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A study of the microbiological populations of mine wastes

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by

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Abstract

The activities of mineral-oxidising microorganisms play a crucial role in global mineral cycles, and are of huge importance to the mining industry. Their activities *in situ* often result in the production of acid mine drainage (AMD), with dire environmental consequences. While the microbial populations of AMD itself are relatively well studied, very little is known about the microbial populations of solid-phase mine wastes and products. Increased understanding of these populations will allow greater understanding of pollution genesis in mine wastes, such as the longevity of the problem. It may also be possible to identify novel organisms that may be important for the biotechnological processing of mineral ores, while increasing the understanding of microbial interactions therein. Therefore, the microbial populations of material from seven mine sites located in the U.K., mainland Europe and the U.S.A. were investigated using culture-based and biomolecular methods. Samples were obtained from heaps at five metal mines and one coal mine. The heaps varied greatly in terms of their mineralogies and ages since deposition. There was considerable variation in the microbial populations between all the materials analysed. The simplest population was observed in water droplets forming on the surface of pyritic rock in an abandoned sulfur mine. This was dominated by *Acidithiobacillus ferrooxidans*, while *Leptospirillum* spp. were the only other microorganisms detected. The microbial populations of spoil and tailings from an abandoned Portuguese mine were restricted to a group of iron-oxidising "*Firmicutes*" and the sulfur-oxidiser, *At. thiooxidans*. Iron-oxidising "*Firmicutes*" also dominated a currently inactive pilot-scale heap bioleaching operation at a copper mine in Utah, suggesting that this group of organisms are important in mineral heaps in semi-arid zones. Enrichment cultures from both the Portuguese tailings and the U.S. bioleach heap were capable of extensive sulfide mineral oxidation. The majority of organisms detected at the remaining four sites could not be cultured with the media used, and therefore, in many cases, their metabolic activities could not be ascertained. However, a novel iron-oxidising bacterium was isolated from the oldest site investigated, and was characterised in detail. This was found to be a member of the *Rubrobacteridae* subclass of the *Actinobacteria*, which has not previously been found to include any iron- or sulfur-oxidising species. Mineral-oxidising *Bacteria* were detected in samples from all seven mine sites, although *Acidithiobacillus* was the only genus to be detected at every site. Microbial biodiversity varied greatly between the sites, and appeared to be primarily dependent on *p*H, although deposit age was also a factor. Biodiversity was enhanced where mine wastes had been landscaped and planted with trees as part of a remediation process. However, this did not appear to restrict the amount of mineral oxidation, and therefore potential genesis of acid mine drainage waters; geochemical data implied that sulfide mineral oxidation was on-going, even in mine wastes that had been deposited at least 120 years ago. The data indicated that mineral oxidation and hence the environmental hazard posed by mine wastes can be a very long-term phenomenon. Further work should be undertaken to overcome problems associated with sample processing, to sample a greater number and diversity of sites and to further characterise novel and potentially useful organisms isolated in this study.

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Dad, this is for you.

All around me is falling down,
The rush of life that makes no sound.
Open wounds I cannot feel,
I fear this flesh can no longer heal.
Untimely poised, and poison sipped,
The fragile heart so crudely ripped.
The rhythm slows but never alters,
The lightshow fades, the heartbeat falters.
Onwards always and now to be,
Unchained of life, in death set free.
All-consuming Time cares less,
As we must all embrace its dark caress.

Abbreviations

16S rRNA	16S (small) ribosomal ribonucleic acid subunit
AAS	Atomic absorption spectrophotometry
ALD	Anoxic limestone drains
AMD	Acid mine drainage
amp	Ampicillin
ARD	Acid rock drainage
BLAST	Basic logical alignment search tool
bp	Base pair
BS	Plate basal salts
cfu	Colony forming units
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
E.U.	European Union
EA	Environment agency (England and Wales, U.K.)
EDTA	Ethylenediaminetetra-acetic acid
E_h	Reduction-oxidation (redox) potential
EPA	Environmental Protection Agency (U.S.A.)
EPS	Extracellular polymeric substances
Fe	Iron (liquid medium)
Feo	Iron overlay (solid medium)
FeSo	Iron/tetrathionate overlay (solid medium)
FeST	Iron/tetrathionate/TSB (liquid medium)
FeT	Iron/TSB (liquid medium)
FeTo	Iron/thiosulfate overlay (solid medium)
FeTT	Iron/thiosulfate/TSB (liquid medium)
FeYE	Iron/yeast extract (liquid medium)
FISH	Fluorescent *in situ* hybridisation
Fp	"*Ferroplasma*" medium (liquid medium)
G+C	Guanine + Cytosine (content of genome)
HBS	Heterotrophic basal salts
IC	Ion chromatography
iFeo	Inorganic iron overlay (solid medium)
Kb	Kilobase
ML	Maximum likelihood
nt	Nucleotide
OTU	Operational taxonomic unit
P	Pyrite (liquid medium)
PCR	Polymerase chain reaction

PIRAMID	Passive *in-situ* remediation of acidic mine/industrial drainage (E.U.-funded research project)
PLS	Pregnant leach solution
PRB	Permeable-reactive barriers
PYE	Pyrite/yeast extract (liquid medium)
R2A	10% R2 agar (solid medium)
RAPS	Reducing and alkalinity producing systems
RDP	Ribosomal Database Project II
RFLP	Restriction fragment length polymorphism
RISC	Reduced inorganic sulfur compound
RO	Reverse osmosis
ROM	Run-of-mine
rpm	Revolutions per minute
S	Sulfur (liquid medium)
SDS	Sodium dodecyl sulfate
sp(p).	Species (plural)
SX/EW	Solvent extraction/electro-winning
t	Tonne (metric)
TE	Trace elements
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
Tris	Tris(hydroxymethyl)methylamine
TSB	Tryptone soya broth
$TSB_{2.5}$/TSB_3/TSB_4	TSB (liquid/solid media)
U.K.	United Kingdom
U.S.A.	United States of America
UBS	Umeå basal salts
v/v	Volume per volume
w/v	Weight per volume
YE	Yeast extract
YE_3/YE_4	Yeast extract (liquid/solid media)
$YE_3\underline{o}$/$YE_4\underline{o}$	Yeast extract overlay (solid media)

