3.6: Microbial species which may be responsible for the *Hha*I 170.00 bp / *Alu*I 112.22 bp T-RFs in Hoole Bank acid tar lagoon. This data was generated using the PAT programme at http://T-RFlp.limnology.wisc.edu/index.jsp (Kent *et al.* 2003), using the default dataset and creating an additional bin of size 50.0 bp with a tolerance of 1.0.

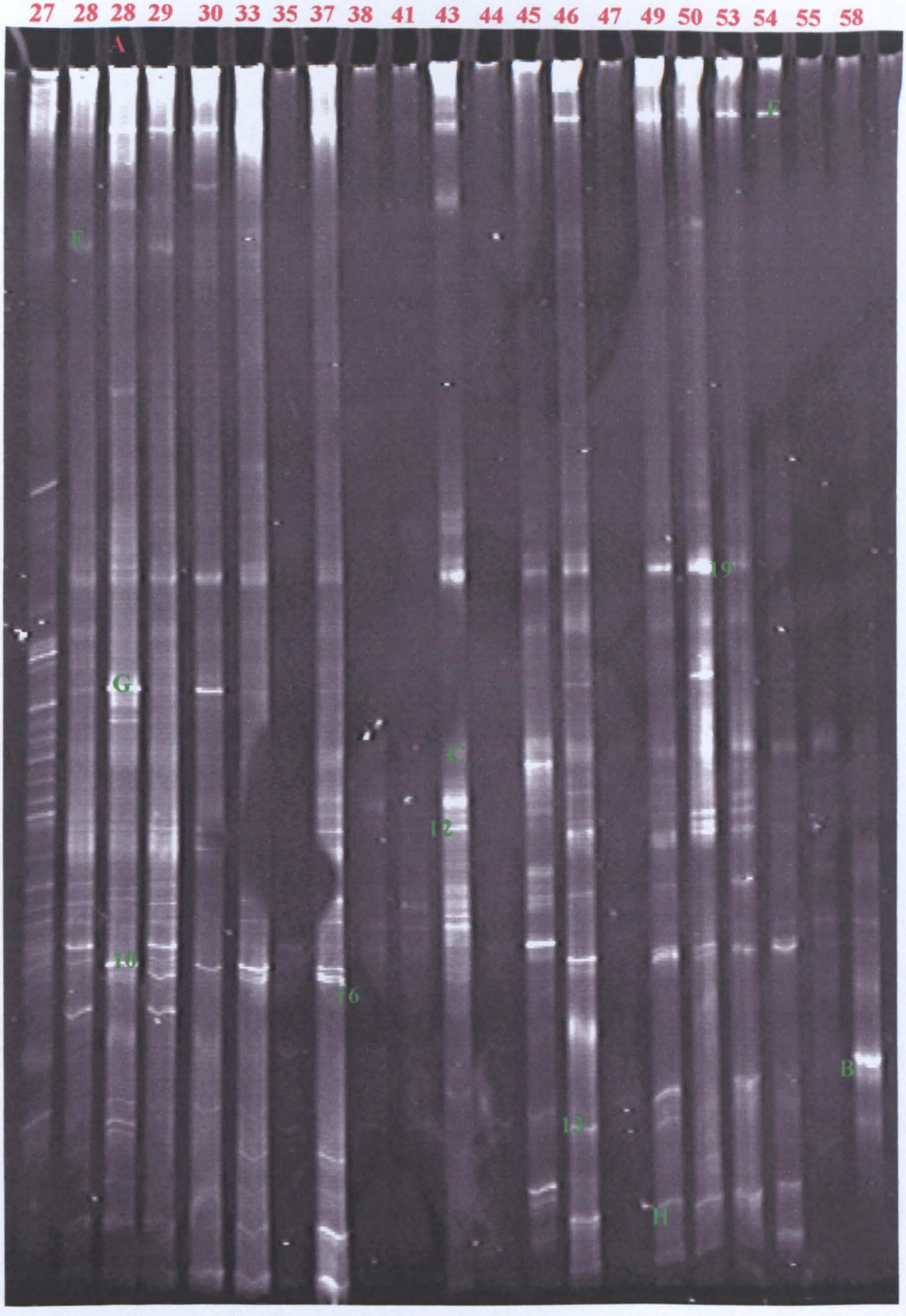


Figure 3.8: A denaturing gradient gel of the PCR amplified V9 hypervariable region of the 16S rRNA gene from Hoole Bank acid tar lagoon samples. The green numbers and letters refer to bands which were excised and sequenced, further details of which are shown in Table 3.7.

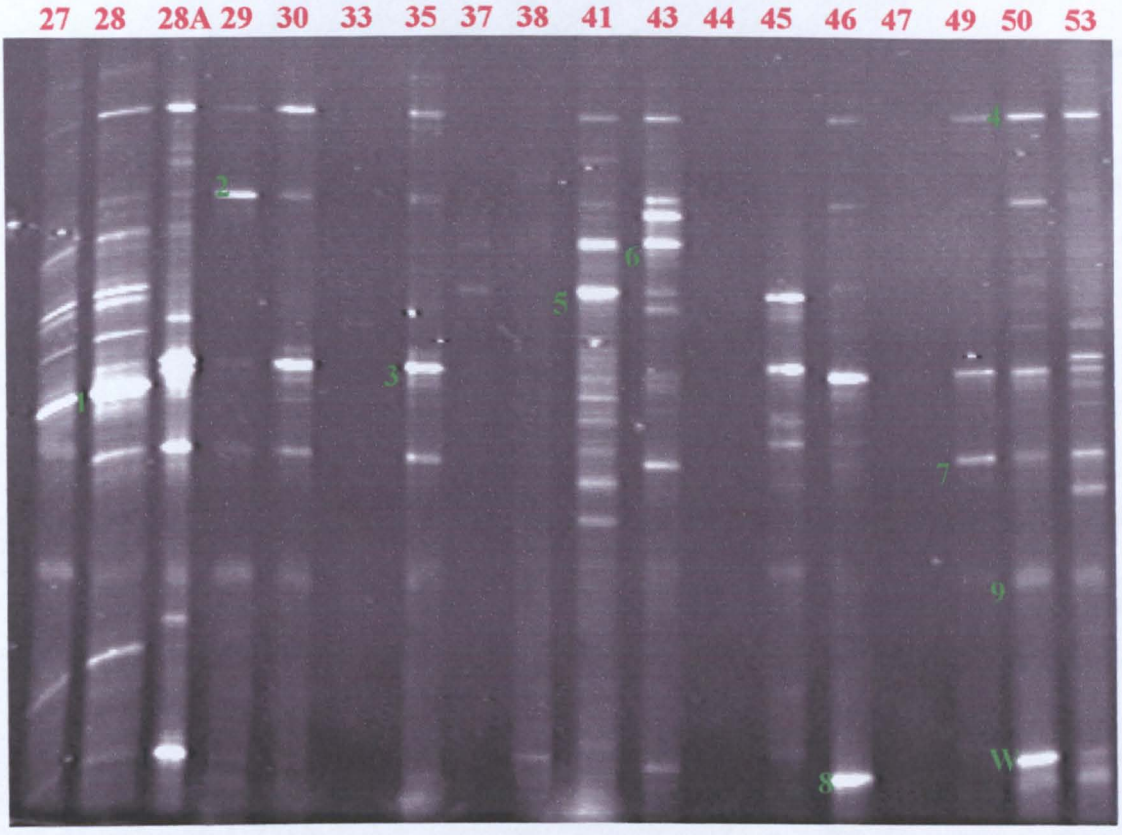


Figure 3.8: The bottom half of a denaturing gradient gel of the PCR amplified V3 hypervariable region of the 16S rRNA gene from Hoole Bank acid tar lagoon samples. The green numbers and letters refer to bands which were excised and sequenced, further details of which are shown in Table 3.7.

Band	BLAST match	NCBI Accession Number	Environment of match
V9-C	Uncultured *Verrucomicrobia* bacterium clone BSR2LF12	AY690115	Naturally acidic mountain stream sediment
V9-F	Uncultured eubacterium WD238	AJ292597	Polychlorinated biphenyl-polluted soil
V3-3	Uncultured bacterium clone GXDC-17	EU250231	Acid mine drainage
V9-G	Uncultured bacterium clone ERF-F6	DQ906078	Tinto River Rhizosphere
V9-H	Uncultured gamma proteobacterium clone A2-4c03	EU236255	Radionuclide contaminated subsurface sediments
V3-1	Uncultured epsilon proteobacterium clone	AY437423	MTBE contaminated shallow aquifer
V3-2	*Euglena mutabilis* strain SAG 1224-9b	AY626044	N/A
V3-4	Uncultured archaeon clone arq3_h8	EF446255	Macroscopic filaments from Rio Tinto
V3-5	Bacterium ML2-86	DQ145977	Milk Lake, Taiwan. A sulphur lake.
V3-6	Uncultured bacterium clone biogas-DI-b70	DQ419703	Biogas
V3-7	*Acidithiobacillus ferrooxidans* strain DBS	EU084696	N/A
V9-E	*Acidocella* sp. DM2	DQ419948	Moderate acid mine drainage
V3-W	Uncultured *Acidiphilium sp.* clone K6-C83	EF612396	Semi arid lead-zinc mine tailings site
V9-B	*Burkholderia* sp. AFF	EF506612	Phenanthrene soil
V3-9	Uncultured soil bacterium clone PAH-Feed-53	DQ123784	PAH-contaminated soil
V3-8	*Chryseobacterium gregarium* Type strain DSM 19109T	AM773820	Phyllosphere of grasses
V9-10	Uncultured bacterium clone FS0612_U6	EU101113	Frasassi sulfidic cave stream biofilm
V9-12	*Pseudomonas sp.* BFSY-1	EU258737	Oil contaminated soil from oil refining plant
V9-13	Uncultured *Bacteroidetes* bacterium clone GalB60	AY193184	Oxidized iron deposits
V9-16	Uncultured bacterium clone ERF-1A1	DQ906050	Metagenome of the Tinto River rhizosphere
V9-19	Uncultured bacterium clone YSK16S-36	EF612999	Acid mine drainage, Yinshan Mine, China

Table 3.7: Representative BLASTN matches of excised bands from denaturing gradient gels of V3 (Figure 3.9) and V9 (Figure 3.8) hypervariable regions of the 16S rRNA gene. N/A = Not applicable; the environment from which the sequence match was isolated/cloned from is not listed.

The results of BLASTN using the megablast algorithm on the NCBI website (Altschul *et al.* 1990) revealed a wide variety of microorganisms present in Hoole Bank acid tar lagoon samples, including a species of alga and an archaeaon. Each band produced many matches with percentage identities greater than 97 %, with most bands having 99 % or 100 % identity over the 200-300 bp sequenced region to other sequences in the NCBI database. In several cases where the excised DGGE band sequence matched multiple sequences with equal identity the highest matching, most appropriate organism or clone was selected. For example, band V9-16 is listed in Table 3.7 as a sequence match with an uncultured bacterium clone from the Rio Tinto rhizosphere, yet this band also matched accession numbers AY683287 (clone from beech and spruce litter), EF221112 (vegetated soil from Antarctica), AY425773 (volcanic deposit) and DQ528761 (a novel *Acidobacterium* species) amongst several others with equal sequence identity.

DGGE bands V9-E (Figure 3.8) and V9-B (Figure 3.8) are of particular interest as these bands were identified through BLASTN as *Acidocella* sp. DM2 and *Burkholderia* sp. AFF respectively (Table 3.7); both of which are very closely related species to microorganisms which were isolated using classical culture methods (Section 3.2.2 and Table 3.2). Furthermore, given the short region of the 16S rRNA gene used for identification it is extremely likely that the organisms isolated using classical culture techniques are the same microorganisms responsible for bands V9-E and V9-B respectively.

Band 2 of the V3 gel (Figure 3.9) was identified as *Euglena mutabilis* strain SAG 1224-9b using the megablast algorithm. Identification of a species of the genus *Euglena* is noteworthy since previous attempts to characterise eukaryotic diversity in the Rio Tinto failed to identify *Euglena* using 18S rRNA DGGE despite microscopic observations of this genus in the samples analysed (Aguilera *et al.* 2006). It is of further interest since *Euglena mutabilis* is well known to tolerate acidic conditions (Lane and Burris 1981; Olaveson and Nalewajko 2000) yet a closely related species, *Euglena gracilis*, was isolated from Hoole Bank acid tar lagoon samples using classical techniques. As has been mentioned previously, the reliability of 16S rRNA to distinguish between different species is not totally reliable, further proven by the remaining BLASTN matches to V3-2 which included *Euglena viridis*, *Euglena deses* and *Euglena stellata* amongst other species of *Euglena* and other uncultured clones.

Therefore it remains a possibility that band V3-2 may be affiliated to *Euglena gracilis*, as opposed to *Euglena mutabilis*. In order to quantify this further, the V3-2 DGGE band sequence was aligned against the chloroplast 16S rRNA gene sequence obtained from the Hoole Bank *Euglena gracilis* isolate using the bl2seq alignment tool on the NCBI website (Tatusova and Madden 1999). The alignment was strong, with an 88 % identity over 171 residues which means that the possibility remains that the DGGE band is a result of the presence of *Euglena gracilis* in Hoole Bank acid tar lagoon samples. However, there also remains the possibility that both organisms are present in Hoole Bank acid tar lagoon samples, with one species isolated using classical culture techniques and the other species identified through molecular analyses.

Of the two remaining cultured isolates it would not be possible to detect the eukaryotic yeast isolate *Rhodotorula* using 16S rRNA DGGE since it possesses no 16S rRNA. However, detection of the bacterial *Gordonia* species would be possible using 16S rRNA DGGE, yet this organism was not detected on either V9 or V3 DGGE gels. The absence of a *Gordonia* species from either 16S rRNA DGGE may simply be that the band affiliated with this microbial species was not sufficiently visible for excision, as was the case for several bands which were visible in a closed UV transilluminator dark box but not using an open UV transilluminator. It is also possible that this species comprised such a small minority of the microbial population in Hoole Bank samples that PCR amplification was biased against this or that little or no genomic DNA was obtained from this organism irrelevant of its abundance within the microbial population.

Acidithiobacillus ferrooxidans was also identified as present in Hoole Bank acid tar lagoon samples (V3-7; Figure 3.9). This is not unusual given that this organism is a known acidophile and has been identified in many acidic environments previously (Gonzalez-Toril *et al.* 2003; Johnson *et al.* 2001; Mahmoud *et al.* 2005). *A. ferrooxidans* is often readily cultured from acidic samples, despite not always being the dominant species (Rawlings *et al.* 1999), yet this organism was not detected using the classical culture techniques employed in this study. This is most likely because specific growth media and long incubation period is required to culture this species.

3.3: General Discussion

The results of microbial diversity analysis at Hoole Bank acid tar lagoon are in agreement with previous studies which reveal a much greater microbial diversity using

molecular techniques than classical culturing techniques (Amann *et al.* 1995). A total of five microorganisms were isolated and identified from 21 samples from Hoole Bank acid tar lagoon, which would indicate a very low level of microbial diversity present. It is important to consider that the classical culture techniques emplyed were chosen specifically to isolate microorganisms which could be cultured readily and using a very limited pH range. Therefore, the limited diversity which was cultured does not represent the likely culturable population. Furthermore, results from molecular microbial community analysis using T-RFLP and DGGE indicate a much higher level of diversity present in some of the samples that that found using classical culture techniques.

There are several reasons for the discrepancy between the number of cultured organisms and the estimates of the number of species produced from T-RFLP and DGGE data, which were discussed earlier (Section 1.2.1 and 1.2.2). Underestimates of microbial diversity based on cultured organisms occur because many of the species present in environmental samples cannot be cultivated using the particular set of culture techniques employed. The lack or over abundance of certain nutrients, cofactors, terminal electron acceptors, synergies and levels of oxygen are just some of the reasons which may cause a particular organism to resist being cultured.

Many microorganisms may rely on the presence of another organism in some way in order for them to grow and survive, yet this study prevented the growth of fungi since these organisms out-competed the growth of other microorganisms. It is highly likely that the inhibition of fungal growth from Hoole Bank acid tar lagoon samples reduced the number of other microorganisms isolated from the samples. Similarly, a very narrow range of growth media were used in attempts to isolate organisms therefore the proportion of organisms present in samples which could grow using the media provided was considerably reduced compared to the total microbial population. All attempts to culture microorganisms were carried out under aerobic conditions, thus excluding a further proportion of the microbial population which were micro-aerophilic or anaerobes.

Of the five species of organisms isolated from Hoole Bank acid tar lagoon samples only one, *Acidocella* 29, would have been considered a likely candidate for isolation prior to culturing. *Acidocella* are known acidophiles and have been found in other acidic

environments previously (Johnson *et al.* 2001; Kimura *et al.* 2006), as well as exhibiting the potential to degrade a range of carbon compounds which are likely to be present at Hoole Bank acid tar lagoon (Dore *et al.* 2003; Roling *et al.* 2006). Of the remaining four isolates, *Rhodotorula* are known to be acidotolerant (Gadanho *et al.* 2006) whilst *Burkholderia* (Tillmann et al. 2005) and *Gordonia* (Chatterjee and Dutta 2003; Kim and Pagilla 2003) species have previously been found in environments similar to Hoole Bank acid tar lagoon.

The isolation of these microbial species, as opposed to the type of acidophilic organisms discussed in Section 1.5 which are commonly isolated from similar environments would suggest that the culture regime employed to isolate organisms was not yielding the maximum number of culturable isolates. However, the methodology employed to isolate organisms was chosen such that any microorganisms which were cultured could be readily grown in order to aid further studies.

A greater number of organisms could have been isolated from Hoole Bank acid tar lagoons relatively simply by using a greater number of growth media and conditions such as overlay plates (Johnson *et al.* 1987), minimal medium supplemented with carbon sources present in Hoole Bank acid tar lagoon samples, using a wider range of pH during media preparation and by using anaerobic growth conditions for example. Preparation of overlay plates (Johnson 1995), a commonly used technique for isolating microorganisms using the removal of toxic organic chemicals from the top agar by an underlayer containing another microorganism, are time consuming in their preparation and do not produce colonies for approximately 7-14 days, therefore they were not used in this study due to time constraints.

The isolation of a unicellular alga was not surprising since visibly green biofilms were present at Hoole Bank acid tar lagoon. However, it was surprising that the species apparently responsible for much of this biomass was *Euglena gracilis*, as opposed to the more acidophilic species *Euglena mutabilis*. It is thought that *E. gracilis* is in fact equally acid tolerant but is far less resistant to heavy metals, and since the presence of heavy metals is often found in highly acidic environments, *E. mutabilis* is the most commonly isolated euglenoid from acid environments (Olaveson and Nalewajko 2000). Isolation of *E. gracilis* is not that surprising since Hoole Bank acid tar lagoon does not contain high levels of heavy metals despite its acidity (Nichol 2000).

All of the organisms isolated were from different Hoole Bank acid tar lagoon samples, with pH ranging from 2.62 to 3.40. However three of the five isolates were from sample group 1, thick sludge external to the lagoon, and the remaining two isolates from sample group 3, green biofilm. However, it is important to mention at this point that several other organisms were cultured from Hoole Bank samples but could not be maintained for more than two generations and therefore could not be successfully identified.

Of note is the isolation of two organisms from Group 3 samples, compared to the relatively low microbial diversity detected using DGGE in these samples (Lanes 29, 45 and 46, Figures 3.8 and 3.9). Initially the limited microbial diversity detected using DGGE was thought to be a result of inefficient genomic DNA extraction due to the biofilm structure. However, these samples produced a greater or comparable number of T-RFs when compared to water samples (Table 3.4) which would suggest that this is not the true reason for the limited diversity seen using DGGE. One possibility is that bands which comprised only a small proportion of the population in these samples were not detected using DGGE due to the limited sensitivity of staining and visualisation but were detected using T-RFLP.

No microorganisms were isolated from sample 27, despite this sample having the highest pH, at 5.2. The most likely reason for the absence of cultured organisms from this sample and several others is that all the media used for isolation were adjusted to pH 3 in order to isolate acidophilic or acidotolerant species for further study. Based on V9-DGGE, sample 27 appears to have the highest level of microbial diversity compared to all the other samples (Figure 3.8). However, this considerably higher level of microbial diversity observed in V9 and V3 DGGE gels does not correspond with T-RFLP data which shows comparable levels of microbial diversity between sample 27 and the remaining 20 samples. One possible explanation for this discrepancy is that T-RFLP data is simply much more sensitive. For example, Figures 3.7a-d show many T-RFs with very small percentage peak areas and only approximately four to six columns with high percentage areas, which more than likely represent species comprising only a minority of the microbial population and predominant, abundant species within the population respectively. Thus, the most prevalent and abundant species with large percentage peak areas seen in the histograms of Figures 3.7a-d are likely to be represented by the brightest bands visible in the DGGE gels, whilst the species which

comprise a minority of the microbial population may only produce faint bands or bands which are not visualised at all.

The T-RFLP profiles generated from Hoole Bank acid tar lagoon samples did not show any strong correlation between sample grouping and microbial diversity, or pH and microbial diversity. Statistical analyses using principal component analysis of interstitial data (Figures 3.5a-h), peak area data (Figures 3.6a-h) and correspondence analysis of both interstitial and peak area data (data not shown) indicate few trends within sample groupings or sample pH. Certain samples cluster closely in Figures 3.5h, 3.6d, 3.6g and 3.6h which may indicate some as yet unknown linking factor but this factor was not determined.

There are advantages and disadvantages of both molecular methods, therefore using both methods has provided a broader picture of the microbial diversity present in Hoole Bank acid tar lagoon. Whilst T-RFLP is more sensitive and provides a greater insight into the number of probable microbial species present in the acid tar lagoon there is no definitive sequence information obtained using this technique. Although *in silico* analyses such as PAT (Kent *et al.* 2003) and TAP (Marsh *et al.* 2000) allow the phylogeny of T-RFs to be inferred, the information obtained from these programmes is purely theoretical. A large number of results were obtained using the T-RFLP profiles from Hoole Bank acid tar lagoon using PAT. For example, sample 46 produced a total of 5600 potential phylogenetic affiliations. Whilst inference of phylogeny is useful, analysis of these data is time consuming given the large number of potential matches which arise as a result of the high number of matches for each T-RF combination, the number of different T-RF combinations which produce matches to species within the database used and the high number of repetitive sequence information contained within the databases. The latter point is particularly relevant to sequences from clone libraries and metagenomic libraries which produce vast amounts of sequence data which is added to the database which ultimately resulted in 20-40 matches to duplicate or slight variants of a clone insert from a particular clone or metagenomic library in some instances.

DGGE is advantageous in this respect since bands can be excised and sequenced in order to determine the phylogeny of the organism responsible for a particular band; however the disadvantage is that staining and visualisation of the gels is less sensitive than T-RFLP, therefore the proportion of DGGE bands which can be identified is

always likely to be significantly less than 100 % of the microbial diversity present as the technique is not sufficiently sensitive. Furthermore, it is known that one band can consist of the 16S rRNA gene fragment from more than one organism (Gafan and Spratt 2005), therefore the level of microbial diversity may also be underestimated using DGGE. Optimisation and alteration of the denaturing gradient may allow detection of species which are less abundant or allow the separation of two closely migrating species.

The multitude of high percentage identity sequence matches using BLASTN highlights several potential pitfalls of molecular microbial diversity analysis using DGGE. Firstly, the phylogenetic identification of organisms present in environmental samples based on the short sequences (200-300 bp) obtained from excised DGGE bands is not definitive. The results also highlight the limits of the 16S rRNA gene as a phylogenetic marker, since the regions of the 16S rRNA gene used in these experiments were both hypervariable regions yet sequences from excised DGGE bands matched several species of microorganisms. Limitations in our current knowledge and data also affect the results obtained, since it is not possible to match a query sequence to any sequences from organisms or clone libraries which have not been entered into the NCBI, or any other database, being used for cross-referencing. Finally, as demonstrated in the example above, the results are also open to user bias and selectivity, particularly when there are multiple equal identity matches. Although, in some circumstances it may be argued that selection of the most appropriate sequence match may be justified.

Chapter Four: Characterisation of "Green 46" – *Euglena gracilis* G46

4.1: Introduction

The following chapter describes some the effects of low pH on an organism which grew on ½ BLB pH 3 medium following inoculation with sample number 46 from Hoole Bank acid tar lagoon. This organism has been deposited in the Culture Collection of Algae and Protozoa (CCAP) under strain number *E. gracilis* G46 1224/46. Sample 46 was from a biofilm present in shallow lagoon water, approximately 20 – 30 cm deep, close to the edge of the lagoon with a pH of 3.07 (Figure 3.1c). This organism was chosen for further study since the biofilm was visibly abundant within several areas of the lagoon and thought to constitute a significant feature of the microbial community present at Hoole Bank acid tar lagoon.

4.2: Results

4.2.1: Identification of "Green 46"

A unicellular alga was cultured from lagoon sample 46, initially on solid ½ BLB medium. Initial microscopic observations showed cells to be approximately 40 µm in length by 6 µm wide. Liquid cultures of this organism were grown at approximately 27 ±2 °C in diurnal conditions prior to phylogenetic identification (Section 2.12.2). Genomic DNA preparations were made from liquid cultures and both 16S and 18S rRNA genes amplified by PCR and sequenced for phylogenetic identification of the organism. Phylogenetic analysis of the "Green 46" chloroplast 16S rRNA gene (NCBI accession number EU263908) using the BlastN algorithm at NCBI showed a 95 % identity to *Euglena gracilis* chloroplast genes, whilst BlastN comparison of the "Green 46" 18S rRNA gene (NCBI accession number EU263909) revealed a 97 % identity to *Euglena gracilis* small subunit rRNA (Figures 4.1 and 4.2).

Stackebrandt and Goebel (1994) suggested that > 97 % homology between 16S rRNA genes is a strong indication that two organisms are the same species, therefore the 97 % sequence identity between the "Green 46" 18S rRNA gene and the *E. gracilis* 18S rRNA gene would suggest that "Green 46" is *Euglena gracilis*. Therefore, from this point onwards "Green 46" will be referred to as *E. gracilis* G46. Following the identification of *E. gracilis* G46 using BlastN, the type strain *Euglena gracilis* Z (CCAP 1224/5Z) was used as a control organism for most experiments involving *E. gracilis* G46. Initially growth was monitored over a range of pH using chlorophyll content as a

measure of cell biomass. *E. gracilis* G46 grew in liquid media at pH 2 whereas the type strain *E. gracilis* Z showed no growth at this pH over a 12 day period (Figures 4.3a and 4.3b).

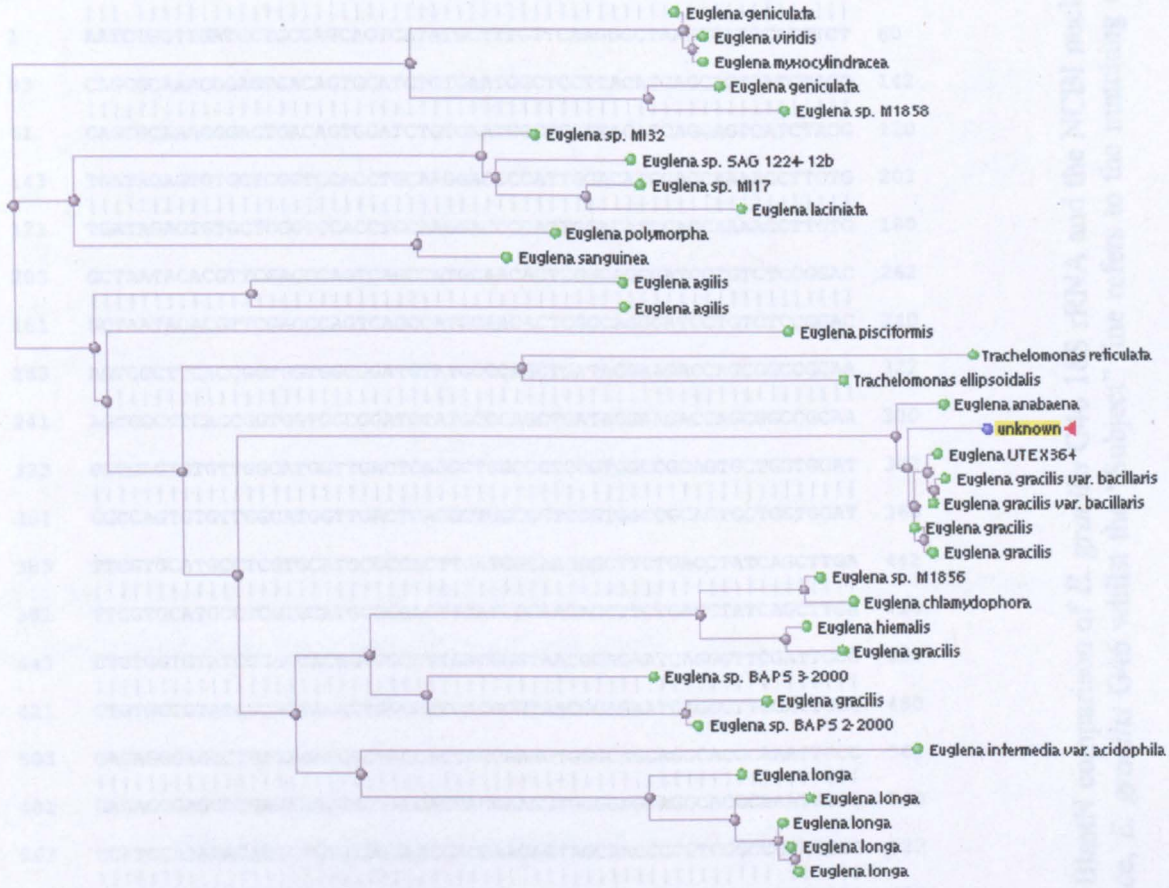


Figure 4.1: Neighbour joining phylogenetic tree showing the position of *E. gracilis* G46 (unknown) in relation to other *Euglenoid* species. Pairwise alignment with maximum sequence difference of 0.1 produced using BLASTN at NCBI.

> gb|M12677.1|EGRRGSS Euglena gracilis small subunit rRNA, complete
Length=2305

Score = 1849 bits (1001), Expect = 0.0
Identities = 1025/1036 (98%), Gaps = 3/1036 (0%)
Strand=Plus/Plus

```
Query  24    AAT-TGGTTGATCCTGCCAGCAGTCATATGCTTTGTTCAAGGGCTAAGCCATGCACGTCT  82
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     AATCTGGTTGATCCTGCCAGCAGTCATATGCTTTGTTCAAGGGCTAAGCCATGCACGTCT  60

Query  83    CAGCGCAAACGGAGTGACAGTGGATCTGTGAATGGCTCCTTACATCAGCAGTCATCTACG  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    CAGCGCAAACGGAGTGACAGTGGATCTGTGAATGGCTCCTTACATCAGCAGTCATCTACG  120

Query  143   TGATAGAGTGTGCTCGGTCCACCTGCAAGGACCCCATTGGACATCCACCAAAACCTTGTG  202
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   TGATAGAGTGTGCTCGGTCCACCTGCAAGGACCCCATTGGACATCCACCAAAACCTTGTG  180

Query  203   GCTAATACACGTTCGACCCAGTCAGCCATGCAACACTCGGCAGGGATCCTGTCTCCGGAC  262
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   GCTAATACACGTTCGACCCAGTCAGCCATGCAACACTCGGCAGGGATCCTGTCTCCGGAC  240

Query  263   AGTCCCTTCACCGGTGGTGGCGGATGTATGCCCAGCTGATACGAAGACCAGCGGCCGCAA  322
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   AGTCCCTTCACCGGTGGTGGCGGATGTATGCCCAGCTGATACGAAGACCAGCGGCCGCAA  300

Query  323   GGCCAGTGTGTTGGCATGGTTGACTCAGGCTGGCCCTCCGTGGCCGCAGTGCTGGTGGAT  382
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GGCCAGTGTGTTGGCATGGTTGACTCAGGCTGGCCCTCCGTGGCCGCAGTGCTGGTGGAT  360

Query  383   TTCGTGCATGCCTCGTGCATGCCCCACTTGATCGCAAGAGCTTCTGACCTATCAGCTTGA  442
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   TTCGTGCATGCCTCGTGCATGCCCCACTTGATCGCAAGAGCTTCTGACCTATCAGCTTGA  420

Query  443   CTGTGGTGTATCGGACCACAGTGGCCTTGACGGGTAACGGAGAATCAGGGTTCGATTCCG  502
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CTGTGGTGTATCGGACCACAGTGGCCTTGACGGGTAACGGAGAATCAGGGTTCGATTCCG  480

Query  503   GAGAGGGAGCCTGAGAGACGGCTACCACTACCAAGGTGGGCAGCAGGCACGCAAATTGCC  562
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GAGAGGGAGCCTGAGAGACGGCTACCACTACCAAGGTGGGCAGCAGGCACGCAAATTGCC  540

Query  563   CCATGCAAAGACAGTCTGTGAGGCAGCGACGAACAGTAGCAACCCCGTCGGCCCTACGTG  622
             ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  541   CCATGCAAAGACAGTCTGTGAGGCAGCGACGAACAGTAGCAACCCCGTCGGCCTTACGTG  600

Query  623   CCGATGGGGCTTGGAATGGACGCTATCCAAAGACAGCCGTAAGTATCAACCGGAGGGCAA  682
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  601   CCGATGGGGCTTGGAATGGACGCTATCCAAAGACAGCCGTGAGTATCAACCGGAGGGCAA  660

Query  683   GTCTGGTGCCAGCAGCTGCGGTAATTCCGGCTCCGAGGGCGTATACTAACATTGCTGCTG  742
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Sbjct  661   GTCTGGTGCCAGCAGCTGCGGTAATTCCAGCTCCGAGGGCGTATACTAACATTGCTGCTG  720

Query  743   TTAAAACACTTGTAGTCTGCCTACGGGCTGCAGGTCTGCTGGGTGGCCGGTTTGTTGTTT  802
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   TTAAAACACTTGTAGTCTGCCTACGGGCTGCAGGTCTGCTGGGTGGCCGGTTTGTTGTTT  780

Query  803   CTCTGGCCAGGGAAGGACCTCGGTTCGACCCTGTGTTGGGCTGCAACGGCTGGACTCAAC  862
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   CTCTGGCCAGGGAAGGACCTCGGTTCGACCCTGTGTTGGGCTGCAACGGCTGGACTCAAC  840

Query  863   CCCCAGTGGTACGTCCCTGCGCCCACCTCTCAGTCGATGGTGAGATCTGCTCCTGCCAAA  922
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   CCCCAGTGGTACGTCCCTGCGCCCACCTCTCAGTCGATGGTGAGATCTGCTCCTGCCAAA  900

Query  923   A-TCTGCTTCACTGCAGGCCAAAGCGGTTTATGCCTCCCGCACTGGCAACGGACACCAAC  981
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   AGTCTGCTTCACTGCAGGCCAAAGCGGTTTATGCCTCCCGCACTGGCAACGGACACCAAC  960

Query  982   AGGGGACCAAGCCTCGATCTGGGTAGTCTACCTCTGGTCCAC-ACCGGAACCCACCGTTT  1040
             |||||||| |||||||| |||||||||||||||||||||||| |||||| |||||||| |
Sbjct  961   AGGGGACCCAGCCTCGAGCTGGGTAGTCTACCTCTGGTCCACCACCGGAGCCCACCGTCT  1020

Query  1041  TCAACACCCTGGAAAA  1056
             || ||||||||||||
Sbjct  1021  TCGACACCCTGGAAAA  1036
```

Figure 4.2: The highest percentage identity match following BlastN comparison of *E. gracilis* G46 18S rRNA and the NCBI nucleotide collection (nr/nt) database. The "Query" line refers to the input sequence, *E. gracilis* G46 whilst the "Subject" line refers to the matching sequence, in this instance *Euglena gracilis* small subunit rRNA

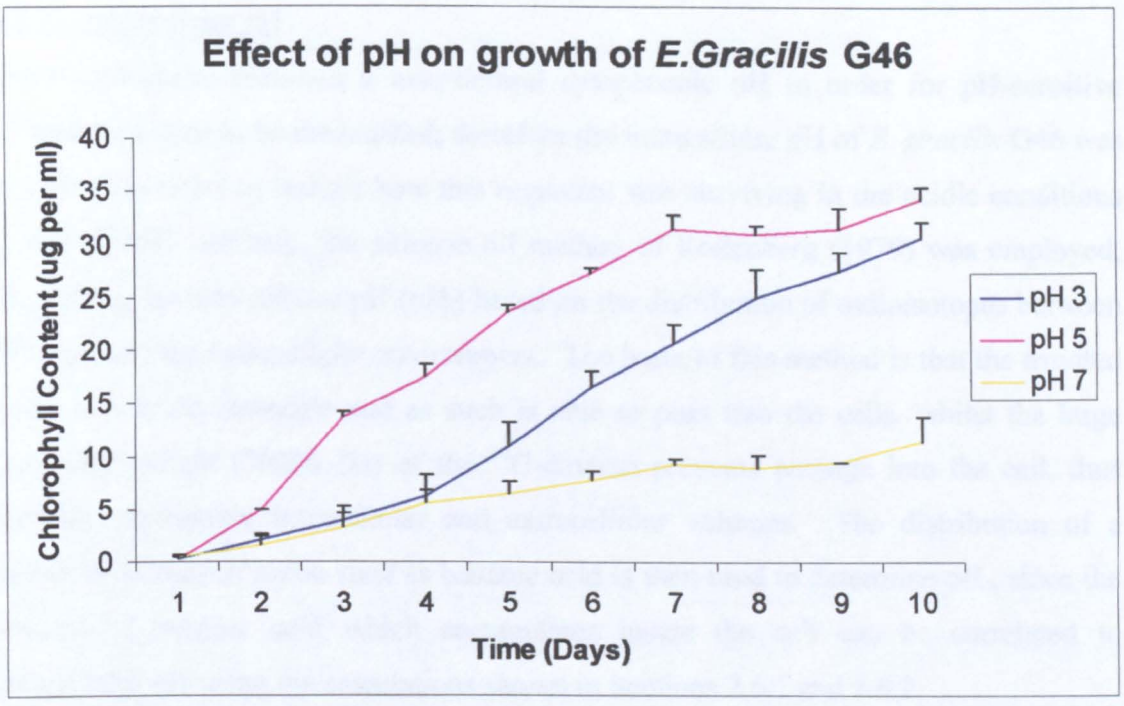


Figure 4.3a: Effect of pH on the growth of *E. gracilis* G46. Data points are the mean of three replicates and the error bars represent one standard error.

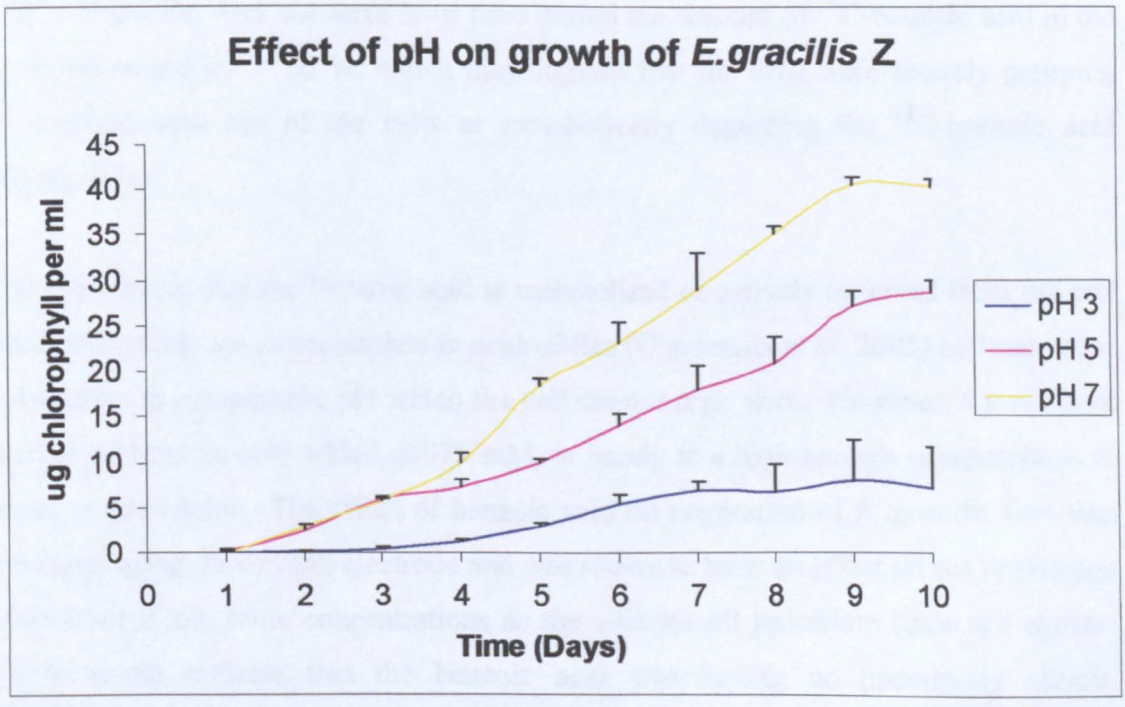


Figure 4.3b: Effect of pH on the growth of *E. gracilis* Z. Data points are the mean of three replicates and the error bars represent one standard error.

4.2.2: Intracellular pH

Most acidophiles maintain a near-neutral cytoplasmic pH in order for pH-sensitive cellular functions to be maintained; therefore the intracellular pH of *E. gracilis* G46 was measured in order to deduce how this organism was surviving in the acidic conditions of the lagoon. Initially, the silicone oil method of Rottenberg (1979) was employed, calculating the intracellular pH (pH_i) based on the distribution of radioisotopes between the cells and the extracellular environment. The basis of this method is that the tritiated water is a small molecule and as such is able to pass into the cells, whilst the large molecular weight (70000 Da) of the ^{14}C-dextran prevents passage into the cell, thus creating measurable intracellular and extracellular volumes. The distribution of a further radioisotope probe such as benzoic acid is then used to determine pH_i, since the amount of benzoic acid which accumulates inside the cell can be correlated to intracellular pH using the calculations shown in Sections 2.6.1 and 2.6.2.

In order to measure pH_i based on the distribution of isotopes, control timecourse experiments were carried out in order to ensure no erroneous uptake or efflux of any of the isotopes was occurring. Three hour time course experiments to measure uptake or efflux of isotopes from the cells showed linear levels of ^{14}C-dextran, $^{3}H_2O$ and ^{14}C-TPP^+. However, over the three hour time period the amount of ^{14}C-benzoic acid in the pellet decreased by > 50 %, which may suggest that the cells were actively pumping ^{14}C-benzoic acid out of the cells or metabolically degrading the ^{14}C-benzoic acid (Figure 4.4).

It is quite likely that the benzoic acid is metabolised or actively removed from the cell since weak acids act as uncouplers in acidophiles (Ciaramella *et al.* 2005) and can cause a reduction in cytoplasmic pH which the cell cannot cope with. However, the minimal amount of benzoic acid added, 0.075 mM, is barely at a high enough concentration to act as an uncoupler. The effect of benzoic acid on respiration of *E. gracilis* G46 was measured using the oxygen electrode and was shown to have no effect on the respiration when used at the same concentrations as the silicone oil procedure (data not shown) which would indicate that the benzoic acid was having no uncoupling effects. Therefore, a five minute incubation time was deemed acceptable since there was little, if any, uncoupling effect of benzoic acid at the concentration used over such a brief period of time.