Genus abbreviations

A.	*Acidiphilium*
Ab.	*Acidobacterium*
Ac.	*Acidocella*
Ad.	*Acidianus*
Al.	*Acidilobus*
Alb.	*Alicyclobacillus*
Am.	*Acidimicrobium*
Amn.	*Acidimonas*
As.	*Acidisphaera*
At.	*Acidithiobacillus*
B.	*Bacillus*
E.	*Escherichia*
"*Fm.*"	"*Ferrimicrobium*"
Fp.	*Ferroplasma*
H.	*Hydrogenobaculum*
L.	*Leptospirillum*
M.	*Metallosphaera*
P.	*Picrophilus*
S.	*Sulfolobus*
Sb.	*Sulfobacillus*
Sc.	*Sulfurococcus*
Sg.	*Stygiolobus*
Ss.	*Sulfurisphaera*
Tm.	*Thiomonas*
Tp.	*Thermoplasma*

Welsh words commonly used in place names

Afon	River
Coch/Goch	Red
Cwm	Valley
Mynydd	Mountain
Nant	Stream

CHAPTER 1: INTRODUCTION

Human exploitation of the Earth's mineral reserves predates recorded history. These activities have left a legacy of severe environmental damage, imparted largely through the formation and uncontrolled release of acid mine drainage (AMD). This is caused by microbially-mediated decomposition of exposed sulfide minerals in mine wastes and voids. While there are many remedial options available to treat AMD and thus ameliorate its environmental impact, the microbial processes responsible for AMD genesis can be harnessed in biotechnological operations for the processing of mineral ores. Such biomining operations provide a more environmentally friendly and more effective means for mineral processing, in many cases, though it is generally considered to be a niche technology. While mining economics at this time may preclude the wholesale adoption of biomining as a replacement for established high temperature mineral processing, increasingly stringent environmental legislation and public pressure, along with an increasing shortage of raw minerals, may see this technology develop as a method for low-cost, effective reprocessing of mine wastes, allowing maximum metal recovery while reducing the potential for the genesis of AMD. Essential to improving current biomining processes, and resolving the potential for their application to the reprocessing of mine wastes, is the in depth study of the microbiota responsible for mineral dissolution. Microbial succession in mine waste heaps from the point of deposition, the effects of mineral type and location and the age of the waste are important areas of study.

1.1 MINES AND MINE DRAINAGE

Acid mine drainage (AMD), generally referred to as acid rock drainage (ARD) in the U.S.A., is the often highly acidic, metal rich effluent draining both active and abandoned mine sites. It is a highly toxic, persistent legacy of sulfide mineral exploitation. The origin and genesis of AMD and its impact on the biosphere are discussed below.

1.1.1 Occurrence of AMD

Extreme acidity in the environment can occur naturally or as a result of human disturbance. Sources of extreme acidity are usually from the contribution of magmatic gasses, such as acidification by hydrochloric and hydrofluoric acid (HCl and HF), or the subsequent oxidation of gasses such as hydrogen sulfide (H_2S) and sulfur dioxide (SO_2) to sulfuric acid (H_2SO_4); or the generation of sulfuric acid from the oxidation of exposed sulfidic minerals. Although natural exposures of sulfidic minerals do occur, as in parts of the Iberian pyritic belt in the Mediterranean, most are of anthropogenic origin.

The vast majority of exploited metal ores occur as metal sulfides. These may occur in igneous and metamorphic rocks, but are typically of hydrothermal origin, as a result of igneous intrusions. The most abundant sulfide mineral in the Earth's crust is pyrite (FeS_2). Other important sulfide minerals include chalcocite (Cu_2S), chalcopyrite ($CuFeS_2$), arsenopyrite (FeAsS), sphalerite (ZnS) and galena (PbS). Since prehistory these materials have been located and exploited, originally in the form of shallow workings using simple tools. Evidence of these workings can be found throughout the world where sulfide ores occur close to the surface. With technological advances, mining developed, exploiting more and more previously inaccessible lodes through deep underground workings and huge opencast excavations. The greatest expansion occurred during the industrial revolution and continues to this day. Often, mineral deposits would first be worked with opencast operations, with less accessible lodes latterly followed underground. This will continue until it becomes uneconomical to continue working a lode. Mines will often close due to the effects of economics rather than complete depletion of the lode.

In mine workings, sulfide minerals on the surfaces of mine shafts, adits and voids are often exposed to moisture and oxygen in the air and are subsequently oxidised, forming effervescent acid-generating salt layers on the rock. In active mines, water that would normally flood workings below the water table is actively removed by pumping. However, when mining activity ceases the water level is allowed to rebound to its natural level, flooding underground workings and areas of open cast voids which are below this

level. The rising waters become contaminated with metals and sulfuric acid as the oxidised mineral salts are dissolved from the rock surface. Waters flooding underground workings and opencast voids will usually find their way into the wider environment, draining from naturally occurring fissures in the rock or along mine adits and shafts.

Coal seams often occur between layers of carboniferous limestone. Therefore, water reaching the underground workings of coal mines usually contains sufficient mineral alkalinity to counter the effects of the sulfuric acid produced during sulfide dissolution, resulting in circum-neutral *p*H flood water. Where there is a lack of, or insufficient, alkaline overburden, as is the case with opencast operations and most deep mineral mines, there is little or no buffering capacity in the flooding waters and these can subsequently become highly acidic.

Mine and mineral processing sites also give rise to huge amounts of waste materials. Excavated material that has a metal content that is too low to be processed economically is dumped in spoil heaps, along with mine overburden. Ores are often processed to concentrate desired metal sulfides prior to smelting. Concentration procedures, such as floatation, involve grinding the ore and treating it to make the target mineral fraction float on addition of specific chemicals and active aeration. This is skimmed off the top of large floatation tanks, and the tailings left over are dumped, usually on site. These tailings heaps typically still contain significant amounts of finely ground sulfide minerals, often dominated by pyrite, depending on the efficiency of the concentration procedure.

In the oxic zone at the surface of waste heaps, sulfidic minerals exposed to moisture and oxygen are oxidised, contaminating water percolating through the heap. Depending on the nature and integrity of the surface bedrock underlying the heap, water draining these heaps may find its way into the wider environment as point or diffuse sources of pollution.