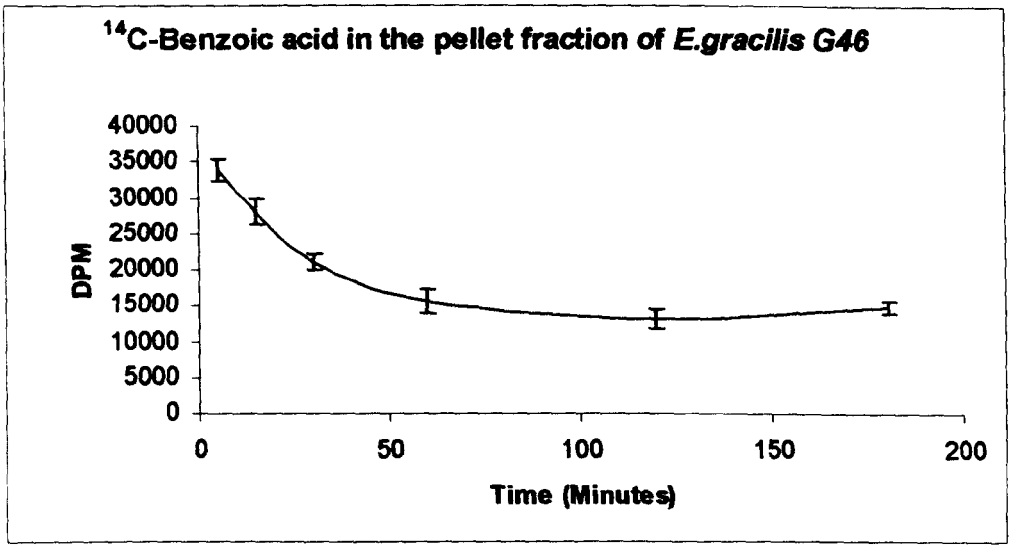


Figure 4.4: ^{14}C-benzoic acid in the pellet fraction of *E. gracilis* G46. The distribution of benzoic acid between the pellet and supernatant is used to calibrate the internal pH and it should not vary after the initial accumulation. Values are the mean of three repeats ± one standard error.

Following the timecourse controls the silicone oil procedure was carried out in full using five minute incubations for each isotope in order to determine the pH_i of *E. gracilis* G46, and *E. gracilis* Z as a comparison. Further control experiments were carried out using *E. gracilis* G46 resuspended in M6 pH 7 medium and *E. gracilis* Z resuspended in pH 3 M6 medium, as well as controls using iodine-killed *E. gracilis* G46 and *E. gracilis* Z (Table 4.1). The silicone oil method produced a mean intracellular pH of 5.47 ± 0.36 (n=16) for *E. gracilis* G46 (Table 4.1).

There is a slight difference in the pH_i of the two strains of *Euglena*, with *E. gracilis* Z having a slightly lower pH_i when measured in M6 pH 3. This is not unexpected after a significant shock in external pH. Since *E. gracilis* Z is routinely grown in pH 7 media it is possible that resuspension in pH 3 media may have temporarily reduced the pH_i and due to the short time period in this experiment it is unlikely that any pH homeostasis mechanisms would have begun. *E. gracilis* Z cells resuspended in pH 7 M6 have a much higher pH_i of 6.52 which would suggest that the pH_i measured in cells resuspended in pH 3 M6 is lower than their usual pH_i during growth, whereas the lower pH_i measured in *E. gracilis* G46 is likely to be much closer to the normal intracellular pH during growth of *E. gracilis* G46 in the acid tar lagoon.

Resuspension Conditions	M6 pH 3	M6 pH 7	M6 pH 3 plus iodine	M6 pH 7 plus iodine
E. gracilis G46	pH 5.47 ± 0.36 (n=16)	Negative ICV	pH 5.42 ± 0.19 (n=5)	Negative ICV
E. gracilis Z	pH 5.35 ± 0.14	pH 6.52 ± 0.64	Not determined	pH 6.9 ± 0.20

Table 4.1: Summary of mean intracellular pH measurements using the silicone oil method. The values represented are the mean of three replicates ± one standard error unless otherwise stated. Where applicable 50 µl of Gram's Iodine was added to the cells to kill them. Negative ICV = Attempts were made to measure pH_i but a negative intracellular volume was obtained therefore it was not possible to determine pH_i.

The purpose of killed controls using iodine was to determine if pH_i was actively maintained at low external pH. There is almost no difference between the pH_i of live and killed *E. gracilis* G46 resuspended in pH 3 M6 media. If active mechanisms were involved in pH homeostasis it would be expected that there would be a reduction in the pH_i of killed cells yet there is almost no difference. One possible explanation for this observation is that *E. gracilis* G46 may possess a highly impermeable cell membrane, a common adaptation in acidophilic organisms, which reduces proton influx into the cells. Active maintenance of pH_i by *E. gracilis* G46 is supported by data in Table 4.1, since the pH_i of killed control cells is significantly higher (pH 6.9) than the living cells at pH 7 (pH 6.52).

Since the intracellular pH of both *E. gracilis* G46 and *E.* gracilis Z measured using the silicone oil technique was lower than normal and given the slight abnormalities in the benzoic acid timecourse, ^{31}P NMR was used as an alternative method to measure the intracellular pH of *E. gracilis* G46. Using the shift of intracellular phosphate this method gave a pH_i of 6.6 for *E. gracilis* G46 grown at pH 3 and 6.7 for type strain *E. gracilis* Z grown at pH 7 (Figure 4.5). The difference in the measured pH_i between the silicone oil method and NMR may be caused by several factors. Firstly, cells were highly concentrated for NMR experiments and it is likely that they became anaerobic quickly and remained anaerobic for the duration of the measurements. Cells were also resuspended in Tris buffer for NMR experiments as opposed to growth media which may also have some effect on the pH_i.

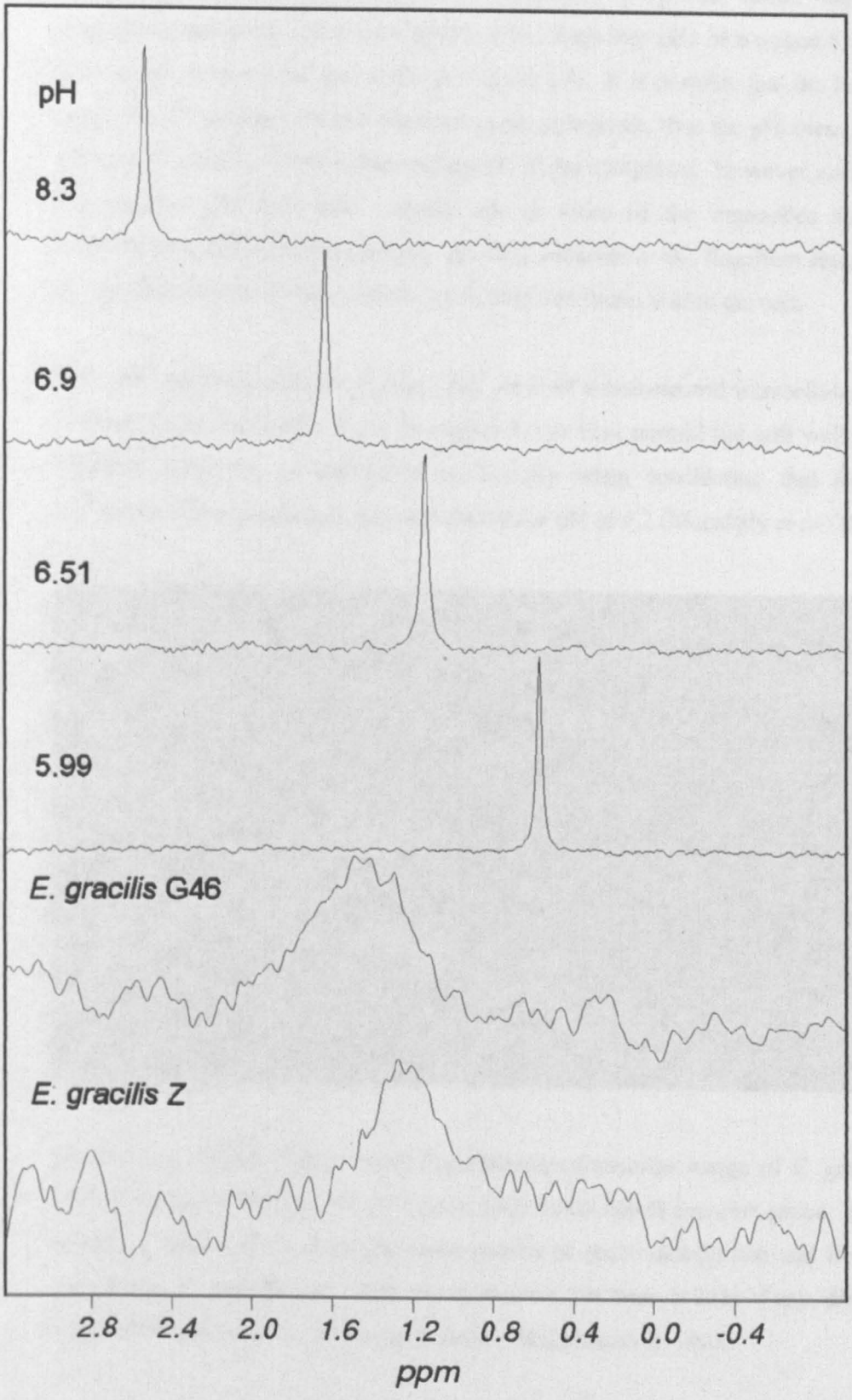


Figure 4.5: Comparison of phosphate shift of *E. gracilis* G46 and *E. gracilis* Z with titrated phosphate solutions ranging from pH 5.99 to pH 8.3. The cell pH was determined by interpolation to be pH 6.6 for *E. gracilis* G46 cells grown at pH 3 and pH 6.7 for *E. gracilis* Z cells grown at pH 7.

Experiments using the LysoSensor Yellow/Blue DND-160 probe highlight the compartmentalisation of these eukaryotic cells which may also be a reason for the discrepancy between the two methods (Figure 4.6). It is possible that the benzoic acid may have entered the cell and remained in the cytoplasm, thus the pH_i measured by the silicone oil method would be the average pH of the cytoplasm. However, some or all of the benzoic acid may have entered one or more of the organelles such as the mitochondria, chloroplasts, eye spot, vesicles, vacuoles or the flagellum reservoir. It is not possible to identify where the benzoic acid distributes within the cell.

Both pH_i measurements are strongly indicative of a near-neutral intracellular pH. The silicone oil measurement of pH_i is slightly lower than normal but still well within the expected range for an acidophile, particularly when considering that the extreme acidophile *Ferroplasma spp.* has an intracellular pH of 4.2 (Macalady *et al.* 2004).

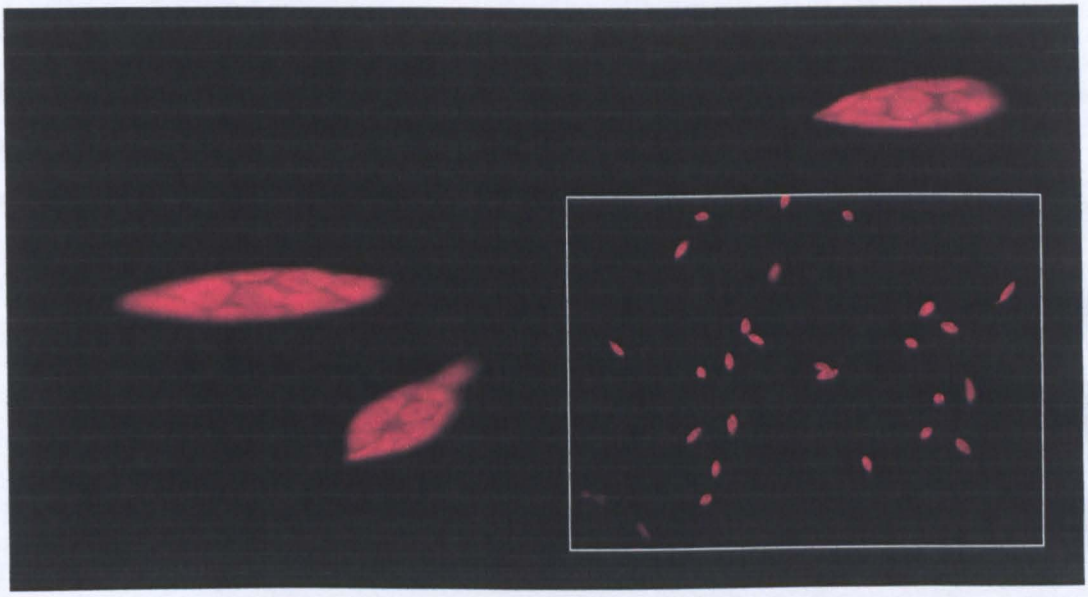


Figure 4.6: **(Main)** False colour fluorescence microscope image of *E. gracilis* G46 after incubation with LysoSensor Yellow/Blue DND-160 fluorescent probe. **(Inset)** *E. gracilis* Z control cells show the same pattern of probe distribution and fluorescence emission as *E. gracilis* G46. This would indicate that there is little, if any, difference in intracellular pH between the two organisms. Magnification: 100X.

4.2.3: Rubisco analysis

In order to further verify the pH_i measurement, exons from the gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), RbcL, were amplified by PCR and sequenced. RuBisCO is one of the most abundant proteins on the planet (Dhingra *et al.* 2004) and is extremely important in photosynthesis; therefore it would be expected that any adaptation to low pH within the cell would be observed in the gene sequence of this protein. In order for the cell to function properly every protein is specialised to function in the specific intracellular conditions of its particular environment. Therefore a particularly low intracellular pH is likely to result in amino acid alterations in peptides and proteins in order to confer acid stability and in order for the protein to function properly in acidic conditions.

Primers designed to amplify the largest and second largest exons of the gene, exons 8 and 5 respectively, were designed based on other RuBisCO sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/). The 363 bp fragment of exon 5 was successfully amplified and sequenced. Sequence analysis showed a highly conserved sequence between *E. gracilis* G46 and other *Euglenoids* (Figures 4.7 and 4.8).

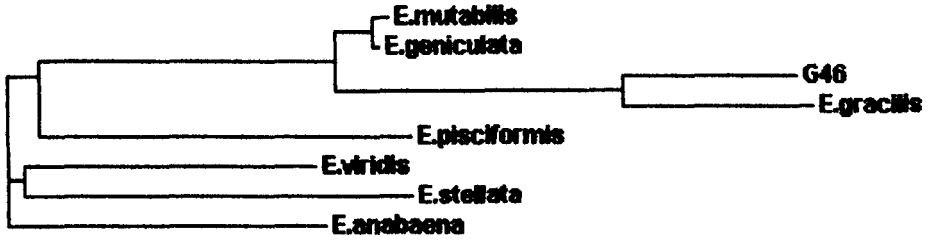


Figure 4.7: Phylogram generated by ClustalW (1.83) (Chenna *et al.* 2003) multiple sequence alignment tool service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw) comparing the DNA sequence of exon 5 from *E. gracilis* G46 (G46) with other *Euglenoid* DNA sequences.

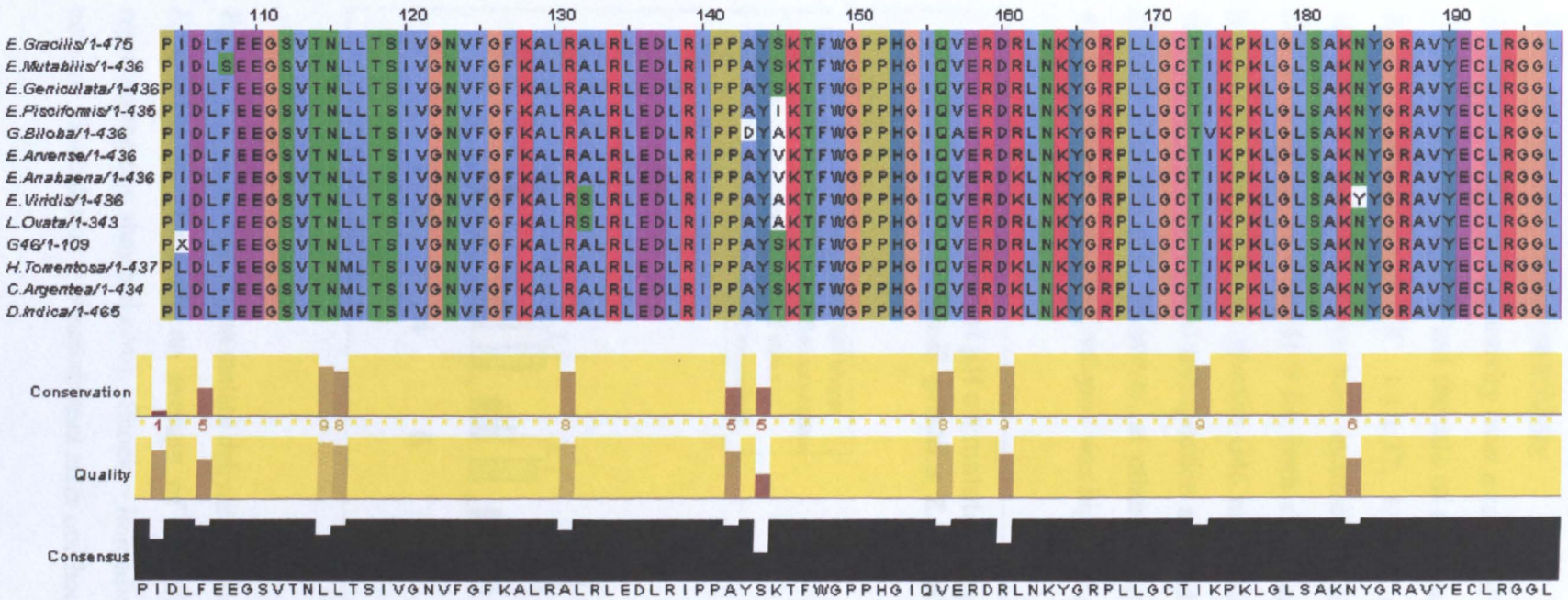


Figure 4.8: KAlign multiple sequence analysis of translated *E. gracilis* G46 RbcL exon 5 against the amino acid sequences of the highest matching TBlastN results.

4.2.4: Malate dehydrogenase acitivity

Measurement of enzyme activity over a range of pH was also carried out. Crude cell-free extract was prepared and the rate of reaction of malate dehydrogenase (L-malate–NAD^+ oxidoreductase; EC 1.1.1.37; MDH) measured over the pH range 3-9, immediately and after a one hour incubation. *E. gracilis* G46 malate dehydrogenase had an optimum rate at pH 9 for both shock and incubation experiments, as did *E. gracilis* Z (Figure 4.9). *E. gracilis* G46 malate dehydrogenase was able to catalyse the reaction at pH 3 after initial resuspension and had almost 10X the specific activity of *E. gracilis* Z at this pH. However, at other acidic pH values (4, 5 and 6), the specific activity of the *E. gracilis* Z enzyme was higher in each case.

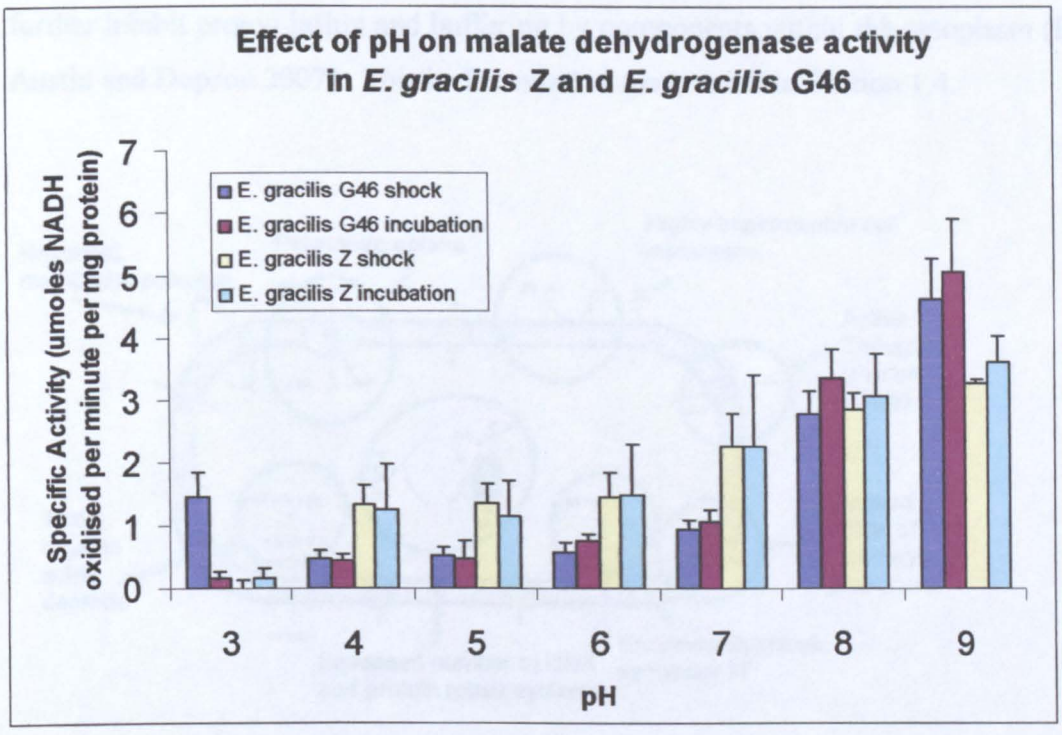


Figure 4.9: Effect of pH on malate dehydrogenase activity in *E. gracilis* G46 and *E. gracilis* Z. Data are an average of two triplicate experiments with error bars representing one standard error. Shock = immediate measurement after resuspension in new pH. Inoculation = Measurement after one hour after resuspension in new pH.

Malate dehydrogenase from neutralophilic organisms has a relatively high optimum pH ranging from pH 7.5 to pH 10 in some cases (Eprintsev *et al.* 2003). Therefore the optimum pH of *E. gracilis* G46 is in agreement with neutralophilic organisms which further suggests that these cells have a near-neutral intracellular pH. *E. gracilis* Z has

been shown to possess 3 isoenzymes of malate dehydrogenase; mitochondrial, supernatant and peroxisomal, all having optimum pH > 7 (Davis and Merrett 1973). Combining all this evidence together it is reasonable to conclude that the intracellular pH of *E. gracilis* G46 isolated from Hoole Bank acid tar lagoon is near neutral.

4.2.5: Maintenance of intracellular pH

Many mechanisms have been suggested by which acidophiles may maintain near-neutral intracellular pH (Figure 4.10) including efflux of protons from the cell, storage of protons within intracellular compartments separate from all other metabolically active parts of the cell, degradation of weak acids, restricting proton entry into the cells, reducing pore size in membrane channels, maintaining a chemiosmotic gradient to further inhibit proton influx and buffering by components within the cytoplasm (Baker-Austin and Dopson 2007). This is discussed in more detail in Section 1.4.

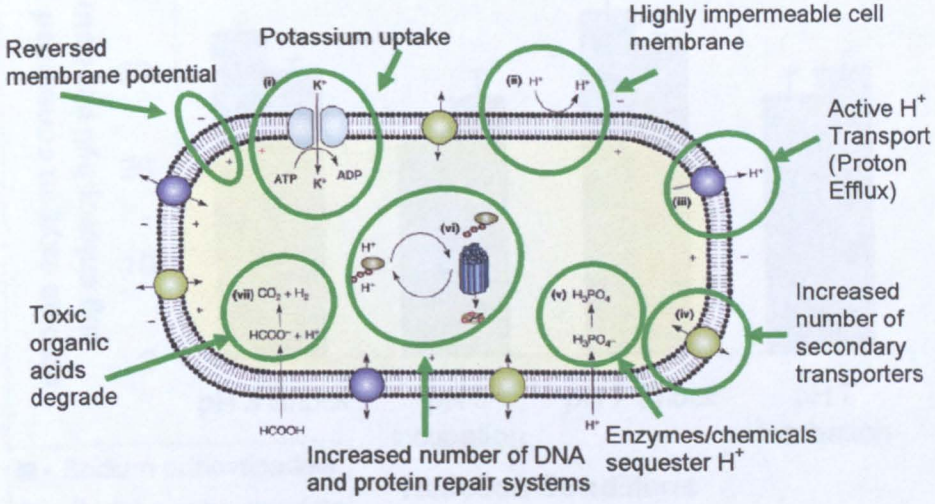


Figure 4.10: Mechanisms of pH homeostasis in acidophiles. Figure adapted from Baker-Austin and Dopson (2007).

4.2.5.1: Proton efflux inhibition

Sodium orthovanadate, Na_3VO_4, is a phosphate analogue which inhibits non-F_1F_0 ATPases, which was employed to inhibit active proton transport from *E. gracilis* G46 in order to determine what contribution, if any, proton pumping from *E. gracilis* G46 has in maintaining a near-neutral intracellular pH. Cells were resuspended in phosphate-free minimal media in order to maximise any effects of the sodium orthovanadate, since

the proposed mechanism for inhibition is the irreversible binding of VO_4^{3-} to the active site of tyrosine phosphatases.

At 1 mM concentrations of sodium orthovanadate a 13 % reduction in respiration was observed immediately after addition of sodium orthovanadate and a 22 % increase after a 60 minute incubation with 1mM sodium orthovanadate in phosphate-free minimal media at pH 3. Cells resuspended in pH 7 phosphate-free minimal media had an 11 % reduction in respiration immediately after the addition of 1 mM sodium orthovanadate, and a 10 % increase in respiration after a 60 minute incubation with 1 mM sodium orthovanadate (Figure 4.11).

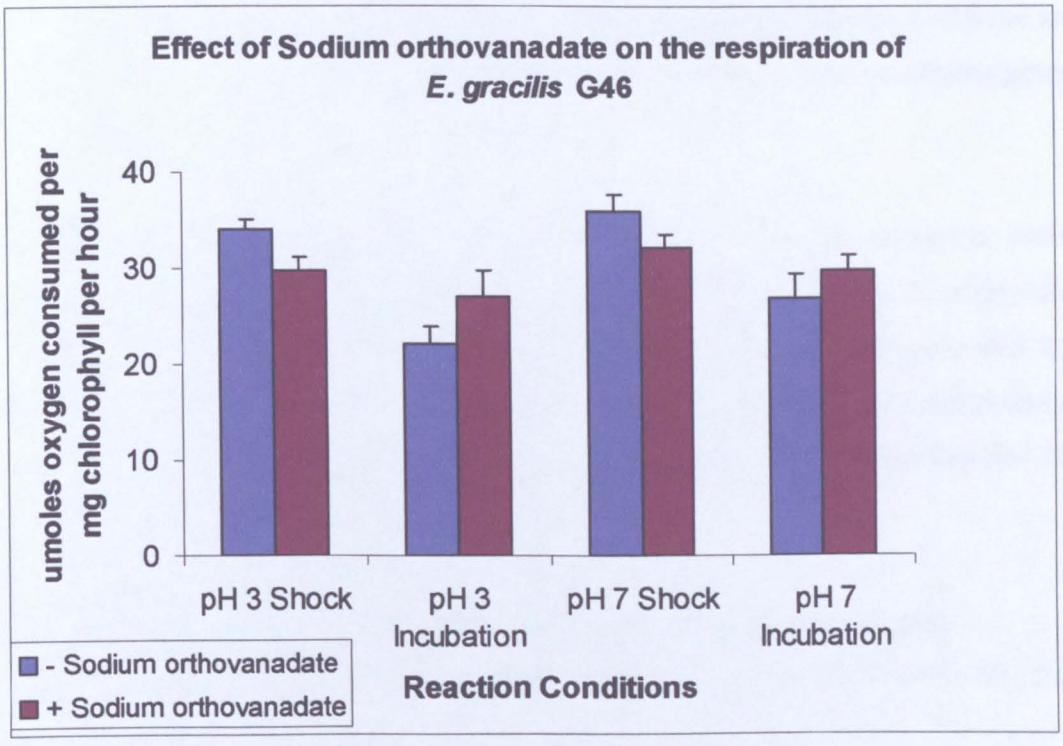


Figure 4.11: The effect of sodium orthovanadate on *E. gracilis* G46. Data are an average of two triplicate experiments with error bars representing one standard error. Shock = Measurement of respiration rate immediately after addition of orthovanadate. Immediate = Measurement of respiration rate 60 minutes after addition of orthovanadate.

If non-F_1F_0 ATPases are heavily involved in pH homeostasis there would be a significant reduction in the respiration levels of cells incubated with sodium orthovanadate at pH 3 in particular. Therefore, the minimal effect of sodium

orthovanadate at both pH suggests that either *E. gracilis* G46 does not rely heavily upon active proton transport to maintain intracellular pH or that an alternative mechanism of pH homeostasis is induced upon the addition of sodium orthovanadate in order to compensate for the reduction of proton efflux. If the latter is true this could account for the initial decrease in respiration since any alternative mechanism would have little or no time to take effect and the increase in respiration observed after incubation with sodium orthovanadate may occur as a result of increased cellular activity in order to compensate for the loss of proton export via non-F_1F_0 ATPases.

4.2.5.2: Reversed transmembrane potential

The membrane potential of *E. gracilis* G46 and *E. gracilis* Z was measured using ^{3}H-TPP$^+$. Membrane potential is an essential component of the proton motive force and is usually inside negative in bacteria and algae. Table 4.2 shows the membrane potential of *E. gracilis* G46 at pH 3 and *E. gracilis* Z at pH 7.

The results very clearly indicate that *E. gracilis* G46 has a reversed membrane potential at pH 3. A reversed membrane potential (see Figure 4.10) is common in acidophiles in order to restrict proton entry into the cells. The chemiosmotic barrier generated by the inside positive $\Delta\psi$ inhibits proton influx. The $\Delta\psi$ is generated by a Donnan potential of positively charged molecules and may be produced by a greater potassium ion influx than proton efflux (Donnan 1924).

4.2.5.3: Effect of valinomycin and gramicidin on transmembrane potential

After measuring $\Delta\psi$ the effects of valinomycin and gramicidin on $\Delta\psi$ were measured as described in Section 2.6.6, the results of which are shown in Table 4.2. Valinomycin is a potassium specific ionophore, whilst gramicidin increases the permeability of the cell membrane allowing inorganic cations to travel through unrestricted, thereby destroying the ion gradient between the cytoplasm and the extracellular environment.

The effect of valinomycin on *E. gracilis* Z is negligible, with only a slight decrease in membrane potential after treatment which would suggest that the contribution of potassium ions to $\Delta\psi$ in *E. gracilis* Z is minimal. The effect of gramicidin is also very minimal which may be because *Euglena* lack a cell wall and instead have a flexible pellicle covering their cell membrane thus the usual target of gramicidin is not present.

Cells	Resting Membrane potential (Δψ) (mV)	+ Valinomycin	+ Gramicidin
E. gracilis G46 (pH 3)	+10 ± 16.7 (n=6)	-9.1 ± 7.7	-4.0 ± 4.0
E. gracilis Z (pH 7)	-109 ± 6.2 (n=6)	-101.2 ± 12.5	-103.0 ± 13.6

Table 4.2: Membrane potential measurements from *E. gracilis* G46 and *E. gracilis* Z cells treated with valinomycin and gramicidin. The values represented are the mean of triplicate experiments ± one standard error.

The effects of valinomycin and gramicidin on the membrane potential of *E. gracilis* G46 are much more pronounced, with a significant reversal of membrane potential from – 10 mV to +9 mV when the cells were treated with valinomycin. This evidence is in accordance with the hypothesis that potassium ion accumulation is used to maintain a reversed membrane potential. Similarly, the effect of gramicidin on *E. gracilis* G46 is more significant than in *E. gracilis* Z, which may also be in part due to some potassium efflux from the cells since gramicidin is not selective.

4.2.6: Effect of pH on photosynthesis and respiration

The effect of pH on the rate of photosynthesis and respiration was measured using an oxygen electrode. Interestingly, no oxygen is evolved from photosynthesis at pH 3 by *E. gracilis* G46 cells yet cultures grown at this pH produce normal amounts of chlorophyll. The maximum rate of photosynthesis in *E. gracilis* G46 occurred at pH 7, whilst the maximum rate of respiration occurred at pH 6. *E. gracilis Z* controls showed maximal rates of photosynthesis and respiration at pH 5 (Figures 4.12 and 4.13 respectively).

At pH 2, the rate of photosynthesis is negative for both strains of *E. gracilis*. Initial assumptions were that the cells resuspended in the low pH solution would have an increased rate of respiration due to high level of cell stress, with cells consuming a much greater amount of oxygen that that produced by photosynthesis. However, the rate of respiration at pH 2 is the lowest recorded, as shown in Figure 4.13, which would indicate that both strains are functioning very minimally at pH 2, with both *E. gracilis* G46 and *E. gracilis* Z showing almost identical responses.

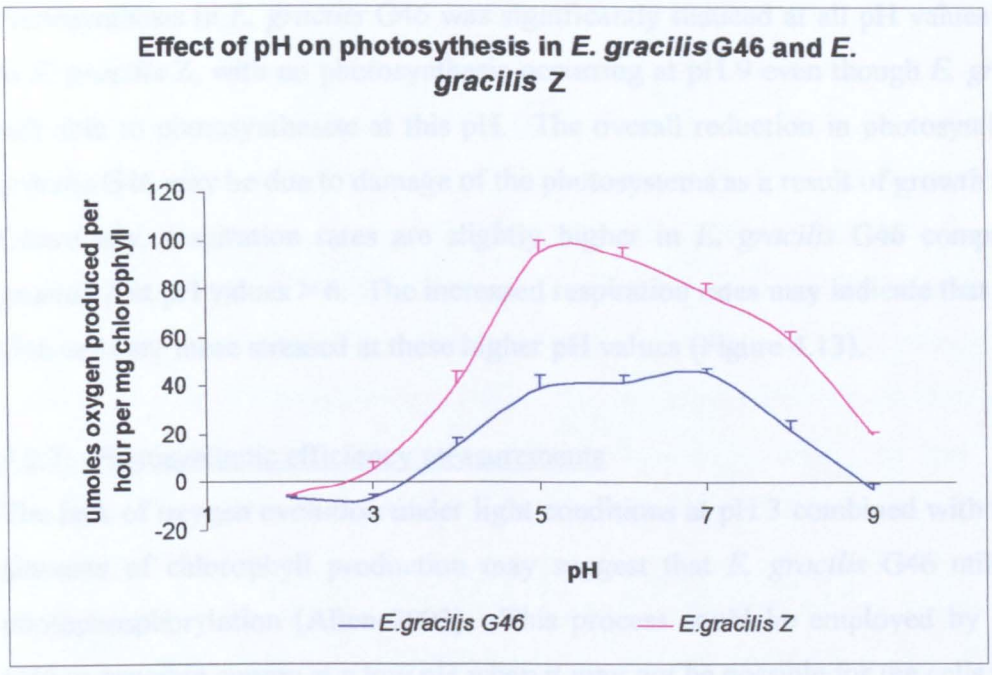


Figure 4.12: Effect of pH on photosynthesis in *E. gracilis* G46 and *E. gracilis* Z. Photosynthesis was calculated as light induced oxygen uptake plus oxygen consumption in the dark. Values represented are the mean of three triplicate experiments ± one standard error.

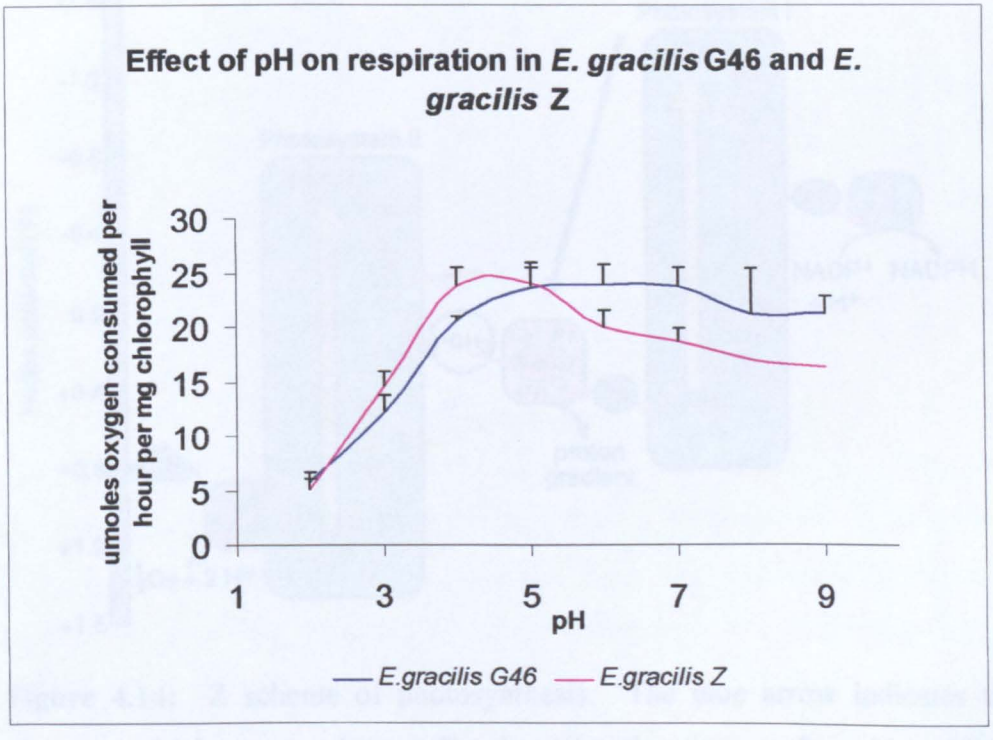


Figure 4.13: Effect of pH on respiration in *E. gracilis* G46 and *E. gracilis* Z. Respiration was measured as oxygen consumption in the dark. Values represented are the mean of three triplicate experiments ± one standard error.

Photosynthesis in *E. gracilis* G46 was significantly reduced at all pH values compared to *E. gracilis* Z, with no photosynthesis occurring at pH 9 even though *E. gracilis* Z is still able to photosynthesise at this pH. The overall reduction in photosynthesis in *E. gracilis* G46 may be due to damage of the photosystems as a result of growth at low pH. Conversely, respiration rates are slightly higher in *E. gracilis* G46 compared to *E. gracilis* Z at pH values > 6. The increased respiration rates may indicate that *E. gracilis* G46 cells are more stressed at these higher pH values (Figure 4.13).

4.2.7: Photosynthetic efficiency measurements

The lack of oxygen evolution under light conditions at pH 3 combined with the normal amounts of chlorophyll production may suggest that *E. gracilis* G46 utilises cyclic photophosphorylation (Allen 2003). This process could be employed by *E. gracilis* G46 to generate energy at a low pH when it may not be possible for the cells to generate energy using the full Z scheme and normal electron transport systems (Figure 4.14). Cyclic photophosphorylation operates around PSI only and no oxygen is evolved.

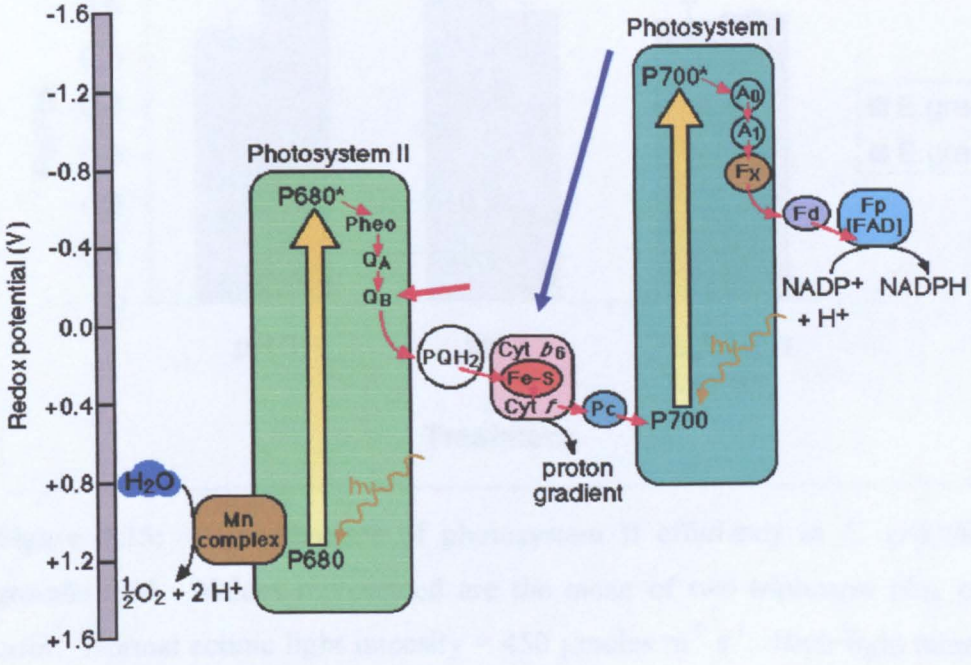


Figure 4.14: Z scheme of photosynthesis. The blue arrow indicates the flow of electrons which occurs when cyclic photophosphorylation takes place. The red arrow indicates where DCMU inhibits photosynthesis.

Following on from the oxygen electrode results which showed that *E. gracilis* G46 evolved no oxygen at pH 3, experiments were designed to measure the efficiency of photosystem II (PSII). The hypothesis being that *E. gracilis* G46 would show a reduced yield with respect to PSII at pH 3. Chlorophyll fluorescence was measured using a pulse modulated fluorescence system with saturating flashes of light from a fibre optic source. This system allows a wide range of parameters to be measured, however in this instance the most applicable is the maximum efficiency of photosystem II (PSII), described by Fv/Fm (Rees *et al.* 1992). This parameter has been widely used as an indicator of photosynthetic efficiency in several algae under stressed conditions, including *E. gracilis* (Doege *et al.* 2000) and *Dunaliella* (Gordillo *et al.* 2001). Results from *E. gracilis* G46 showed a very slightly reduced Fv/Fm at pH 3 compared to pH 7 (Figure 4.15), however both values were between 0.5 and 0.6 which is well within the normal range, suggesting that PSII is present and functioning normally.