The *p*H of water draining waste heaps will be a product of the potential for acid genesis, i.e. sulfide mineral content, versus the potential for neutralisation (alkali genesis). Therefore, drainage from pyritic coal spoil and most mineral waste heaps will become acidic in the absence of sufficient alkaligenic material.

1.1.2 Sulfide mineral dissolution and the genesis of AMD

Sulfide minerals are susceptible to oxidative dissolution wherever they are exposed to both air and moisture. While the rate at which this occurs abiotically is relatively slow, especially at low *p*H, the rate of dissolution is greatly increased in the presence of certain microorganisms.

The dissolution of sulfide minerals involves the oxidation of sulfide sulfur moiety, causing disruption of the crystal structure. As this process involves the transfer of electrons to the oxidant via the cathodic site, in theory the rate-limiting step of sulfide mineral oxidation is the conductivity of the mineral (Rimstidt and Vaughan, 2003). However, in practice the limiting factor in sulfide mineral dissolution is the availability of the aqueous oxidant (Singer and Stumm, 1970; M^{c}Guire *et al.*, 2001). In acidic environments, ferric iron is a more effective oxidant than oxygen and is the most important oxidant in the context of AMD genesis (M^{c}Kibben and Barnes, 1986).

Sulfide minerals can be divided into two categories: those that are acid-soluble and those that are acid-insoluble. The oxidation of acid soluble minerals follows either the polysulfide or hydrogen sulfide pathway, whereas the oxidation of acid-insoluble minerals follows the thiosulfate pathway (Schippers *et al.*, 1996; Sand and Gehrke 2006). Acid-soluble minerals include sphalerite (ZnS), chalcopyrite ($CuFeS_2$) and galena (PbS). The dissolution of such minerals occurs through either ferric iron or ferric iron and proton attack, resulting in the release of free metal ions and elemental sulfur, via a polysulfide intermediate. For example, the dissolution of sphalerite occurs thus:

$$ZnS + Fe^{3+} + H+ \rightarrow Zn^{2+} + \tfrac{1}{2}H_2S_n + Fe^{2+} \qquad \textbf{[1.1]}$$

$$\tfrac{1}{2}H_2S_n + Fe^{3+} \rightarrow (\tfrac{1}{4}n)S_8 + Fe^{2+} + H^{+} \qquad \textbf{[1.2]}$$

In the absence of ferric iron, dissolution may follow the hydrogen sulfide pathway (Sand and Gehrke, 2006):

$$ZnS + 2H^{+} \rightarrow Zn^{2+} + H_2S \qquad \textbf{[1.3]}$$

Pyrite (FeS_2), molybdenite (MoS_2) and tungstenite (WS_2) are examples of acid-insoluble sulfide minerals. These minerals are oxidised by ferric iron via the thiosulfate pathway, resulting in free metal ions, thiosulfate and proton acidity. The oxidation of pyrite occurs thus:

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + 6H^+ + S_2O_3^{2-} \quad \textbf{[1.4]}$$

Thiosulfate is unstable in acid conditions in the presence of ferric iron and is quickly oxidised to form tetrathionate:

$$2Fe^{3+} + 2S_2O_3^{2-} \rightleftharpoons 2FeS_2O_3^{+} \rightarrow 2Fe^{2+} + S_4O_6^{2-} \quad \textbf{[1.5]}$$

In the presence of pyrite or ferric iron in acidic conditions, tetrathionate formed in equation [1.5] will further decompose. This may be through rearrangement reactions, forming tri- and pentathionate or via hydrolysis to form elemental sulfur and other reduced inorganic sulfur compounds (RISCs) (Schippers *et al.*, 1996; Druschel *et al.*, 2003).

All of the above reactions will occur abiotically and in the absence of oxygen. However, the activities of certain microorganisms dramatically increase the overall rate of mineral dissolution by as much as a factor of a million-fold (Singer and Stumm, 1970). This is the result of two key microbial actions: (i) the oxidation of ferrous iron, and (ii) the oxidation of RISCs. A hypothetical scheme for the oxidative dissolution of pyrite by acidophilic prokaryotes is shown in Figure 1.1.

In acidic environments (*p*H <3.5), ferrous iron is very stable and will oxidise only very slowly. Therefore, abiotic mineral dissolution occurs very slowly, due to the slow regeneration of ferric iron. However, under aerobic conditions some microorganisms oxidise ferrous iron very rapidly:

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad \textbf{[1.6]}$$

This provides a supply of ferric iron to the mineral oxidation pathways and prevents sulfide oxidation becoming limited by the concentration of available oxidant.

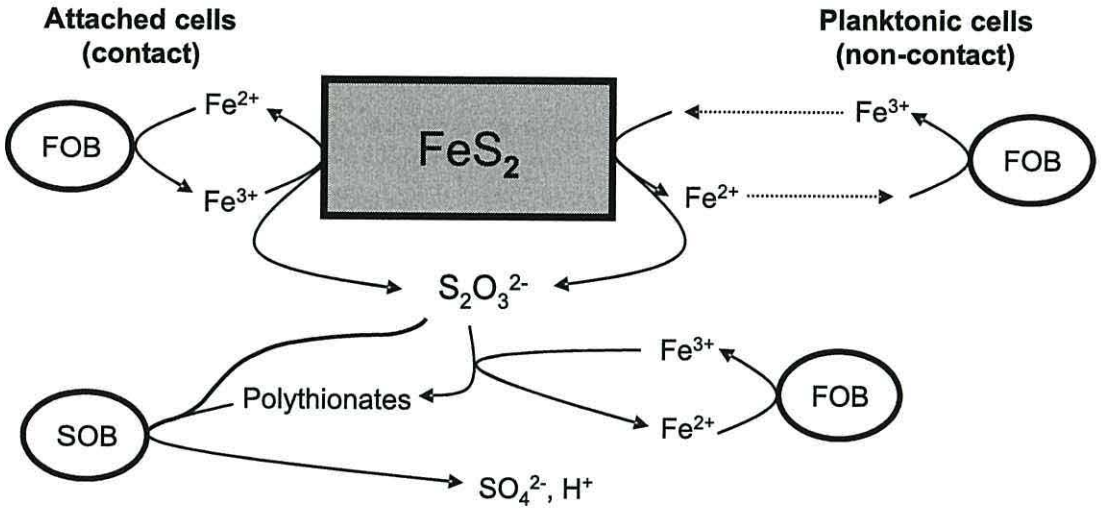

Figure 1.1 Hypothetical scheme for the oxidative dissolution of pyrite (FeS_2) by acidophilic bacteria, either by cells attached to the mineral surface or free-swimming (modified after Hallberg and Johnson, 2001). (FOB, iron-oxidising prokaryotes; SOB, sulfur-oxidising prokaryotes.)