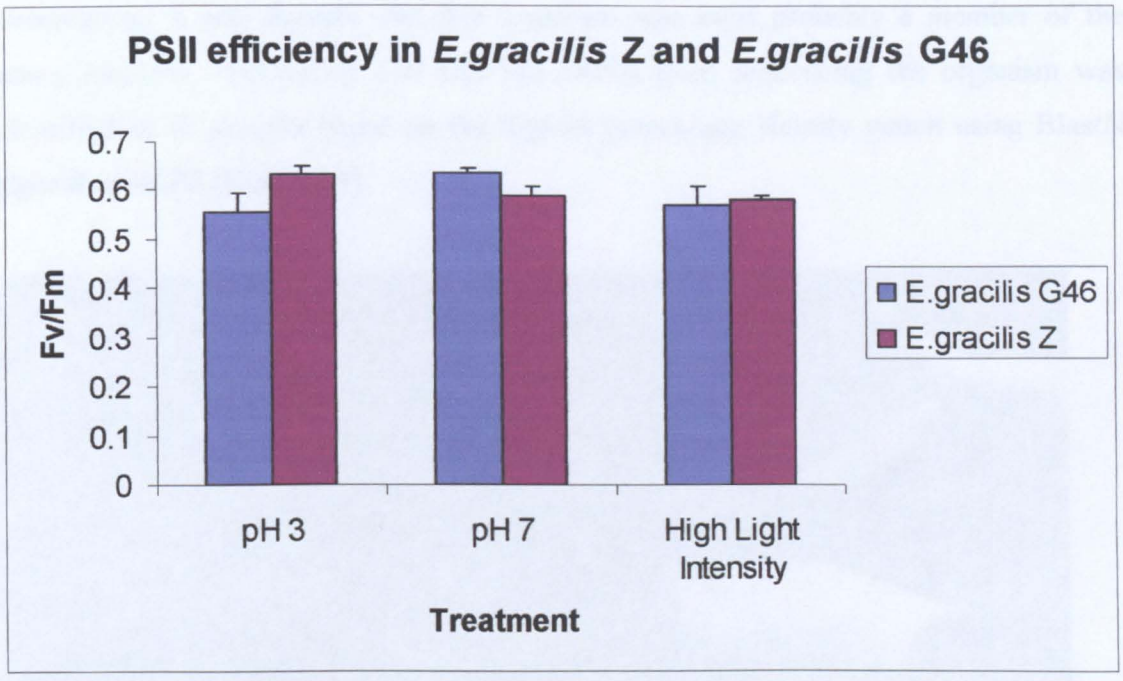


Figure 4.15: Measurements of photosystem II efficiency in *E. gracilis* Z and *E. gracilis* G46. Values represented are the mean of two triplicates plus one standard error. Normal actinic light intensity = 450 μmoles m^{-2} s^{-1}. High light intensity = 1500 μmoles m^{-2} s^{-1}.

4.2.8: Photosystem II inhibition

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is an inhibitor of PSII which was also used in a further attempt to deduce if *E. gracilis* G46 was using cyclic

photophosphorylation. If *E. gracilis* G46 uses cyclic photophosphorylation DCMU would have no effect on photosynthesis when measured using the oxygen electrode (see Figure 4.14). However, DCMU must be dissolved in ethanol therefore control experiments were needed to ensure that ethanol alone had no effect on photosynthesis. Unfortunately, respiration rates were dramatically increased in the presence of ethanol, presumably as the cells used this as a carbon source. High respiration rates also correlated with severe repression of photosynthesis therefore this experiment had to be abandoned.

4.3: General Discussion

Isolation of an alga from Hoole Bank acid tar lagoon was not unexpected after initial sampling revealed a visibly abundant photosynthetic biofilm present in several areas around the lagoon (Figure 4.16). Initial microscopic observations of colonies grown on ½ BLB plates revealed large, motile photosynthetic organisms. From these observations it was thought that this organism was most probably a member of the genus *Euglena*. Following 16S and 18S rRNA gene sequencing the organism was identified as *E. gracilis* based on the highest percentage identity match using BlastN algorithm NCBI (Figure 4.1).

Figure 4.16: A visibly abundant biofilm was present at Hoole Bank acid tar lagoon.

Euglena gracilis has been isolated from acidic environments previously (Hallberg *et al.* 2006; Nixdorf *et al.* 2001) therefore it is not entirely unusual to have isolated this organism from the Hoole Bank lagoon samples. However, it is more common to

isolate *Euglena mutabilis* from acidic environments since it is much more tolerant to metal ions than *Euglena gracilis* (Olaveson and Nalewajko 2000) and many acidic environments such as acid mine drainage and the Rio Tinto, are extremely rich in metal ions, such as iron and copper. Chemical analysis of Hoole Bank acid tar lagoon samples (Nichol 2000) revealed relatively low concentrations of various metal ions which may explain why the acidotolerant *E. gracilis* was isolated from the lagoon as opposed to the more acidophilic *E. mutabilis.*

Phylogenetic analysis by comparison of the 16S and 18S rRNA gene sequences from *E. gracilis* G46 to other sequences in the NCBI database revealed the highest percentage identity match to *E. gracilis* species in both instances. The resulting pair-wise alignment at NCBI generates a phylogenetic tree which clusters *E. gracilis* G46 with other *Euglena gracilis* (Figure 4.1) species yet ClustalW analysis using several *Euglena* species for comparison places *E. gracilis* G46 closest to *E. mutabilis* in neighbour joining phylogenetic trees. However, BlastN analysis of the *E. gracilis* G46 18S rRNA gene sequence specifically against *Euglena mutabilis* sequences in the database produces only approximately 350 bp of homologous sequence. Therefore based on this further evidence the phylogenetic assignment of *E. gracilis* G46 to the species *gracilis* is more than likely correct.

Euglena gracilis is phylogenetically widespread, as can be seen by the position of several *E. gracilis* organisms in distinct branches of the 18S rRNA phylogenetic tree (Figure 4.17). This is also supported by the fact that the genus *Euglena* is a polyphyletic genus with a recent study dividing *Euglena* into four independent clades (Marin *et al.* 2003). In order to optimise phylogenetic tree construction a greater number of input sequences could be used as well as obtaining the full length 18S rRNA gene sequence from *E. gracilis* G46, since only approximately 1400 bp of sequence was obtained.

E. gracilis has been shown to grow at acidic pH values previously (Cook 1971; Olaveson and Nalewajko 2000) yet the mechanisms of tolerance to low pH in algae have been studied very little. Colman and Balkos (2005) measured the intracellular pH of *E. gracilis* Klebs over the external pH range 3.5 - 7.5 essentially using the silicone oil method as detailed in Section 2.6 with the exception that ^{14}C-DMO was used as a probe at pH > 5.5. The near-neutral pH_i of *E. gracilis* G46 measured by NMR at an external

pH of 3.0 is in agreement with the measurements of pH_i made by Colman of *E. gracilis* Klebs which was also shown to maintain a near-neutral intracellular pH over the external pH range 3.5-7.5 (Colman and Balkos 2005).

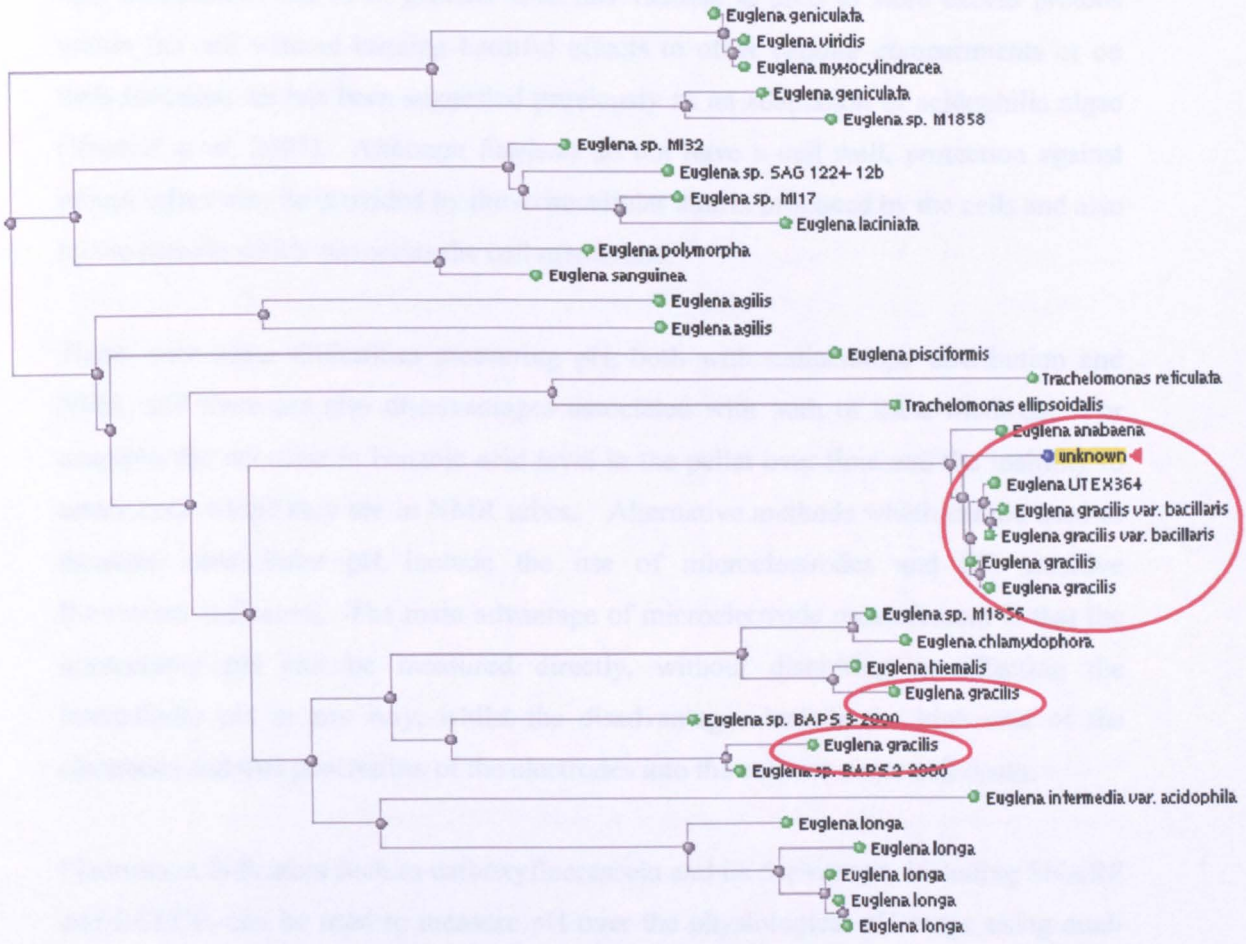


Figure 4.17: Phylogenetic tree based on 18S rRNA with organisms representing *E. gracilis* highlighted by red circles. "Unknown" highlighted in yellow represents *E. gracilis* G46.

Other algal species have been isolated from acidic environments including a new species of *Euglena* isolated from an acidic hot mud pool from a volcanic area (Sittenfeld *et al.* 2002). The Rio Tinto has abundant photosynthetic biofilms which contain *E. gracilis* amongst other algal species including *Dunaliella, Cyanidium, Zygnemopsis, Chlamydomonas, Chlorella* and *Klebsormidium* (Aguilera *et al.* 2007; Amaral Zettler *et al.* 2002). The presence of biofilms is also very common in acid mine drainage systems where the growth of algae has been shown to support growth of other microorganisms (Rowe *et al.* 2007).

The effects of intracellular compartmentalisation on measured pH_i of *E. gracilis* G46 have already been discussed briefly in Section 4.5.2. However, it is of note that *Euglena gracilis* possess a contractile vacuole which is usually used to prevent the cells from bursting in osmotically stressful conditions (Kivic and Vesk 1974). However it may be possible that in *E. gracilis* G46 this vacuole is used to store excess protons within the cell without causing harmful effects to other cellular compartments or on their functions, as has been suggested previously as an adaptation of acidophilic algae (Nixdorf *et al.* 2001). Although *Euglena* do not have a cell wall, protection against proton influx may be provided by the extracellular matrix produced by the cells and also by the pellicle which surrounds the cell membrane.

There were some difficulties measuring pH_i both with radioisotope distribution and NMR, and there are also disadvantages associated with both of these methods. For example, the decrease in benzoic acid level in the pellet over time and the inability to aerate cells whilst they are in NMR tubes. Alternative methods which can be used to measure intracellular pH include the use of microelectrodes and pH sensitive fluorescent indicators. The main advantage of microelectrode measurement is that the intracellular pH can be measured directly, without disturbing or affecting the intracellular pH in any way, whilst the disadvantages include the high cost of the electrodes and that penetration of the electrodes into the cell can cause cell death.

Fluorescent indicators such as carboxyfluorescein and its derivatives, including SNARF and BCECF, can be used to measure pH over the physiological pH range using dual-emission or dual-excitation ratiometric techniques (Bassnett *et al.* 1990; Rottenberg 1979). The use of fluorescent indicators requires a fluorescence spectrophotometer or flow cytometer as well as approximate knowledge of the pH being measured in order to choose the most suitable indicator. Difficulties may also arise as a result of the natural fluorescence from chloroplasts.

The cell membrane potential of *E. gracilis* G46 measured at pH 3 and was found to be + 10 mV compared to -109 mV in *E. gracilis* Z at pH 7. Again, intracellular compartmentalisation may have some effect on the measurement of $\Delta\psi$ in *E. gracilis* as the $^{3}H\text{-}TPP^{+}$ may distribute across some cellular compartments including the mitochondria and chloroplasts. However, any intracellular compartmentalisation is highly likely to be the same in both strains of *Euglena*, therefore the contrasting $\Delta\psi$

measurements is certainly significant. The reversed membrane potential is a common adaptation in acidophiles in order to reduce proton influx into the cell (Konings *et al.* 2002). One hypothesis regarding the genesis of the inside positive membrane potential is the accumulation of potassium ions within the cell (Baker-Austin and Dopson 2007). The genome sequences of several extreme acidophiles have revealed an increased number of membrane bound cation transporters which further supports this theory (Futterer *et al.* 2004; Tyson *et al.* 2004).

The reversal of membrane potential in *E. gracilis* G46 after treatment with valinomycin also supports this hypothesis. If the accumulation of potassium ions was not involved in maintenance of the reversed membrane potential (inside positive) in *E. gracilis* G46 it would be predicted that valinomycin would have no effect on the membrane potential yet the membrane potential is flipped from + 10 mV to – 9 mV after treatment with valinomycin. Gramicidin also has some effect on membrane potential in *E. gracilis* G46 which may also be due to potassium efflux from the cells through the channels created by gramicidin activity.

Exon 5 of RuBisCO from *E. gracilis* G46 shows a high degree of conservation with other *Euglenoid* species, which again concurs with the evidence for a near-neutral intracellular pH as there are no obvious adaptations to acidic conditions. Malate dehydrogenase from *E. gracilis* G46 showed a high optimum pH in agreement with previous studies from *Euglena* and with other neutralophilic bacteria further confirming the measurement of intracellular pH. Also noticeable was a comparatively reduced activity over the pH range 3 to 7 in *E. gracilis* G46 compared to *E. gracilis* Z. Interestingly, the specific activity of malate dehydrogenase from *E. gracilis* G46 at pH 3 was 10 times higher than malate dehydrogenase from *E. gracilis* Z under shock conditions. This may imply that *E. gracilis* G46 is able to stabilise intracellular proteins for a short time at low pH. At slightly less acidic pH values there is an indication that malate dehydrogenase from *E. gracilis* Z is more active than malate dehydrogenase from *E. gracilis* G46 at the slightly less acidic values.

The overall effect of pH on photosynthesis and respiration is a much reduced rate in *E. gracilis* G46 when compared to the wild-type strain *E. gracilis* Z, with no oxygen evolution from *E. gracilis* G46 during light conditions at pH 3. The reduced rates of photosynthesis and respiration may be due to cell stress, however there is no indication

of a reduction in growth at this pH; in fact growth of *E. gracilis* G46 at pH 3 is comparable to the growth of *E. gracilis* Z at pH 7. Therefore, it is possible that the reduced rates of respiration and photosynthesis may intimate the use of cyclic photophosphorylation alongside non-cyclic photophosphorylation. This would mean cells were generating some ATP without producing oxygen through cyclic photophosphorylation which may also lead to a reduction in normal respiration via the electron transport chain and thus a low oxygen uptake (Figure 4.14).

Clearly, cyclic photophosphorylation and "classical" respiration are still occurring in *E. gracilis* G46 under most conditions since oxygen levels within the oxygen electrode decrease under dark conditions and at pH > 3 oxygen is evolved under light conditions. Cyclic photophosphorylation generates ATP without consuming oxygen however non-cyclic photophosphorylation is necessary in order to generate $NADPH^+$ therefore this is some explanation for the compromise between the two photosynthetic pathways occurring at pH > 3. However, there is a clear switch at pH 3 when non-cyclic photophosphorylation is completely switched off. This observation led to the measurement of photosystem II (PSII) efficiency using a pulse modulated fluorescence system since PSII is not required during cyclic photophosphorylation. The hypothesis being that proteins or other components involved in photosystem II may be damaged by the low external pH.

The maximum photosynthetic efficiency, Fv/Fm, measured in *E. gracilis* G46 and *E. gracilis* Z were within the normal expected range for healthy cells (Doege *et al.* 2000), and only show a slight decrease when exposed to high light intensity conditions. The normal values for Fv/Fm indicate that *E. gracilis* G46 do not utilise cyclic photophosphorylation out of necessity since PSII is present and functional. However, the data does not provide sufficient evidence to unequivocally determine if cyclic photophosphorylation is occurring or not.

The lack of oxygen evolution under light conditions at pH 3 may also be explained by increased respiration at this pH due to the slight pH shock after resuspension in fresh media. The pH of cultures was measured regularly and found to be approximately pH 4.0 after 7 days of growth. Although the increase in the pH of the culture medium may be a deliberate or an accidental effect of metabolism, it does mean that cells resuspended in fresh culture medium will be exposed to some pH shock which could

cause an increase in respiration greater than that caused by resuspension in culture medium of a higher pH.

Attempts to inhibit PSII in order to ascertain more information on the effect of low pH on respiration and photosynthesis were unsuccessful due to the lack of suitable solvent for the inhibitor DCMU. Therefore, the exact mechanisms behind the lack of oxygen evolution at pH 3 remain unclear.

Chapter Five: Characterisation of *Acidocella* 29

5.1: Introduction

This chapter describes some of the effects of low pH on an organism which grew on AC pH 3 medium following inoculation with sample number 29 from Hoole Bank acid tar lagoon. Sample 29 was from a predominantly green biofilm present in shallow lagoon water close to the edge of the lagoon with a pH of 3.4, and was chosen for further study based on the predominance of the biofilm at Hoole Bank acid tar lagoon (Figure 5.1).

Figure 5.1: Sample 29 at Hoole Bank acid tar lagoon

5.2: Results

5.2.1: Identification of *Acidocella* 29

Following genomic DNA extraction and amplification of the 16S rRNA gene, the Gram negative isolate which grew on acidiphilium media from sample 29, AC29, was identified through BlastN as an *Acidocella* species (Figure 5.2). *Acidocella* are obligately aerobic chemoorganotrophs, and are commonly found in extremely acidic mineral environments (Kishimoto 1995). Some species have also been found to be capable of degrading some aromatic compounds (Dore *et al.* 2003; Hallberg *et al.* 1999). *Acidocella* 29 was shown to be capable of growth over the pH range 2.5 – 6.0 however no growth was observed at pH $\leq$ 2.4 and $\geq$ 6.1. The growth rate of *Acidocella* 29 was faster at pH 5 than at pH 3, with no growth at pH 7 as expected (Figure 5.3).

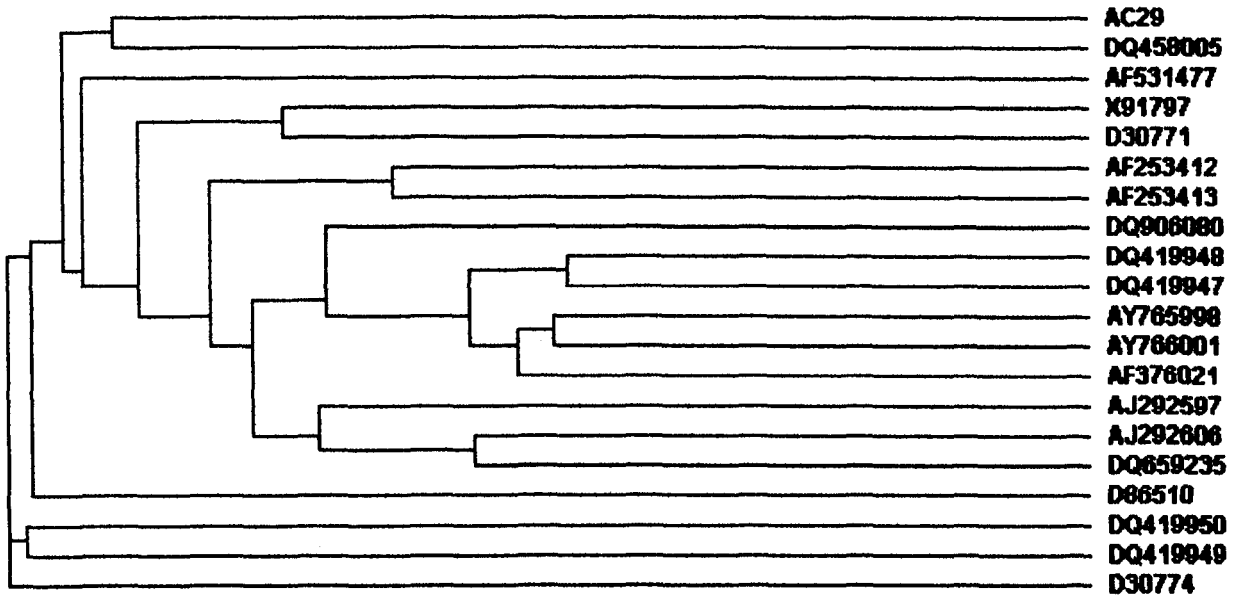


Figure 5.2: Neighbour joining phylogenetic tree constructed using ClustalW (1.83) based on the highest percentage identity matches from BlastN at NCBI. Accession numbers used refer to sequences deposited with NCBI. The *Acidocella* 29 16S rRNA gene sequence is deposited in the NCBI database under accession number EU263910.

DQ458005 uncultured bacterium clone from Dexing Copper Mine, China;
AF531477 *Acidocella* sp. IS10; **X91797** *Acidocella* sp; **D30771** *Acidocella aminolytica*; **AF253412** *Acidocella* sp. WJB-3 (Hallberg *et al.* 1999);
AF253413 *Acidocella* sp. LGS-3 (Hallberg *et al.* 1999); **DQ906080** uncultured clone from Tinto River Rhizosphere; **DQ419948** *Acidocella* sp. DM2; **AY765998** *Acidocella* sp. MZ1 (Hallberg *et al.* 2006); **AY766001** *Acidocella* sp. CCW30 (Hallberg *et al.* 2006); **AF376021** *Acidocella* sp. NO-12 (Johnson *et al.* 2001);
AJ292597 uncultured eubacterium WD238 (Nogales *et al.* 2001); **AJ292606** uncultured eubacterium WD295 from polychlorinated biphenyl-polluted soil (Nogales *et al.* 2001); **DQ659235** uncultured bacterium clone DBS from acid mine drainage in China; **D86510** Acidocella sp; **DQ419950** Acidocella sp. DM4 from moderate acid mine drainage; **DQ419949** Acidocella sp. DM3 from moderate acid mine drainage; **D30774** *Acidocella facilis* (Kishimoto 1995).

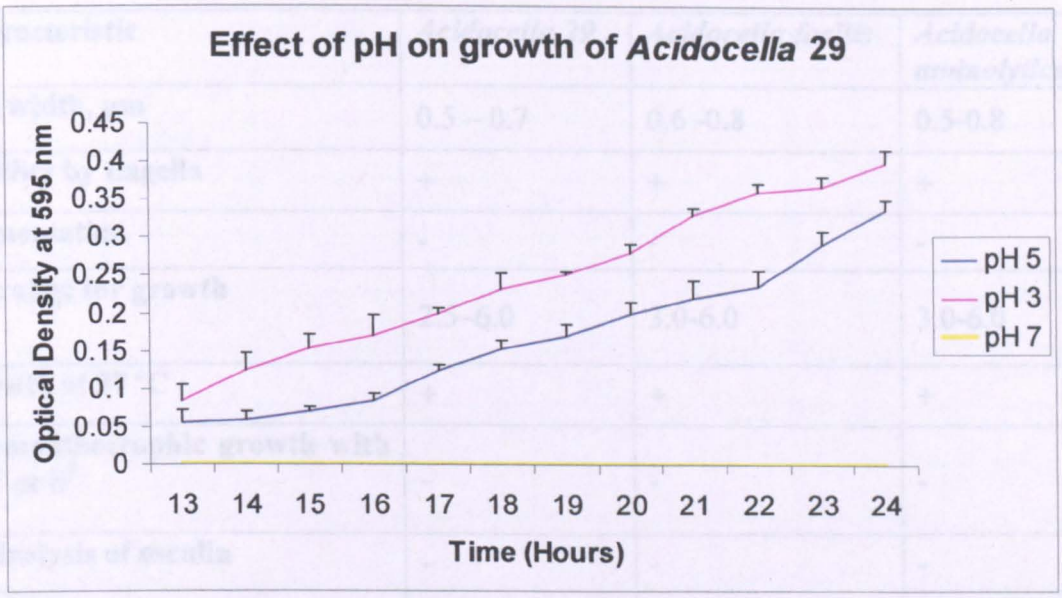


Figure 5.3: Growth curve of *Acidocella* 29. Values shown are the mean of three triplicates plus one standard error of the mean.

5.2.2: Biochemical characterisation

Biochemical characterisation of *Acidocella* 29 was carried out using an API 20NE Biochemical Identification Kit. *Acidocella* 29 was shown to be oxidase negative, catalase positive and esculin was not hydrolysed (Table 5.1). In order to further compare *Acidocella* 29 to other *Acidocella* species, *Acidocella* 29 was grown on M9 minimal media supplemented with various carbon sources to determine which carbon substrates could support growth of *Acidocella* 29 (Table 5.1).

The results shown in Table 5.1 indicate that *Acidocella* 29 is biochemically similar to *A. facilis* and *A. aminolytica* in all key traits. There is more diversity in the pattern of carbon source utilisation in the three species of *Acidocella*. Since other strains of *Acidocella* have been shown to degrade hydrocarbons and use these as carbon sources (Dore *et al.* 2003), the ability of *Acidocella* 29 to utilise several hydrocarbons including benzene, toluene, xylene, ethylene, decane, and hexadecane was tested (Table 5.2). Although the results of this experiment showed no growth on these substrates at any of the three concentrations (1 %, 0.1 % and 0.01 %) tested, there is a strong possibility that the growth environment was anaerobic. Sealing the cultures was necessary to prevent toxic hydrocarbons escaping, which may have prevented growth of the obligately aerobic *Acidocella* 29 (Section 2.18).

Characteristic	*Acidocella 29*	*Acidocella facilis*	*Acidocella aminolytica*
Cell width, µm	0.5 – 0.7	0.6 -0.8	0.5-0.8
Motility by flagella	+	+	+
Pigmentation	-	-	-
pH range for growth	2.5–6.0	3.0-6.0	3.0-6.0
Growth at 37 °C	+	+	+
Chemolithotrophic growth with Fe^{2+} or S^0	-	-	-
Hydrolysis of esculin	-	-	-
Oxidase	-	d	-
Catalase	+	+	+
Carbon source utilisation: **Lactose, ethanol**	+	+	-
Sorbitol, inositol, alanine, lysine, spermine	+	-	+
Glycerol	-	+	d
Creatine	nd	-	d
L-Arabinose, D-xylose, D-ribose,D-glucose, D-galactose, D-fructose, arabitol, mannitol, succinate, diaminobutane,DL-4-aminobutyrate, DL-5-aminovalerate, arginine	+	+	+
L-Rhamnose, maltose, cellobiose, starch, methanol, formate, acetate, lactate, glutamate, glycine	-	-	-
Pyruvate	-	d	nd
Citrate, cis-aconitate, α-ketoglutarate, fumarate, malate	+	+	nd
Gluconate	+	nd	+
Casamino acids, peptone, yeast extract	+	+	+

Table 5.1: Comparison of *Acidocella* 29 with other *Acidocella* species adapted from Bergey's Manual of Systematic Bacteriology (Hiraishi 2005).

For *A. amnolytica* and *A. facilis* : + 90% or more of the strains are positive; d 11-89% of the strains are positive; nd not determined. For *Acidocella* 29: + all four replicates are positive; +/- = one or more replicate negative; nd not determined.

Carbon source ↓	0.01 %	0.1 %	1.0 %
AC pH 3 (+ve control)	-	-	-
M9 pH 3 (-ve control)	-	-	-
Benzene	-	-	-
Toluene	-	-	-
Xylene	-	-	-
Ethylene	-	-	-
Decane	-	-	-
Hexadecane	-	-	-

Table 5.2: Table showing the hydrocarbon utilisation of *Acidocella* 29 at increasing hydrocarbon concentrations. Cultures were sealed with parafilm and placed in a sealed jar to prevent toxic hydrocarbons escaping which may have prevented growth since *Acidocella* are obligate aerobes.

Since there is evidence that 100 mM Al^{3+} increases the growth yield of *Acidocella* (Hiraishi 2005) and some species of *Acidocella* are known to be resistant to certain heavy metals (Ghosh *et al.* 1997), the effect of aluminium, copper, nickel, cadmium and zinc on the growth of *Acidocella* 29 was tested. *Acidocella* 29 was grown in AC pH 3 medium in the presence of each metal sulphate at 10 mM and 100 mM concentrations. The qualitative effects of these metals on the growth of *Acidocella* 29 are shown in Table 5.3.

Metal sulphate	10 mM	100 mM
Aluminium	+	++
Zinc	-	-
Nickel	-	-
Cadmium	-	-
Copper	+	-

Table 5.3: Effects of metal sulphates on the growth of *Acidocella* 29.
- no growth; + growth unaffected; ++ growth increased

The addition of 100 mM Al^{3+} increased the growth of *Acidocella* 29 as suggested by other literature (Hiraishi 2005), whilst at 10 mM concentrations of both aluminium and

copper sulphate the growth of *Acidocella* 29 was unaffected. The presence of the heavy metals zinc, nickel and cadmium were shown to inhibit growth suggesting that *Acidocella* 29 does not possess any genes which confer resistance to heavy metal ions.

5.2.3: Intracellular pH of *Acidocella 29*

Determination of intracellular pH was attempted using the silicone oil method (Section 2.6) to separate cell pellets from their external media allowing the measurement of intra- and extracellular volumes. The distributions of a small labelled molecule, in this case tritiated water, present in both internal and external volumes and a large membrane impermeable molecule, ^{14}C labelled dextran, are used to calculate intra- and extracellular volumes (Section 2.6.1). This information combined with the distribution of a weak acid probe, ^{14}C-benzoic acid in this instance, is used to determine pH_i using the equations shown in Section 2.6.2.

Time course experiments were carried out using ^{14}C-dextran, $^{3}H_2O$ and ^{14}C-benzoic acid in order to ensure there was no active uptake or efflux of the isotopes after the initial distribution between cells and medium (Figure 5.4 – 5.6). No detectable uptake or efflux of any of the isotopes was observed over a one hour time period.

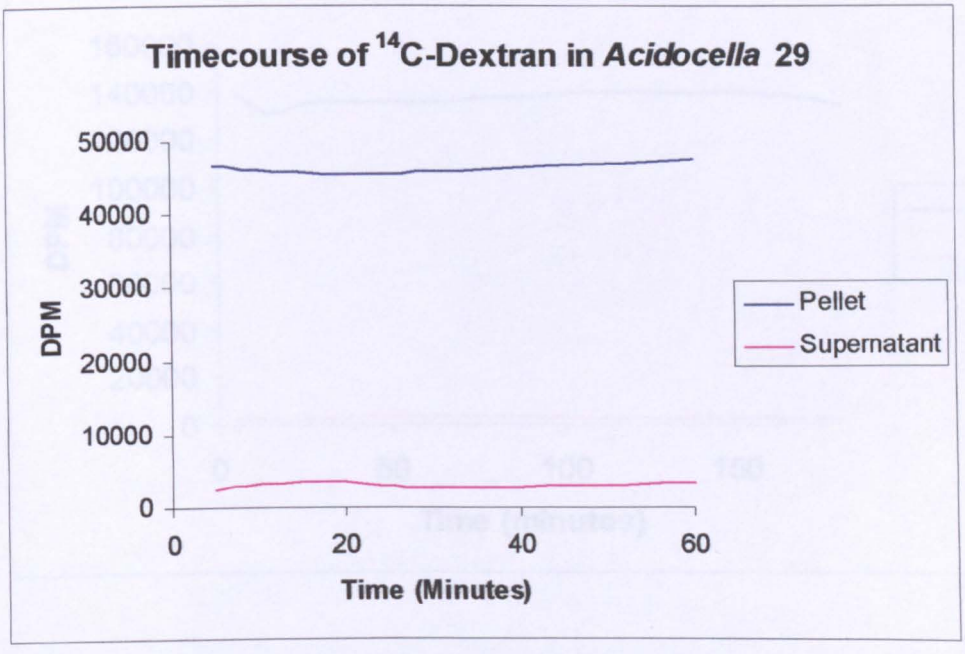


Figure 5.4: Timecourse of ^{14}C-dextran in *Acidocella* 29. No obvious uptake or efflux of ^{14}C-dextran was detected after 1 hour.

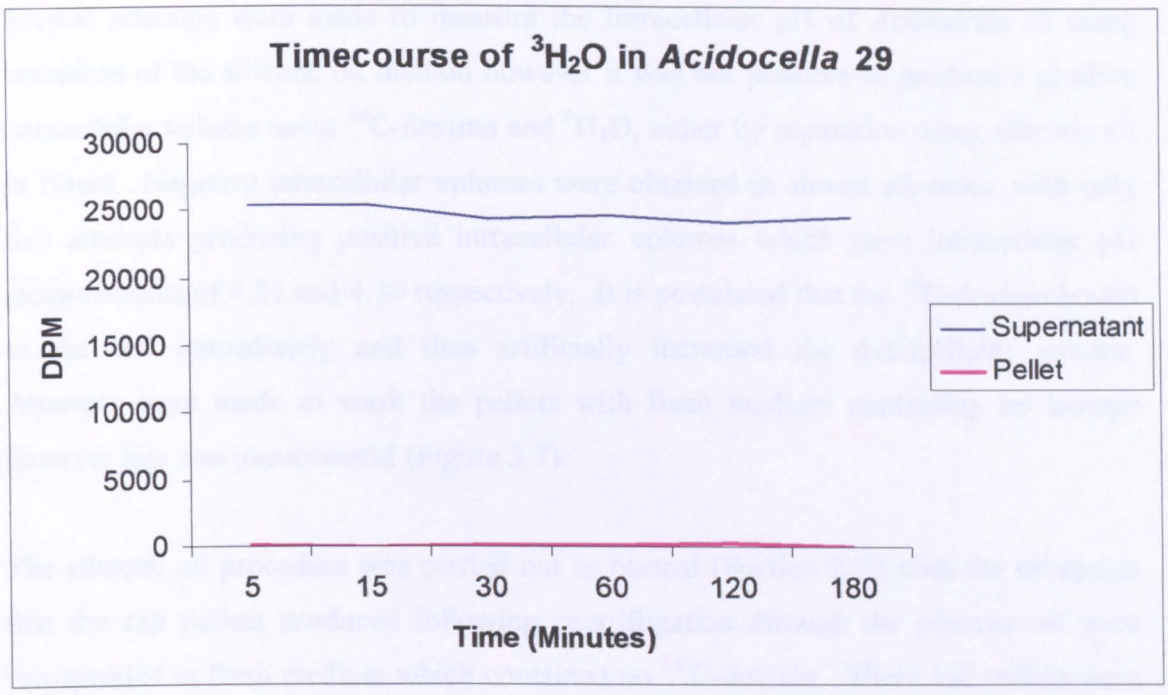


Figure 5.5: Timecourse of ^3H_2O in *Acidocella* 29. No obvious uptake or efflux of ^3H_2O was detected after two hours.

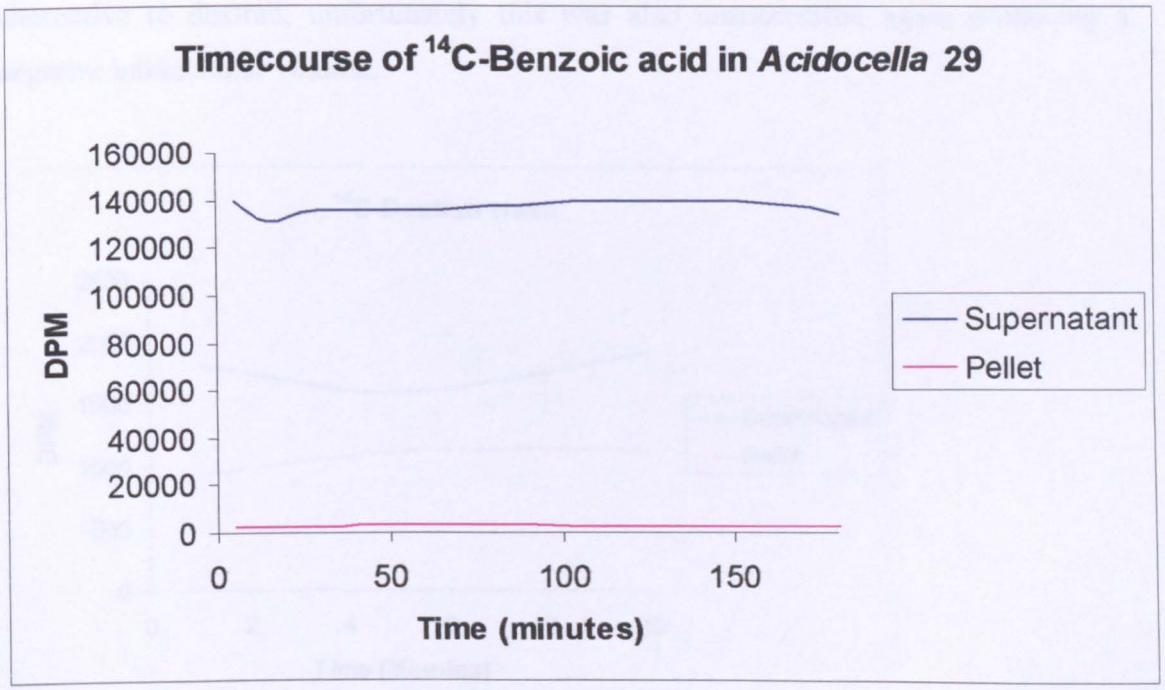


Figure 5.6: Timecourse of ^{14}C-benzoic acid in *Acidocella* 29. No obvious uptake or efflux of ^{14}C-benzoic acid was detected after one hour.

Several attempts were made to measure the intracellular pH of *Acidocella* 29 using variations of the silicone oil method however it was not possible to produce a positive intracellular volume using ^{14}C-dextran and $^{3}H_2O$, either by separation using silicone oil or filters. Negative intracellular volumes were obtained in almost all cases, with only two attempts producing positive intracellular volumes which gave intracellular pH measurements of 4.21 and 4.10 respectively. It is postulated that the ^{14}C-dextran bound to the cell immediately and thus artificially increased the extracellular volume. Attempts were made to wash the pellets with fresh medium containing no isotope however this was unsuccessful (Figure 5.7).

The silicone oil procedure was carried out as normal (Section 2.6), with the exception that the cell pellets produced following centrifugation through the silicone oil were resuspended in fresh medium which contained no ^{14} C-dextran. These cell pellets were incubated in the fresh medium for one, five and ten minutes in an attempt to remove excess ^{14}C-dextran from the cells. Following the incubation in ^{14}C-dextran free medium, the cells were centrifuged and the cell pellet placed in scintillation vials for scintillation counting as per Section 2.6. ^{14}C-PEG (Polyethylene glycol) was used as an alternative to dextran; unfortunately this was also unsuccessful, again producing a negative intracellular volume.

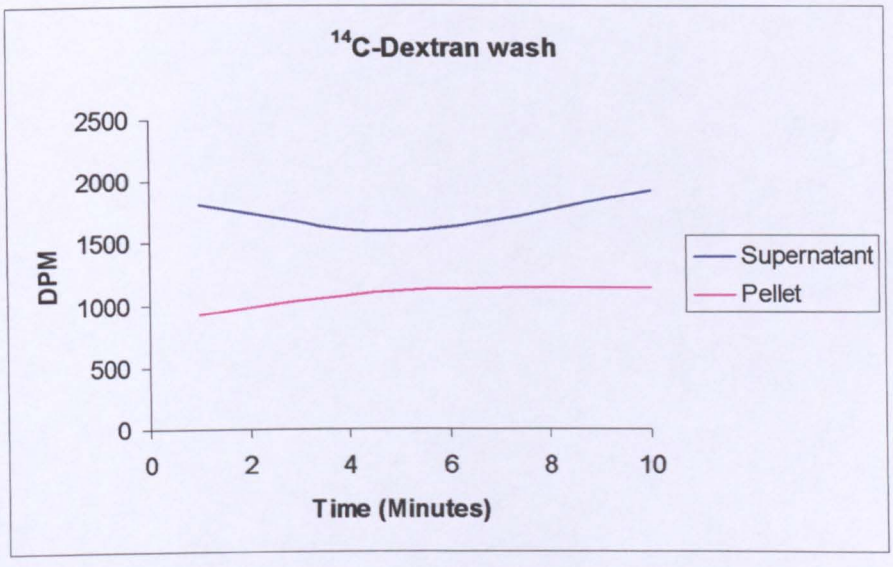


Figure 5.7: Dextran wash. No significant removal of ^{14}C-dextran was observed after washing the pellets in dextran-free media. If the labelled dextran was washed from the cells a significant decrease in the pellet DPM would be expected, however there is a slight increase which indicates that the procedure was unsuccessful.

Therefore, the intracellular pH of *Acidocella* 29 was measured using both ^{31}P and ^{1}H NMR (Section 2.8) and determined to be approximately 4.0 (Figures 5.8 and 5.9). The intracellular pH was also measured in cells resuspended in pH 7 buffer in order to eliminate the possibility that the signal observed was not due to cells which have lysed or become leaky. Figure 5.8 clearly shows the phosphate peak at the same shift in both instances, which also infers that intracellular pH is maintained at approximately pH 4.0 during higher external pH. Following NMR experiments the respiration of cells was measured in an oxygen electrode to ensure their viability and was shown to be normal.

In order to validate or contradict this evidence a further measurement of intracellular pH was attempted using a fluorescent probe in combination with flow cytometry (discussed in more detail in Section 2.7). Although not specifically designed to measure intracellular pH the probe chosen was the LysoSensor Green DND-189 since this was suitable for use with a FACSort machine as it is excited at 490 nm and because it had a relatively low pK_a at 5.2. Since this stain is almost non-fluorescent except when inside acidic compartments and should not fluoresce at $pH \geq 5$, *E. coli* were used as a negative control. However, negative control samples had a greater median fluorescence than the stained *Acidocella* 29 samples at pH 3, 5 and 7, therefore it was not possible to determine a pH_i for *Acidocella* 29 using this method (Figure 5.10).