Some microorganisms can oxidise reduced sulfur compounds such as polysulfides, elemental sulfur and polythionates, generating sulfuric acid. For example:

$$H_2S + 2O_2 \rightarrow 2H^+ + SO_4^{2-} \qquad \textbf{[1.7]}$$

$$\tfrac{1}{2}H_2S_n + (2n)O_2 \rightarrow (\tfrac{1}{2}n)SO_4 + 2H^+ \qquad \textbf{[1.8]}$$

$$S_8 + 12O_2 + 8H_2O \rightarrow 16H^+ + 8SO_4^{2-} \qquad \textbf{[1.9]}$$

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2H^+ + 2SO_4^{2-} \qquad \textbf{[1.10]}$$

$$S_4O_6^{2-} + 3O_2 + 2\tfrac{1}{2}H_2O \rightarrow 6H^+ + 4SO_4^{2-} \qquad \textbf{[1.11]}$$

The oxidation of elemental sulfur may be of particular importance to the rate of mineral dissolution. Elemental sulfur has been shown to build up on the surface of pyrite during oxidative dissolution in the absence of sulfur-oxidising microorganisms. While there is little evidence that this inhibits mineral dissolution, the greatest dissolution rates are observed when sulfur-

oxidising microorganisms are present, and there appears to be little sulfur build-up on the mineral surface (M^{c}Guire *et al.*, 2001). Sulfur-oxidising organisms such as *Acidithiobacillus caldus* may play a key role in determining rates of proton production in acidic ecosystems (Edwards *et al.*, 2000a).

Two distinct methods of microbiologically-mediated mineral dissolution have been described. These are known as the 'direct' and 'indirect' bioleaching mechanisms, and the intricacies and relative importance of each have been the subject of considerable debate. Much of this can be attributable to a lack of clarity on the definition of each mechanism (Rawlings, 2002). In indirect leaching, the microorganisms' sole roles are assumed to be the recycling of soluble ferrous iron to ferric. Initially, this implied that mineral dissolution would occur independently of whether the microorganism was in contact with the mineral or not. The direct mechanism seemed to lack an unequivocal definition, but in the strictest interpretation implied that mineral oxidation occurred through direct interactions of microbial enzymes and the mineral surface (Ehrlich, 2002; Sand *et al.*, 1995). It is widely accepted now that such a mechanism does not exist, and instead there are two sub-categories of the indirect mechanism: the 'contact' and 'non-contact' mechanisms (Rawlings, 2002; Rohwerder *et al.*, 2003). In the non-contact mechanism, planktonic organisms oxidise ferrous iron in solution. The resultant ferric iron ions must then come into contact with the mineral surface to cause dissolution. The contact mechanism is based on the fact that most of the microbial cells in a mineral oxidising population are attached to the mineral surface (Gehrke *et al.*, 1998). Microorganisms attach to the sulfide mineral surface via extracellular polymeric substances (EPS), which provide a controlled reaction zone for the mineral dissolution reactions.

The exact mechanisms of bioleaching are, and will continue to be, the cause of much debate. However, it is likely that both contact and non-contact mechanisms occur simultaneously during mineral leaching. What is known is that in acidic systems, sulfide mineral leaching is accelerated by ferric iron. Certain microorganisms are able to catalyse the oxidative dissolution of sulfide minerals by rapidly oxidising ferrous iron and reduced inorganic sulfur compounds, creating and maintaining an acidic environment that is rich in (soluble) ferric iron and other dissolution products.

1.1.3 The characteristics of AMD

The composition of AMD is dependent on the geochemistry, climate and ambient conditions of both its source and receiving environment. Its composition will often be the subject of both temporal and spatial variation as a result of changing conditions, especially those of receiving water bodies where hydrological factors are very important (e.g. Sánchez-España *et al.*, 2004; Herr and Gray, 1996). The principal components of AMD are protons, iron (ferrous and ferric) and sulfate, due to the relative abundance of pyrite and the central role of iron and sulfur oxidation in oxidative dissolution. Typical concentrations vary from 3 to 200 mg L^{-1} iron and 400 to 5000 mg L^{-1} sulfate, but may be much higher. High concentrations of aluminium and manganese are generally found in AMD, and elevated concentrations of other trace metals and metalloids, including arsenic, barium, cadmium, copper, molybdenum, nickel, lead, selenium and zinc may also occur. These elements are primarily solubilised by the oxidation of sulfide minerals, such as arsenopyrite (FeAsS), and by the dissolution of silicate minerals in acidic liquors; the greater solubility of most metals at low *p*H results in elevated concentrations of these metals in AMD waters (Johnson, 2003). AMD usually has a high solute potential due to the high concentration of dissolved solutes; conductivity is often greater than 1800 µS cm^{-1} and sometimes on a par with that of sea water, at greater than 50'000 µS cm^{-1} (Younger 2002).

Mineral content, iron and sulfur oxidation, the formation of secondary minerals and the ambient temperature all affect the *p*H of AMD. Ferrous iron oxidation is an acid-consuming reaction, involving the consumption of one proton for each ferrous iron ion oxidised (Eq. 1.6). However, dissolution of pyrite by ferric iron attack and the subsequent oxidation of sulfide and RISCs to sulfate are acid-generating reactions (Eqs. 1.4; 1.7-1.11). The higher the sulfur content of the mineral, the greater the number of protons produced per mole of mineral following complete oxidation.