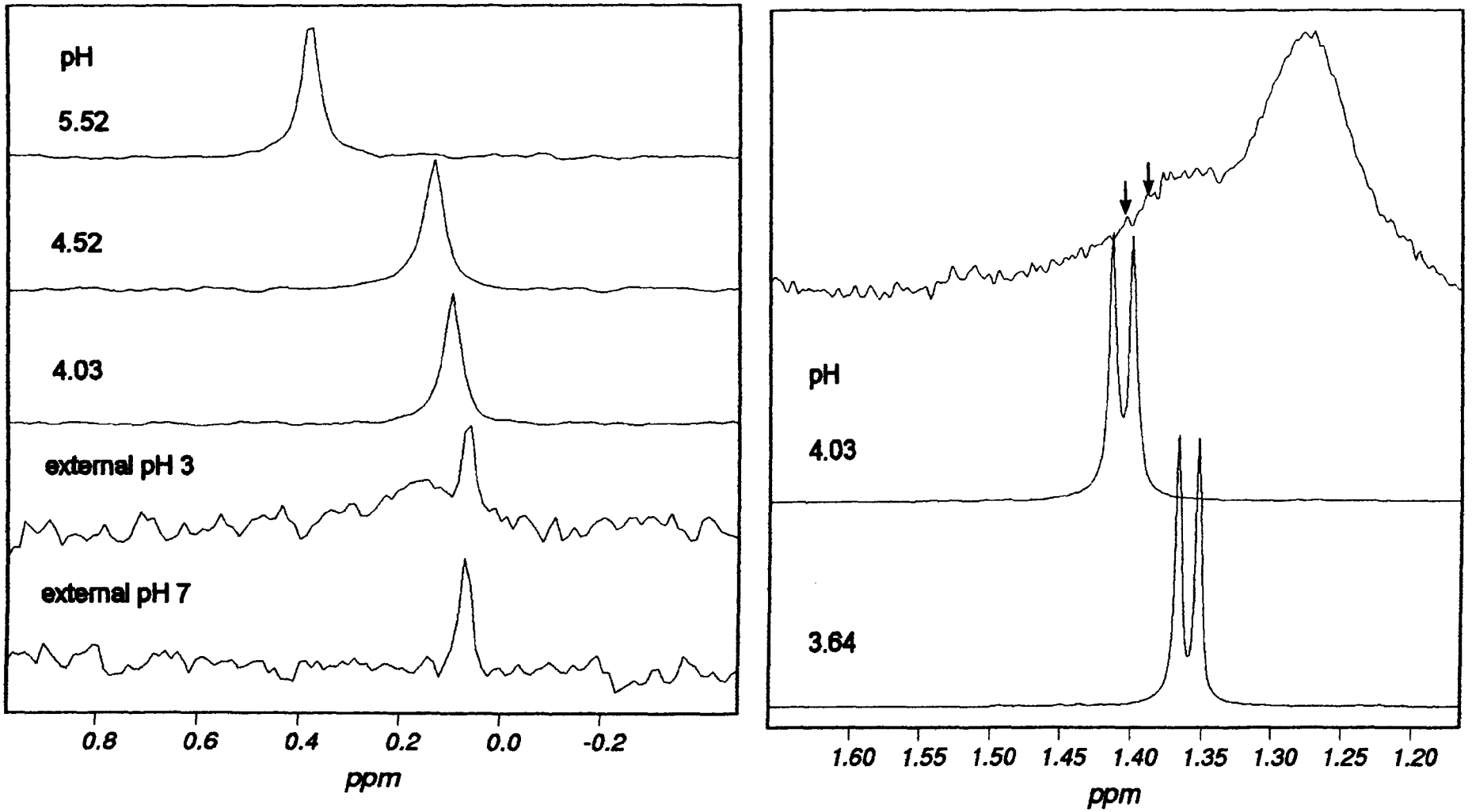
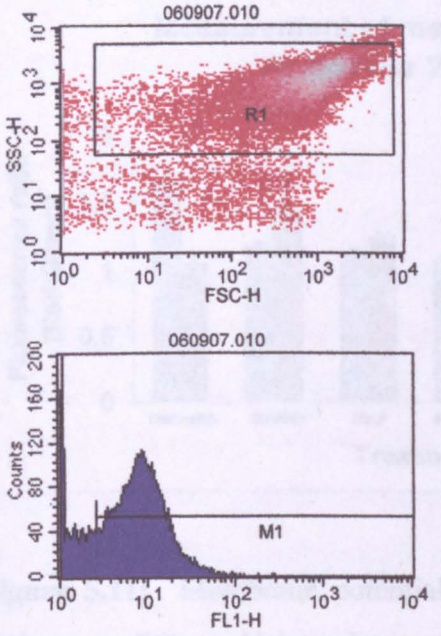


Figure 5.8 (Left): ^{31}P NMR. Comparison of phosphate shift of *Acidocella* 29 with titrated phosphate solutions ranging from pH 4.03 to pH 5.52. **Figure 5.9 (Right):** ^{1}H NMR. Comparison of proton shift in *Acidocella* 29 with titrated lactic acid solutions randing from pH 3.64 to pH 4.03. Cell pH was determined by interpolation to be pH 4.0 for both ^{31}P and ^{1}H NMR samples.



File: 060907.010
Sample ID: E.coli 1
Acquisition Date: 06-Sep-07
Gate: G1
Total Events: 24990

Marker	Left, Right	% Gated	% Total	Geo Mean	Median
All	1, 991C	100.00	80.96	5.52	6.44
M1	3, 991C	77.62	62.84	8.14	8.06

Figure 5.10: Screenshots from CellQuest Pro acquisition software used with FACSort showing fluorescence with the negative *E. coli* control stained with LysoSensor Green DND-189

5.2.4: Measurement of membrane potential of *Acidocella* 29

It was not possible to determine a value for $\Delta\psi$ in *Acidocella* 29 using the silicone oil method since a positive intracellular volume could not be determined, as discussed earlier in Section 5.2.3. Therefore fluorescence activated cell sorting (FACS) was used in combination with the *Bac*Light membrane potential kit in order to provide an indication of the membrane potential.

Although the *Bac*Light kit ($DiOC_2$ stain) is designed to determine membrane potential, the fluorescence intensity response does not appear to be proportional to proton gradient density in Gram-negative organisms ("Molecular Probes Product Information Sheet MP34950"), *E. coli* were used as a control organism in order to compare the responses of *Acidocella* 29 to another Gram negative organism. Stained cells were compared to stained cells treated with CCCP, which abolishes the membrane potential by eliminating any proton gradient (Figure 5.11).

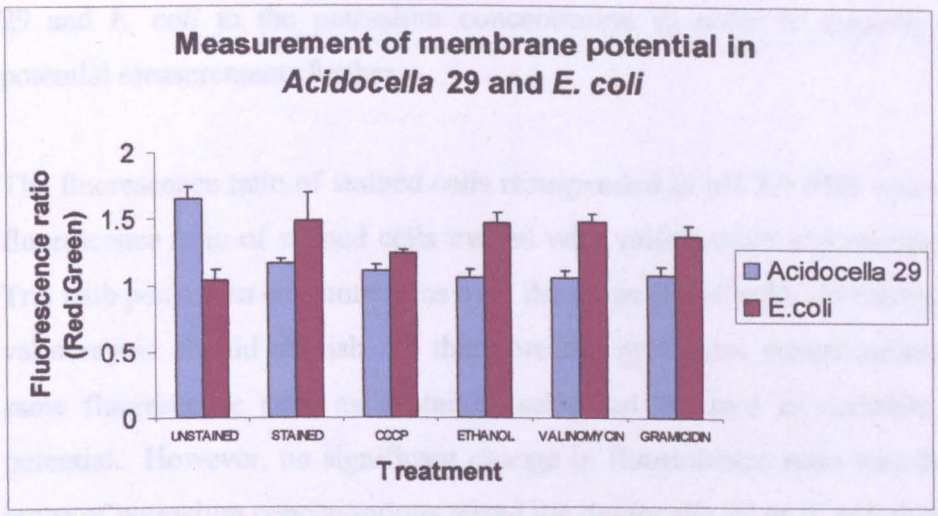


Figure 5.11: Membrane potential of *Acidocella* 29 and *E. coli* when subjected to various conditions. Values shown are the mean of three triplicate experiments plus one standard error.

The addition of $DiOC_2$ stain results in a significant shift and a decrease in the fluorescence ratio compared to unstained cells (Figures 5.11 and 5.12). This effect may be due to the solvent, DMSO, used with the stain as the DMSO may have an independent effect on *Acidocella* 29. Alternatively, it may be possible that the $DiOC_2$ is exerting an effect on the membrane potential of the cells in some way.

CCCP is a lipid soluble weak acid which is able to diffuse freely into the cell. Once in the cell CCCP enters the mitochondria in its protonated form and leaves in its anionic form thus destroying any $\Delta\psi$. *Acidocella* 29 exhibit a much smaller decrease in fluorescence ratio upon addition of CCCP compared to *E. coli*. This may suggest that the membrane potential in *Acidocella* 29 is close to zero or reversed. The contrasting effect seen upon addition of the stain between *Acidocella* 29 and *E. coli* may also indicate a reversed membrane potential in *Acidocella* 29, since the fluorescence ratio decreases considerably in *Acidocella* 29 compared to the considerable increase in *E. coli* after stain is added to the cells (Figure 5.12).

Valinomycin functions as a potassium-specific transporter and facilitates the movement of potassium ions through lipid membranes across an electrochemical potential gradient, suggesting that a potassium gradient may also be involved in the composition of $\Delta\psi$ in *Acidocella* 29. Attempts were made to calibrate the membrane potential of *Acidocella*

29 and *E. coli* to the potassium concentration in order to quantify the membrane potential measurements further.

The fluorescence ratio of stained cells resuspended in pH 3.0 PBS was compared to the fluorescence ratio of stained cells treated with valinomycin and resuspended in pH 3.0 Tris with potassium concentrations over the range 0 – 60 mM. In theory, the addition of valinomycin should abolish $\Delta\psi$ therefore the potassium concentration which has the same fluorescence ratio as unstained cells can be used to correlate the membrane potential. However, no significant change in fluorescence ratio was detected over the range of potassium concentrations tested for *Acidocella* 29 or *E. coli* therefore it was not possible to quantify the membrane potential of *Acidocella* 29 (Figure 5.13).

Gramicidin increases the permeability of the bacterial cell wall in some microorganisms, allowing inorganic cations to travel through unrestricted, thereby destroying the ion gradient between the cytoplasm and the extracellular environment. The effects of valinomycin and gramicidin on *Acidocella* 29 are similar, with a 10 % decrease in fluorescence ratio in cells treated with valinomycin and an 8 % decrease in the fluorescence ratio in cells treated with gramicidin (Figure 5.11). More significantly, valinomycin caused a greater decrease in the fluorescence ratio than CCCP, which may suggest that a proton gradient is not the only component of the membrane potential in *Acidocella* 29. The reduction in the fluorescence ratio in cells treated with gramicidin is approximately equal to cells treated with CCCP.

5.2.5: Effect of pH on respiration in *Acidocella* 29

The effect of pH on respiration in *Acidocella* 29 was tested using a Clark type oxygen electrode. Respiration rate was highest at pH 4, close to the optimum pH for growth (pH 5) of *Acidocella* 29 (See Figure 5.3). Respiration rates at pH 2 and 3 were also relatively high, which is in agreement with the growth data at these pH values whilst there was a significant decrease in respiration rate at pH > 5 which is in agreement with the lack of growth which occurs at pH > 6 (Figure 5.14). In some samples, no respiration occurred after resuspension in pH 7 AC media, further evidence for the lack of viability of *Acidocella* 29 at pH > 6.0. The reduced rates of respiration above pH 5 may suggest that cells quickly become inviable and die, therefore the decrease in respiration may be due to cell death at higher pH.

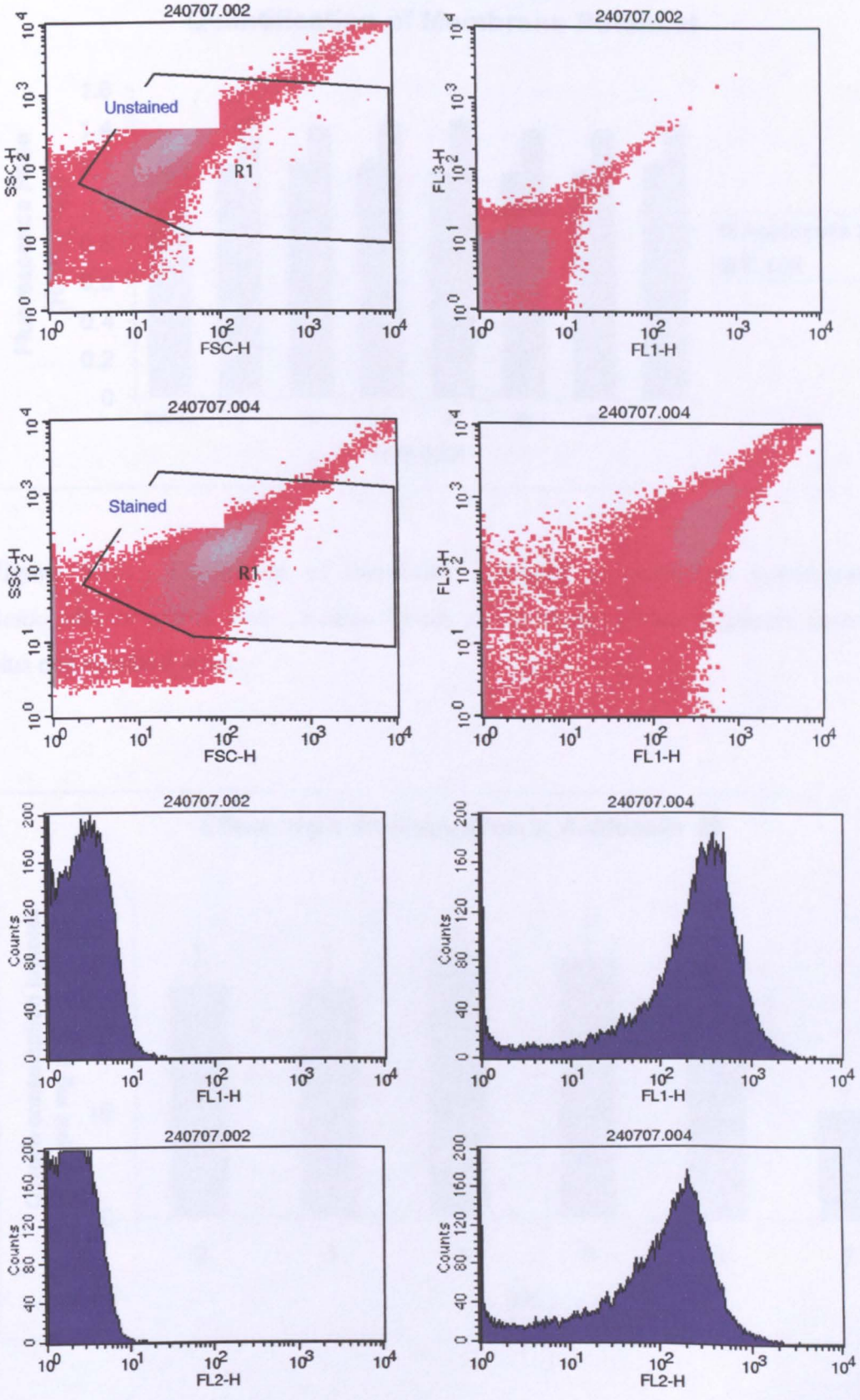


Figure 5.12: Screenshots from CellQuest Pro comparing fluorescence in stained and unstained *Acidocella* 29

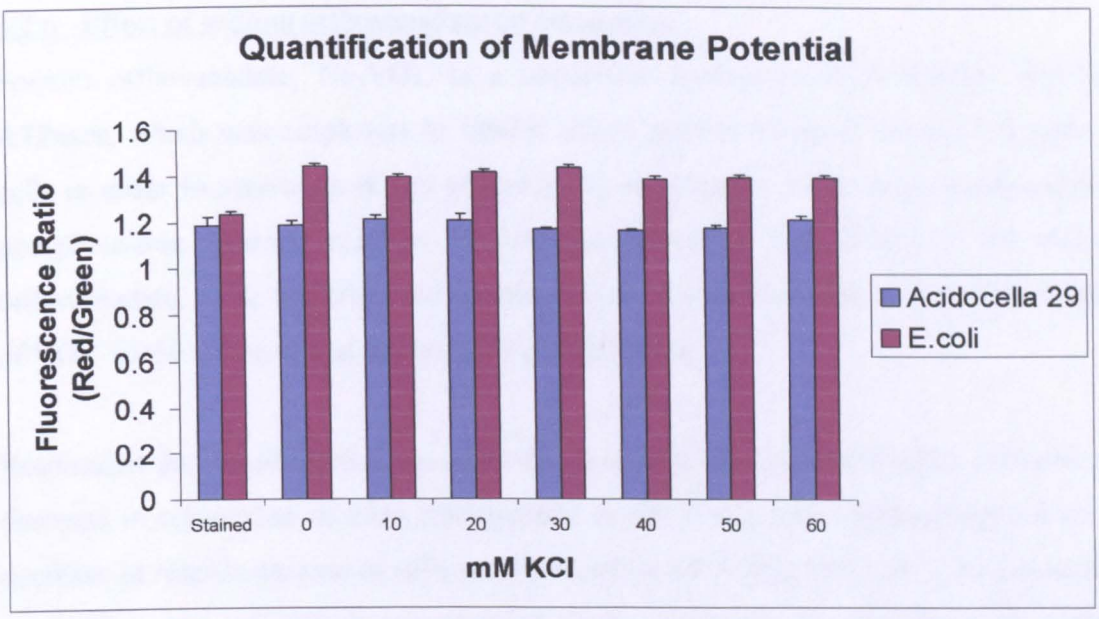


Figure 5.13: Calibration of membrane potential to potassium concentration in *Acidocella* 29 and *E. coli.* Values shown are the mean of two triplicate experiments plus one standard error.

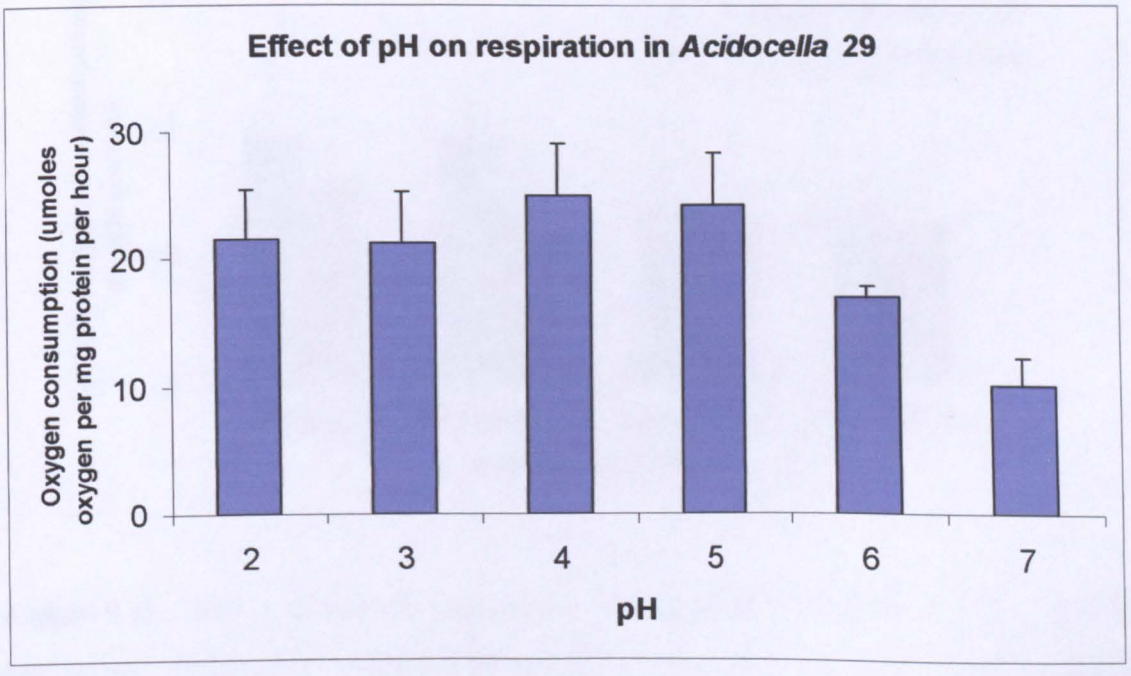


Figure 5.14: Effect of pH on respiration in *Acidocella* 29. Values represented are the mean of three triplicate experiments plus one standard error.

5.2.6: Effect of sodium orthovanadate on respiration

Sodium orthovanadate, Na_3VO_4, is a phosphate analogue which inhibits non-F_1F_0 ATPases, which was employed to inhibit active proton transport from *Acidocella* 29 cells in order to determine if any proton pumping occurs. Cells were resuspended in phosphate-free minimal medium in order to maximise any effects of the sodium orthovanadate, since the proposed mechanism for inhibition is the irreversible binding of VO_4^{3-} to the active site of the tyrosine phosphatases.

Respiration decreased in all cases upon addition of sodium orthovanadate, however the decrease in respiration of cells resuspended at pH 7 was very slight compared to the decrease in respiration rate of cells resuspended in pH 3 (Figure 5.15). The decrease in respiration rate of pH 3 resuspended cells following the addition of sodium orthovanadate would indicate that proton pumping is inhibited and therefore is involved to some extent in proton pumping and possibly pH homeostasis.