Mineral acidity results from the effects of hydrolysis by metals such as ferric iron and aluminium to form solid hydroxide precipitates and will also affect the *p*H of AMD (Sánchez-España *et al.*, 2004). The formation of secondary minerals will affect *p*H by the consumption or generation of

protons, depending on the mineral formed. For example, the precipitation of ferric iron as ochre or "yellow boy" and the formation of aluminium hydroxide generates protons, and effectively act as an acidic buffer as the *p*H rises:

$$Fe^{3+} + 3H_2O \rightleftharpoons Fe(OH)_3 + 3H^+ \quad \textbf{[1.13]}$$

$$Al^{3+} + 3H_2O \rightleftharpoons Al(OH)_3 + 3H^+ \quad \textbf{[1.14]}$$

Therefore, the acidity of AMD and impacted waterways is affected by the concentrations of ferric iron and aluminium, which act as buffers at *p*H 2.7 and 4.5 respectively (Sánchez-España *et al.*, 2004).

Secondary mineral formation on the surface of sulfide minerals may also act to reduce acid generation by affecting the rate of sulfide dissolution (Edwards *et al.*, 2000b).

The dissolution of basic minerals such as calcite ($CaCO_3$), dolomite ($CaMg(CO_3)_2$) and, to a lesser extent, aluminosillcates such as chlorite, will impart alkalinity. The extent to which this neutralises net acidity will depend on the abundance and rate of dissolution of basic minerals in the environment. If there is sufficient alkalinity to overcome both proton and mineral acidity, mine drainage may have circum-neutral *p*H, though this may be only a short term situation, as the dissolution of carbonates tends to proceed more rapidly than sulfide minerals and the former are often depleted before the latter.

Ambient temperature will affect both the chemical and biological processes that occur during sulfide mineral leaching. The process of mineral dissolution is a chemical one, via an oxidant such as ferric iron. Therefore, as the temperature increases the rate of mineral dissolution will increase in a curvilinear trend. However, the regeneration of the ferric iron oxidant at low *p*H is biologically mediated. The iron oxidation rate for an organism will increase with temperature to an optimum, after which point the reaction rate will decrease rapidly. This is due to the effect of high temperature on cell membrane integrity and protein conformation, and thus cell viability. However, different microorganisms have different optimum temperatures (see section 1.4), and so a range of mineral-oxidising organisms will be found over different ambient temperatures. Therefore, as ambient temperature increases,

so will the rate of mineral dissolution to a point where life becomes unsustainable (Rawlings, 2005).

The ambient temperature will also affect both *p*H and total dissolved solutes directly, and therefore solute potential. Where the ambient temperature is high, evaporation will cause an increase in the concentrations of dissolved solutes. The acute effects of this can be seen at the former São Domingos copper mine in Portugal. At this site a pool at the base of a large tailings deposit is subject to evaporation during the hot dry summer months, resulting in *p*H values as low as 0.18 and total iron concentrations as high as 180 g L^{-1} (Johnson, unpublished). Additionally, the oxidation of pyrite and other sulfide minerals is an exothermic reaction and, where rates of pyrite dissolution are high, this can cause a significant increase in temperature. One such example is the ore body at the Richmond Mine, part of the Iron Mountain in California, U.S.A., which contains over 95% pyrite. The temperature of the waters within the mine was as high as 47°C and maybe even hotter further inside. The *p*H in some pools of mine water was calculated to be as low as -3.6, the lowest recorded anywhere, and soluble iron and sulfate concentrations were found to be as high as 200 g L^{-1} and 760 g L^{-1}, respectively (Nordstrom *et al.*, 2000). In highly pyritic coal spoil these exothermic reactions can lead to spontaneous combustion of carboniferous material and spoil heaps can smoulder for years (Johnson and Hallberg, 2003).

1.1.4 The environmental response to AMD

There is some debate as to whether mine voids or mine wastes have the greatest impact in terms of pollution generation. Younger (2001) found that in Scotland, 72% of total contaminant loading came from mine voids. This is in contrast to observations of the Iberian Pyritic Belt where 60% of AMD was found to come from waste rock piles and tailings impoundments (Sánchez-España *et al.*, 2004).

The impact of acid mine drainage is primarily on the receiving watercourses, where its effects are complex. It is a multi-factor pollutant, and affects ecosystems through a number of direct and indirect interactions that can be both chemical and physical (Gray, 1997). Chemical effects are the

result of *p*H, dissolved solutes and salinity, whereas physical effects are caused by the precipitation of secondary minerals and metal oxides. AMD will affect different ecosystems in different ways, and it is difficult to distinguish which component will have what effect, or which, if any, may be more important.

Metals can be classified according to the ions they form. Hard metals preferably form ionic bonds and are easily displaced and mobile. Soft metals tend to form covalent bonds and bind to soft ligands such as sulfide and sulfur donors (Hodson, 2004). These also tend to accumulate in organisms and are generally more toxic. The concentrations at which metals become toxic may not much higher than the normal background levels required for growth (Riesen *et al.*, 2005). The effects can be due to interactions of the metals with enzymes and other biomolecules or the generation of free radicals. Aquatic organisms are especially susceptible to the effects of increased metal concentrations in the environment as they are less able to control their uptake. Pollutants may enter the body through cell membranes, skin, or across the gills.

The impact of acidification of the aquatic environment is probably indirect. The effects of acid rain on mountain streams have been shown to vary significantly depending on the underlying bedrock. A twenty-fold variation in ecological response was found between acidic and circum-neutral *p*H waters (Dangles *et al.*, 2004). The effects were caused by increases in calcium and aluminium concentrations due to the dissolution of calciferous minerals in the bedrock, and this was more acute where the stream was most acidic. *p*H also has an effect on the bioavailability of many metals entering the biosphere by affecting ionic state and solubility.

Sulfate itself is generally considered to be non-toxic. The only limit for sulfate in the U.K. is the drinking water standard of 250 mg L^{-1}, and this is rarely used as a limit for discharges (Hugh Potter, Environment Agency, Personal Communication). However, it does contribute to solute potential, and while this may not be a major problem in temperate climates, in arid and semi-arid zones this is a significant threat to already scarce water resources.

The physical effects of AMD can be as a result of solar absorbance by suspended solids or their deposition on surfaces. For example, the deposition

of ferric hydroxides on leaf surfaces has been shown to inhibit colonisation by decomposers such as fungi and aquatic insects (Gray and Ward, 1983). It has been argued that the effects of precipitates on stream communities may be greater than the activities of dissolved metal ions (M^{c}Knight and Feder, 1984).

Ecosystems respond differently to different levels of stress caused by AMD. This will also depend on whether exposure is continuous or intermittent. Gerhardt *et al.* (2004) found that invertebrate biodiversity was high in an area receiving occasional flows of AMD, presumably due to the transition of an acid-intolerant biota to a tolerant one. Parsons (1977) found that benthic and planktonic species had become adapted to a continuous flow, and varied in abundance and diversity, whereas in an area affected by infrequent effluent flow diversity was higher, but abundance was low.

Many photosynthetic primary producers are principally affected by AMD. Algae seem to be especially susceptible to metal precipitates such as alumina, resulting in their absence or decreased abundance in affected streams (Niyogi *et al.*, 2002; M^{c}Knight and Feder, 1984). This leads to a reduced nitrification potential and low dissolved organic carbon levels (Niyogi *et al.*, 2003). The activity of AMD has also been shown to have a detrimental effect on detritovores, affecting litter turnover. This means there are lower levels of organic matter processing and this affects nutrient cycling in impacted environments (Arp *et al.*, 1999; Dangles *et al.*, 2004).

The larval stages of many organisms are acutely sensitive to AMD. In one study, the toxicity of the drainage was such that it required diluting a thousand times in order to allow the survival of toad larvae (Porter and Hakanson, 1976). Fry, Salmon smolts, and other fish have all been shown to be sensitive to AMD, with reduced abundance or complete absence often reported (e.g. Barry *et al.*, 2000; Grippo and Dunson, 1991; Parsons, 1977). Besides the toxic effect of AMD on the higher organisms, by reducing primary production and nutrient turnover, it affects the entire food chain from the bottom up.

The effects of AMD are often felt considerable distances from its source. While the dilution of AMD due to confluence of affected and unaffected waterways is important in the amelioration of toxic effects (e.g. Soucek *et al.*, 2000), mine sites often occur in clusters and so the total AMD

loading of the environment may be high due to the combined effluents from several mines. For example, the Odiel River basin in southern Spain receives AMD from most of the mines in the Iberian Pyritic Belt. At its mouth, the *p*H is ~3.0 with elevated levels of many toxic metals, making it one of the most polluted river systems in the world (Sánchez-España *et al.*, 2004). Concentrations of pollutants in the Odiel, as with many receiving watercourses, follow seasonal trends. For example during winter the concentrations of dissolved pollutants in the Odiel River are at their lowest, due to intense rains. They then increase again during summer, peaking in autumn (Olías *et al.*, 2004).

The extent to which AMD may inflict these potential effects on the biosphere is dictated not only by its composition, but also the manner in which it is released into the environment. In general, mine effluents occur as chronic, steady sources of pollution. Fluctuations in flow rates from a mine site will largely be determined by climatic events such as rain and flash floods (e.g. Gerhardt *et al.*, 2004). However, acute, and potentially catastrophic, releases do occasionally occur. These are usually as a result of the sudden failure of an adit plug or blockage or the displacement of waste heaps or tailings dams.

An often cited example from the U.K. is the sudden discharge of AMD from the former Wheal Jane tin mine in Cornwall. In late 1991 an adit along which mine water was draining was sealed. Mine waters subsequently accumulated behind the plug, and in 1992, this failed and 50 x 10^3 m^3 of highly contaminated mine drainage was suddenly discharged into the Nangiles adit. These waters flowed into the Carnon River and eventually into the Fal Estuary, resulting in an orange plume of ochre that reached the western approaches of the English Channel. Paradoxically, despite the scale and severity of the release, the environmental impact was not great. This was due to the fact that the Carnon River was already badly polluted by effluents from other mines in the area and as such there was little biota left to harm, and that the volume of the Fal water column allowed sufficient dilution of the release that the over all impact was essentially aesthetic (Younger, 2002).

Tailings dams retain tailings waste dumped underwater in large ponds in order to reduce mineral oxidation. In 1998 a large section of a tailings dam at Las Frailes mine collapsed, releasing up to 7 x 10^6 m^3 acidic sludge into the

Guardiamar River. More silver and lead, and half as much copper and zinc, were released in this single event than the mine produced annually, resulting in mass fish kills along the river (Achterberg *et al.*, 1999). The effects of this are still apparent (Solá *et al.*, 2004).

The pollution caused by mining operations is not necessarily limited to the products of oxidative mineral dissolution. A gold mine near Baia Mare in Romania used large quantities of cyanide in the ore processing procedure to extract and concentrate gold particles, and the resulting tailings still contained high concentrations of free and bound cyanide. In January 2000 a tailings dam breached as a result of large volumes of snow melt water, releasing some 100 x 10^3 m^3 liquid waste, containing 1000 tons of cyanide, into the local river system. This flowed along the Tisa River, into the Danube and eventually into the Black Sea, causing the death of aquatic life and animals living close to the water's edge (Soldán *et al.*, 2001).

1.2 MINING, BIOTECHNOLOGY AND THE ENVIRONMENT

Microorganisms play a key role in the iron and sulfur cycles, and their actions have a fundamental effect on the environment. While these actions are responsible for the majority of pollution emanating mine sites, they can also be harnessed in biotechnological applications. This may be for the purpose of remediation, or to enhance the productivity of mining operations. The necessity for remediation, remedial options and the application of biotechnology in the mining context are described below.

1.2.1 The remediation of mine-impacted environments

The negative impacts of mining-related activities have been acknowledged for thousands of years. The Song Dynasty (1000 BC) recorded the effects on the lungs due to rock crushing, and cases of occupational lead poisoning. Vitruvius, a Roman architect in the last century BC noted the health threats posed by pollution near mines, and Hippocrates described how certain metal rich waters were "*bad for every purpose*". In the 16th century AD, George Agricola described 'Schneeberger' disease among workers at a silver mine in Germany (cited in Davies *et al.*, 2005). This is now known to have been lung cancer caused by exposure to radon gas and uranium metal dust. Indeed, an

early legal case from 1620 brought against the Newbattle Abbey coal workings, near Dalkeith, Scotland complains of "*unwholesome, cankered and infectious*" waters flowing from the mine.

1.2.1.1 The need to remediate

In the U.K. alone, 1000 km of rivers and waterways are thought to be impacted by mine drainage (Kroll *et al.*, 2002) and Younger (2002) has suggest that over 5000 km of waterways in Europe are probably polluted by mine drainage. Almost 10% of the stream reaches in the Northern Appalachians sub region in the U.S.A. were acidic during spring baseflow, due to AMD, with nearly 10,000 km streams affected in this region (Herlihy *et al.*, 1990; Demchak *et al.*, 2004).