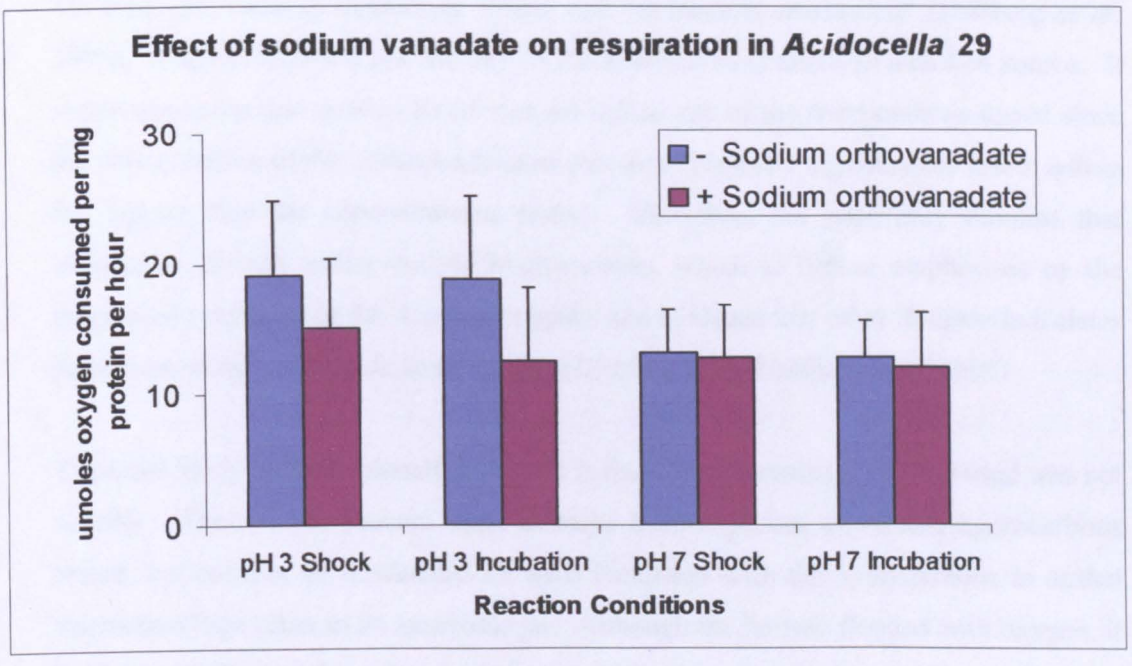


Figure 5.15: Effect of sodium vanadate on the respiration of *Acidocella* 29. Values represented are the mean of three triplicate experiments plus one standard error. Shock = Measurement of respiration rate immediately after addition of orthovanadate. Immediate = Measurement of respiration rate 60 minutes after addition of orthovanadate.

5.3: General Discussion

The isolation of a species of *Acidocella* from Hoole Bank acid tar lagoon is not surprising given that *Acidocella* have been found in several moderately acidic environments previously including acidic drainage waters from copper mines (Johnson *et al.* 2001), coal storage piles (Dore *et al.* 2003), natural petroleum seeps (Roling *et al.* 2006) and acidic sediment (Kimura *et al.* 2006). In this instance *Acidocella* 29 was isolated from an abundant green biofilm present in several shallow areas around the edge of the acid tar lagoon.

Acidocella 29 isolated from Hoole Bank acid tar lagoon appears to be characteristic of the genus, showing very similar morphological and biochemical characteristics and growth requirements to other characterised species (Table 5.1). *Acidocella* 29 can tolerate a lower pH than *A. aminolytica* and *A. facilis* but does not appear to show any further unique characteristics.

Unlike some other species of *Acidocella* isolated from hydrocarbon containing environments such as *Acidocella* PFBC and "*Acidocella aromatica*" (Hallberg *et al.* 1999), *Acidocella* 29 did not use any of the hydrocarbons tested as a carbon source. It is not surprising that *Acidocella* 29 did not utilise any of the hydrocarbons tested since the concentration of the compounds used was most probably significantly lower within the lagoon than the concentrations tested. Therefore, the possibility remains that *Acidocella* 29 may utilise certain hydrocarbons, which is further emphasises by the nutritional versatility of the *Acidocella* genus and evidence that other *Acidocella* isolates have been shown to degrade some of these hydrocarbons (Hallberg *et al.* 1999)

The most likely initial explanation for this is that the experimental method used was not suitable. The need to prevent other cultures from exposure to volatile hydrocarbons meant that cultures of *Acidocella* 29 were incubated with the hydrocarbons in sealed microcentrifuge tubes in an anaerobic jar. Although the jar was flooded with oxygen, it is highly likely that the cultures were anaerobic since the microcentrifuge tubes were sealed with parafilm. It is also possible that the abundance of such hydrocarbons is limited in the biofilm from which this species of *Acidocella* was isolated. Alternatively, these hydrocarbon compounds may undergo chemical transformation within the lagoon or the biofilm specifically and *Acidocella* 29 may be adapted to utilise these transformed hydrocarbons.

The growth yield of *Acidocella* 29 was higher in the presence of 100 mM Al^{3+} in agreement with studies on other *Acidocella* species (Hiraishi 2005); however *Acidocella* 29 appears to have little or no resistance to heavy metal ions. Heavy metal resistance is thought to be mediated through one or more plasmids (Ghosh *et al.* 1997; Ghosh *et al.* 2005), therefore the evidence presented would suggest that *Acidocella* 29 does not possess any of these plasmids. Measurements of the levels of various metal species from Hoole Bank acid tar lagoon are within normal limits therefore the absence of metal ion resistance is not unexpected. Maintenance of plasmids is energetically costly therefore it is highly unlikely that *Acidocella* 29 would maintain a plasmid which did not confer any benefits. Despite the fact that *Acidocella* 29 does not appear to require heavy metal resistance, it is obvious that the cell needs to survive in a low pH environment.

Survival in a low pH environment most commonly requires maintenance of a neutral intracellular pH in order for cell machinery to function properly. Maintenance of pH_i is thought to occur through several mechanisms which have been discussed previously (Section 1.4). Although maintenance of a near neutral pH_i is the most common way to survive in acidic conditions, it is not the only way, with some acidophiles adapting their intracellular components in order to function at a low pH (Dopson *et al.* 2004). Therefore the intracellular pH of *Acidocella* 29 was measured in order to determine if *Acidocella* 29 actively maintained a near neutral pH_i or had adapted its intracellular components to function at a low pH.

Initial attempts to measure pH_i using the distribution of radioisotopes with the silicone oil method for separation were unsuccessful. Negative intracellular volumes were obtained in almost all cases, with only two attempts producing positive intracellular volumes which gave intracellular pH measurements of 4.21 and 4.10 respectively. Attempts to overcome this problem using alternative isotopes, washing steps and filters instead of separation through silicone oil, were all unsuccessful. Various alternative methods were considered in order to try to obtain the intracellular and extracellular volumes. These included measuring the volume of the cells by observing the cells by eye through a microscope and measuring cell dimensions using a haemocytometer, using a cytocrit tube to determine the volume of a specific number of cells and calculate the volume of a single cell, measuring the dry weight of a known number of cells and calculating the intracellular volume based on known weight assumptions and finally

using an alternative organism of similar size and shape with the silicone oil method in order to produce values for the ICV and ECV. Unfortunately, only the last method described would provide an extracellular volume and although it was the most feasible option no suitable organism was available at the time therefore ^{31}P and ^{1}H NMR were employed as an alternative method.

The intracellular pH measured with ^{31}P NMR was estimated to be below pH 4.0, therefore ^{1}H NMR was carried out as the shift in lactate peak is more accurate in this range of pH than the shift in the phosphate peak in ^{31}P NMR since the pK_a of lactate is much lower than that of phosphate. Measurements with ^{1}H NMR were in agreement with the ^{31}P NMR, estimating the intracellular pH at approximately 4.0 (Figures 5.8 and 5.9). This is highly unusual, therefore the respiration rate of the cells was monitored following NMR to ensure that cells were viable and had not lysed. The results of oxygen electrode experiments showed normal respiration for *Acidocella* 29. The intracellular pH of further samples resuspended in pH 7 buffer was measured using ^{31}P NMR to demonstrate that the shift in the phosphate peak was not a result of cell lysis or leakage.

Because the pH_i estimated by NMR was unusually low further efforts were made to measure intracellular pH using another method to ensure the measurement made was indeed true. Fluorescent probes can be used as indicators of pH, however these are usually designed to function over a "normal" pH range, between pH 6.0 and 8.0. Therefore, a probe used to measure the pH of lysosomes was chosen due to its low pK_a of 5.2. Unfortunately the LysoSensor Green DND-189 was not suitable for pH_i measurements in *Acidocella* 29 since it fluoresced in negative control samples therefore the only measurement of intracellular pH in *Acidocella* 29 is from NMR measurements.

Although *Acidocella* species have been the subject of some investigation there are currently no records of intracellular pH measurement in this genus. The lowest recorded intracellular pH to date is that of the thermoacidophilic archaea *Picrophilus torridus and P. oshimae*, with an intracellular pH of 4.6 (Vossenberg *et al.* 1998) which was measured using the distribution of radioisotopes in a similar technique to the silicone oil method (Section 2.6). The intracellular pH of *Acidiphilium acidophilum* (formerly *Thiobacillus acidophilus*), a species of the most closely related genus to

Acidocella, has been measured. *A. acidophilum* is reported to have a pH_i of 6.0 (Bond and Banfield 2001).

NMR has been used to measure the intracellular pH of several organisms including *Ferroplasma* spp. which has an estimated pH_i of 5.6 (Macalady *et al.* 2004), the acidophilic alga *Cyanidium caldarium* (Enami *et al.* 1986), and *Saccharomyces cerevisiae* (Gillies et al. 1981). The method used in this work was very similar to the method used by Macalady *et al.*, therefore the pH_i of *Acidocella* 29 may simply be as low as measured. There is almost no shift in the phosphate peak between cells resuspended in pH 3 buffer and cells resuspended in pH 7 buffer which confirms that the phosphate peak observed in initial ^{31}P experiments is intracellular phosphate and that the cells have not lysed. Although the results from ^{1}H NMR are slightly less conclusive given the high noise level of the signal the lactate peaks are distinguishable following computational adjustments and are in agreement with the pH_i measurements from ^{31}P NMR (Figures 5.8 and 5.9).

Microorganisms which have been shown to have a low intracellular pH as opposed to those which maintain a near-neutral intracellular pH are often inviable above a certain pH and have been shown to lyse. The reduction in respiration rate of *Acidocella* 29 above pH 5 may be caused as a result of cell lysis at these higher pH values or because the higher external pH is damaging the intracellular components which are adapted to low pH (Figure 5.14). This evidence combined with the inability of cells to grow above pH 6 provides further supportive evidence for the low intracellular pH of *Acidocella* 29.

The effect of sodium vanadate on *Acidocella* 29 suggests that it may utilise proton pumping at pH 3 since the decrease in respiration after treatment with sodium orthovanadate is significantly greater at pH 3 at than at pH 7. There is also a greater percentage reduction in respiration at pH 3 following incubation with sodium orthovanadate than the shock experiments at the same pH which is indicative of continued and increased proton pumping inhibition. There is a very slight decrease in respiration rate at pH 7 when cells are treated with sodium orthovanadate, however the decrease is extremely small and almost identical in shock treated cells and cells incubated with sodium orthovanadate which would infer that the sodium orthovanadate has little if any effect on proton pumping at pH 7. This further supports the use of

proton pumping in pH homeostasis in *Acidocella* 29 since little, or no, effect of sodium orthovanadate is expected at higher pH values.

The evidence from sodium orthovanadate experiments which suggests that proton pumping has a role within *Acidocella* 29 may conflict with the intracellular pH measurements of *Acidocella* 29 since it is less likely that intracellular pH mechanisms are utilised in an organism with a pH_i of approximately 4.0. However, *Acidocella* 29 is routinely grown in pH 3 medium yet the pH_i of *Acidocella* 29 is not pH 3, which is what would be expected if no pH homeostasis occurred. Therefore it is possible that removal of protons from the cell by proton pumping is one of the few pH homeostasis mechanisms utilised by *Acidocella* 29.

Chapter Six: Conclusions and Future Work

Characterisation of the microbial diversity present at Hoole Bank acid tar lagoon represents the first work of its kind in this particular environment. Whilst the microbial diversity of similar environments such as acid mine drainage, the Rio Tinto and hydrocarbon-polluted sites have been studied previously there is a distinct difference between these sites and Hoole Bank acid tar lagoon. Hoole Bank acid tar lagoon comprises extremes of low pH and high hydrocarbon content, compared to the single extreme of either acidity or hydrocarbon pollution of other environments.

As expected, the microbial diversity present at Hoole Bank acid tar lagoon was shown to be much higher when measured with the molecular techniques of T-RFLP (Table 3.4) and DGGE (Figures 3.8 and 3.9) than the classical culturing methods employed which isolated only five microorganisms from 21 samples (Table 3.2). Yet, this is a somewhat unfair comparison between the methods since the classical culture techniques employed in this study were far from extensive. A wide range of alterations and improvements can be made to the isolation process in order to increase the number of microorganisms cultured from Hoole Bank acid tar lagoon samples. Using a wider range of solid media such as overlay plates and medium supplemented with appropriate carbon sources present at Hoole Bank acid tar lagoon are likely to improve the culture efficiency. Overlay plates contain an acidophilic heterotroph within their bottom layer which removes compounds which are toxic to oligotrophic acidophiles from the top layer of the agar (Johnson 1995). Similarly, supplementing growth media with a greater range of carbon sources such as volatile hydrocarbons including toluene, benzene, ethylbenzene and xylene and C_{15}-C_{30} hydrocarbons may facilitate the growth of organisms adapted to utilise the carbon sources present in Hoole Bank acid tar lagoon. Increasing the pH of growth media used to include solid medium at pH 4 and pH 5 may also increase the number of isolates from Hoole Bank acid tar lagoon samples, since several lagoon samples had pH values ≥ 3.

An increased number of organisms may have been isolated from Hoole Bank acid tar lagoon samples by using micro-aerophilic and anaerobic growth conditions in order to isolate organisms with different requirements for oxygen. Changing the growth medium and conditions used to specifically isolate sulphate reducing organisms is also likely to lead to an increased number of cultured microorganisms from Hoole Bank acid

tar lagoon samples given the high sulphur content and anaerobic conditions found within the lagoon.

Denaturing gradient gel electrophoresis (DGGE) enabled the identification of a number of microorganisms present within Hoole Bank acid tar lagoon which were not cultured, along with further confirmation of the presence of some organisms which were isolated using culturing techniques (Table 3.7). Indeed, this is the first identification of the genus *Euglena* using either 18S or 16S rRNA DGGE, which represents a step forward in molecular microbial diversity analysis and also provides an alternative route for molecular microbial analysis of algal populations using 16S rRNA from chloroplasts as opposed to 18S rRNA. Previous attempts to use DGGE to assess the algal community present in the Rio Tinto where species of *Euglena* were identified using microscopy failed to reveal the presence of *Euglena* using 18S rRNA DGGE (Aguilera *et al.* 2006). The identification of organisms from excised DGGE bands also allows culturing techniques to be designed specifically to isolate organisms known to be present at Hoole Bank acid tar lagoon such as *Acidithiobacillus ferrooxidans*.

DGGE estimates of microbial diversity were lower than those produced using Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and could be improved further by optimising the denaturing gradient and gel conditions further. Unfortunately it was not possible to visualise as many bands on the DGGE gels using an open transilluminator and the naked eye as it was to visualise bands in a closed transilluminator system and a digital camera due to limitations of the equipment used. This meant that many of the bands present on DGGE gels were not excised and thus not identified. Therefore, further optimisation of this process, including the narrowing the denaturing gradient used or utilising different PCR primers for specific microorganisms such as nitrate reducers, would yield a greater amount of information, particularly regarding microbial species which are likely to represent a smaller proportion of the microbial population.

Whilst comparisons between Hoole Bank acid tar lagoon and other acidic environments such as acid mine drainage and the Rio Tinto have been made previously, the microbial community at Hoole Bank acid tar lagoon was shown to comprise a much greater number of bacterial species than other acidic environments, using both DGGE and T-RFLP.

T-RFLP provided the most detailed understanding of the total number of microbial species present within each Hoole Bank acid tar lagoon sample. The abundance and proportion of each microorganism within the total microbial community was also inferred based on the data directly obtained from T-RFLP (Figures 3.7a-d). However, no phylogenetic information can be determined directly from T-RFLP data. The number of T-RFs in each sample according to T-RFLP varied from as little as 4 to 50 in samples 49 and 50 respectively (Table 3.4). Two methods were used to analyse T-RFLP data in order to statistically compare microbial diversity present within Hoole Bank acid tar lagoon. T-align (Smith *et al.* 2005) was followed by principal component analysis (PCA) and correspondence analysis in order to reveal any patterns in microbial diversity which could be displayed in a visual format. PCA of both interstitial and percentage area data indicated very little correlation between sample groups and microbial diversity or sample pH and microbial diversity as shown in Figures 3.5a-h and Figures 3.6a-h. However, certain samples do group more tightly in certain figures (Figures 3.5h, 3.6d, 3.6g and 3.6h) which may indicate a linking factor between these samples. Further analysis of the samples obtained from Hoole Bank acid tar lagoon, including analysis of the chemicals and metals present and their concentration, may provide further information which may suggest a relationship between the microbial diversity present in these samples and a common component present in these sampling locations at Hoole Bank acid tar lagoon.

The phylogeny of one particularly abundant T-RF (170.00 bp forward *Hha*I) was inferred using the phylogenetic assignment tool (PAT) (Kent *et al.* 2003) based on the combination of that T-RF with the 112.22 bp fragment produced from *Alu*I forward digest as this was equally abundant, both in terms of percentage peak area and number of occurrences. Several possible phylogenetic affiliations were made based on *in silico* analysis of the 16S rRNA gene sequences in the database and included *Acidovorax sp.* OS-6, *Alicycliphilus denitrificans* and *Diaphorobacter nitroreducens* KSP4 (Table 3.6). Although this information is useful as it may allow future culture techniques to be tailored in order to try and isolate these organisms, the vast amount of information produced by PAT is time consuming to analyse and does not provide definitive confirmation of phylogenetic assignment in most cases. Improvements to the T-RFLP data obtained could be made by carrying out a greater number of repeats in order to provide a greater amount of data for T-align and PCA which would allow improved

statistical analysis. A third restriction digest would also significantly improve the ability to reduce the number of possible phylogenetic affiliations generated by PAT.

A clone library of 16S rRNA genes or a metagenomic library of total genomic DNA obtained from Hoole Bank acid tar lagoon would provide a great deal more information than T-RFLP or DGGE. Whilst phylogeny cannot be assigned directly from T-RFLP and only bands which are visible for excision can be identified with DGGE, a 16S rRNA clone library would provide a greater insight into the phylogeny of all microorganisms present at Hoole Bank acid tar lagoon. This could also be extended to include 18S rRNA genes such that eukaryotic diversity including yeasts, algae and fungi could be assessed. Similarly, a metagenomic library of genomic DNA from Hoole Bank acid tar lagoon could be screened for functionality such as degradation of a specific compound. Fluorescence *in situ* hybridisation would also be useful to identify samples which contain specific organisms, such as those already isolated, in order to ascertain interactions of particular species with other organisms. This would be especially useful in the biofilms, where more complex interactions may be occurring.

Of the five microorganisms which were isolated from Hoole Bank acid tar lagoon, the unicellular alga *Euglena gracilis* G46 was chosen for further study due to the predominance of the visibly green biofilms from which this organism was isolated at Hoole Bank acid tar lagoon. Unlike the type strain of *E. gracilis* Z which was used as a control, *E. gracilis* G46 was capable of growth at pH 2 (Figure 4.3a) and possessed a lower optimum pH for growth (Figure 4.3a). The effect of pH on photosynthesis and respiration was measured (Figures 4.12 and 4.13 respectively), with *E. gracilis* G46 showing significantly decreased levels of photosynthesis compared to the wild type organism at pH values ≥ 3.

The intracellular pH of *E. gracilis* G46 was measured and compared to the wild type strain, *E. gracilis Z*, in order to determine how *E. gracilis* G46 survives in such acidic conditions. The pH_i measured using the distribution of radioisotopes with the silicone oil technique was estimated at pH 5.47 at an external pH of 3 (Table 4.1), whilst NMR estimated the intracellular pH of *E. gracilis* G46 at pH 6.6 (Figure 4.5). Although there is some discrepancy between these values, both methods indicate that *E. gracilis* G46 actively maintains a near-neutral intracellular pH, as opposed to adapting its intracellular components to function in an acidic environment. This is further supported

by the conserved sequence of ribulose-1,5-bisphosphate carboxylase/oxygenase (Figure 4.8) which does not exhibit any adaptations to low pH. In order to further clarify the intracellular pH of *E. gracilis* G46 the use of microelectrodes which can be directly inserted into the cells would provide a value for pH_i during normal cellular conditions. The use of a more advanced fluorimeter would also provide a greater insight into the effect of pH on photosynthesis of *E. gracilis* G46, particularly if the fluorescence of photosystem I was measurable, at cryogenic temperatures. .

The second organism chosen for further study from the five organisms isolated from Hoole Bank acid tar lagoon was the acidophilic bacterium *Acidocella* 29, which like *E. gracilis* G46, was also isolated from a biolfilm sample from Hoole Bank acid tar lagoon. Although *Acidocella* 29 exhibited similar traits to other *Acidocella* species (Table 5.1), attempts to demonstrate utilisation of various hydrocarbons present at Hoole Bank acid tar lagoon were negative (Table 5.2). Whilst it may be the case that *Acidocella* 29 does not utilise any of the hydrocarbons tested, limitations in the experimental set up may have influenced the outcome of this test. The outcome of this experiment may be different if repeats were carried out in an Therefore, repeats of this aerobic environment.

Attempts to measure the intracellular pH of *Acidocella* were made using the silicone oil technique but were ultimately unsuccessful, continually producing negative intracellular volumes. Therefore, ^{1}H and ^{31}P-NMR were used to measure pH_i, producing an unexpectedly low pH_i of 4.0 (Figures 5.8 and 5.9) in an external pH of 3.0. Further investigation into this is warranted given the unusually low intracellular pH, perhaps using alternative fluorescent dyes in combination with flow cytometry as attempted in the experiment reported in Figure 5.10. Attempts to measure the specific activity of malate dehydrogenase from *Acidocella* 29 were unsuccessful (data not included); however further investigation into the effect of pH on activity of intracellular enzymes such as malate dehydrogenase may provide more information about the internal pH of *Acidocella* 29. Similarly, DNA or amino acid sequence analysis of intracellular proteins may also allow some inference of intracellular pH based on the presence or absence of adaptations to an acidic environment.

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