The longevity of AMD genesis is one of the key aspects to the problem. Unlike other industries where cessation of operations will lead to a significant reduction in pollution, the reverse is often true for the mining industry. Uncontrolled oxidative dissolution of exposed sulfide minerals will lead to continuing pollution on a time scale often greater than the entire life of the mine. For example, the abandoned Richmond Mine in California, U.S.A. still contains around 8 Mt sulfide minerals. Despite optimal conditions for AMD generation, at an oxidation rate of 20 x 10^6 mol y^{-1}, sulfide oxidation could continue for over 3000 years (Nordstrom and Alpers, 1999). The Richmond Mine is just a single mine: the Iberian Pyritic Belt, which has been exploited by more than 80 mines, is thought still to host 1700 Mt metal sulfides, with 158 x 10^6 m^3 of mine wastes (Sánchez-España *et al.*, 2004). Demchak *et al.* (2004) studied abandoned, but non-flooded underground coal mines in the U.S.A. over 40 years. While the quality of mine drainage had generally improved over this period, all were still discharging acid waters. Roughly 15% of the mines had shown no improvement in water quality at all, while nearly 7% had actually deteriorated.

In the U.S.A., the Clean Water Act (CWA) and the Surface Mine Control and Reclamation Act (SMCRA) were brought in in 1977. These required mine operators to meet certain criteria for effluent discharge during operation and to mitigate the environmental impact of mine effluents and wastes and restore mine sites to their former contours, post closure. However,

mines abandoned prior to 1977 were not covered by these acts. As part of the SMCRA, a tax was levied on coal mining, which was put into a fund to pay for the remediation of abandoned sites. In the European Union, there is much legislation regarding the protection of the environment from most industrial pollution. However, mine drainage is much less coherently regulated and it is often difficult to clearly assign liability (Kroll *et al.*, 2002). For example, prior to 1999 there was no legal requirement to treat mine wastes after closure in the U.K. and many E.U. directives explicitly exclude extraction and mining activities.

Public awareness of mine-related pollution has increased since the 1990's. This has mainly been due to dramatic events such as the Wheal Jane discharge, and the tailings dam collapses in Spain and Romania. While awareness is important, there is the danger that in the knee-jerk demand for legislation to protect the environment, subsequent legislation may be skewed towards preventing accidental releases. This misses the point somewhat, as the majority of environmental damage is done through chronic mine drainage discharges, with the majority emanating from abandoned sites (e.g. Demchak *et al.*, 2004). E.U. Directive 2006/21/EC "management of waste from extractive industries" came in the wake of the Baia Mare disaster, and states that steps must be taken to make sure waste is managed in such as way as to minimise environmental impact. However, it seems mainly to be concerned with accident prevention post-closure, does not cover drainage from mine voids, and it is not clear how it relates to abandoned mines.

However, more legislation is requiring the clean up and protection of the environment generally and clauses exempting extractive industries are being removed. The Environmental Protection Act (1990) in the U.K. requires the clean-up of land posing unacceptable risk to human health or the environment, coupled with a possible 150% tax-relief on expenditure incurred in reclaiming land considered to be contaminated. The main aim of the E.U. Water Framework Directive (WFD) is to achieve good ecological and chemical status of all water catchment areas. Additionally, many of the constituents of AMD may render impacted water unsuitable for municipal uses. In the face of public and legislative pressure, research focus has shifted towards the development of suitable remediative technologies.

Treatment options can either remediate AMD or prevent its initial formation. The two options are not mutually exclusive, and a good pollution control strategy will include both. Preventative measures are aimed at the minimisation of pollutant release from a mine site. Remedial measures are aimed primarily at treating mine waters before they enter the environment, but also at treating areas already affected. In some cases, it may not be necessary to actively intervene at all. This may be due to unusually good quality mine waters, or sufficient dilution by the receiving water-course. Continual monitoring for changes in effluent quality or ecosystem health are all that is necessary in these situations. Such continual monitoring is in place at the former South Crofty tin mine in Cornwall, U.K. (Younger, 2002). Figure 1.2 shows an overview of the options available for the prevention and treatment of AMD.

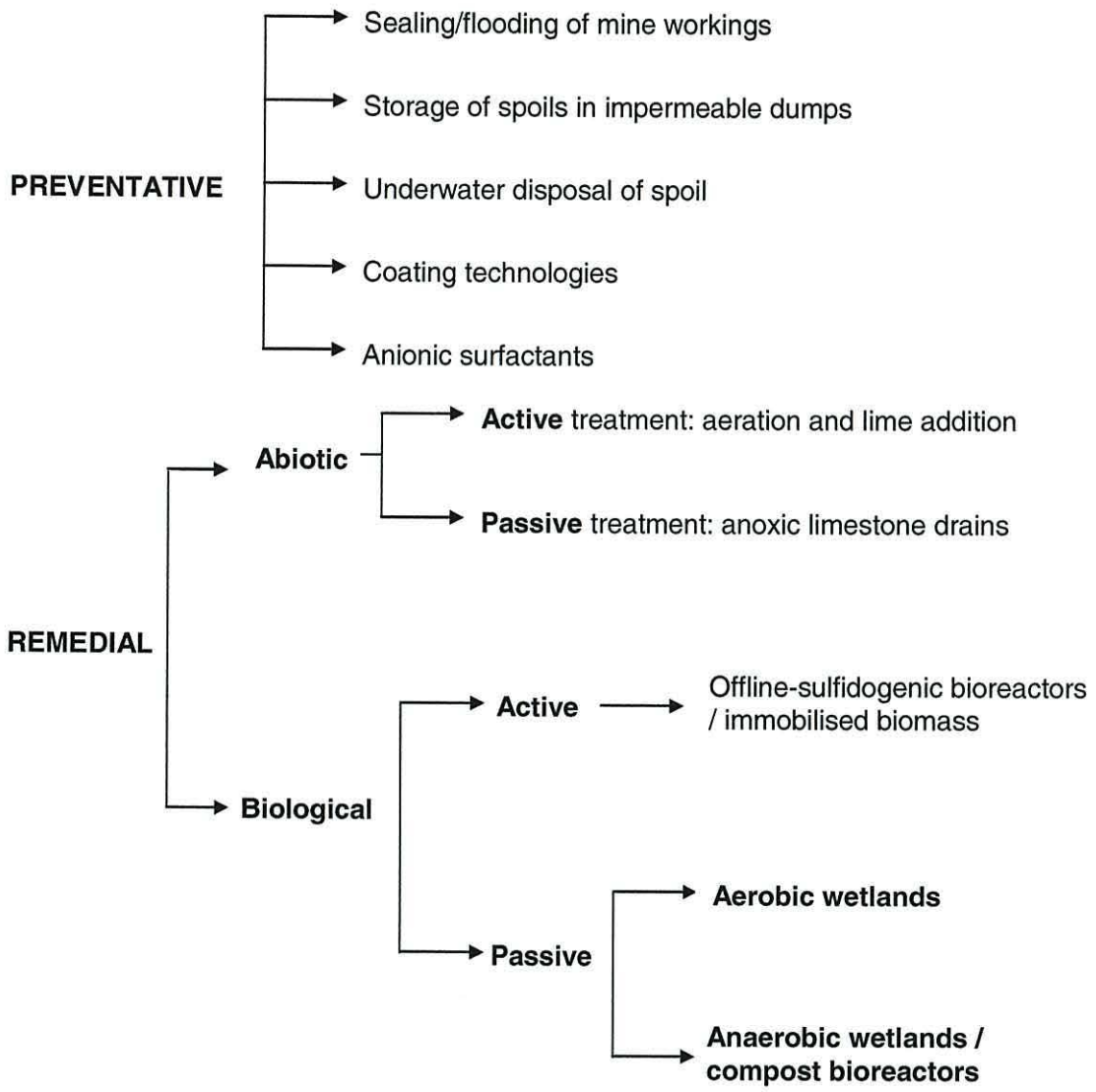

Figure 1.2 Overview of the options available for the prevention and treatment of AMD (modified after Johnson and Hallberg, 2005).

1.2.1.2 Preventative options

Preventative measures usually try to prevent exposed sulfide minerals coming into contact with oxygen and/or moisture, or to reduce the activity of mineral-oxidising microorganisms. Low permeability barriers have been shown to be effective at reducing pollution genesis by limiting water and oxygen influx into waste heaps (e.g. Harries and Ritchie, 1990). Measures aimed at reducing microbial activity have also been evaluated. The use of the detergent sodium dodecyl sulfate (SDS) has been shown to be effective against leaching microorganisms in waste heaps (Schippers *et al.*, 2001). However, this option is costly and it is not thought that the effects last longer than a few months

(Younger 2002). The addition of lime layers to heaps during their construction has been shown to reduce the levels of metal output. By increasing the *p*H, this encourages the precipitation of metals such as iron within the heap. The formation of hardpans due to this precipitation may form a natural barrier to oxygen and moisture, further reducing the output of polluted effluents (Schippers *et al.*, 2001). Preventative measures may be applied retrospectively, often as part of wider regeneration programme of a site that may also include landscaping and revegetation.

These methods may be applicable to mine wastes, and are usually required of the mine operator to safeguard waste piles during and after operation. However, they are inappropriate for mine voids and may not be feasible for waste piles at abandoned sites. Preventative methods for subsurface voids, post-closure, are usually limited to sealing points of water entry into mine networks, or diverting water flow. Where prevention is not a viable, effluent treatment is the only option left to mitigate the detrimental affects of the mine on the ecosystem.

1.2.1.3 Remedial options

Remedial methods can be broadly divided into two types, but they all aim to treat acidity and raise *p*H and remove metals by precipitation as hydroxides, carbonates or sulfides, or by adsorption. Active treatments involve carefully controlled treatment plants, requiring continual process monitoring and control. Passive treatments more often have a biological basis, and require less management once set up than active technologies.

The steps involved in abiotic active treatments include aeration, addition of alkaline materials, such as lime ($Ca(OH)_2$) or caustic soda (NaOH), followed by accelerated sedimentation using a clarifier or lamellar plate thickener with the addition of one or more chemical flocculants or coagulants. Rapid aeration, often involving a system of baffles, promotes the oxidation of soluble metals, which is further accelerated by the addition of alkali. This reduces acidity and raises the *p*H. This leads to the rapid oxidation of ferrous iron, the precipitation of ferric iron and aluminium as hydroxides and the co-precipitation of other metals and metalloids such as arsenic. Following sedimentation the water is discharged, leaving behind a high density sludge.

Biologically-based active treatment systems include the use of sulfidogenic bioreactors or, potentially immobilised biomass (e.g. Kolmert and Johnson, 2001). Sulfidogenic bioreactors rely on microbially produced hydrogen sulfide (H_2S) to precipitate chalcophilic metals. Sulfide produced in a sulfidogenic bioreactor is contacted with the contaminated water in a separate system, and metals are precipitated as insoluble sulfides.

1.2.1.3.1 Active systems

Active systems are the logical treatment of choice in many situations, such as where flow rate is high and where there is limited space for a passive treatment system. A full-scale active system has been installed by the British company "Unipure" to treat the effluent from the Wheal Jane tine mine. This system has a high through-put, treating up to 350 L s^{-1}. Between October 2000 and January 2001 the plant treated 4.4 Mm^3 of AMD, removing 1000 t metal in the process (Younger, 2000). This was at a cost of £20M.

1.2.1.3.2 Passive systems

Not all situations lend themselves to active treatment. This may be for several reasons, such as prohibitive start-up and running costs or site accessibility. Passive systems have several advantages over active systems in that they are relatively cheap to install, operate and maintain and being based on natural processes find favour as 'green' technologies. However, they are more difficult to monitor and usually allow little or no system control. Passive wetland-based systems are complicated and difficult to predict, and require large areas of suitable space. They are sensitive to changes in the environment, often showing seasonal variation in performance (e.g. Mitsch and Wise, 1998). Projecting the sizing and performance requires complex mathematical models, and may never be accurate (e.g. Tarutis Jr. *et al.*, 1999). While the term 'passive' may seem misleading, passive systems have been defined as:

> "...an engineering intervention which prevents, diminishes and/or treats polluted waters at source, using only naturally available energy sources (such as topographical gradient, microbial metabolic energy, photosynthesis and chemical energy), and which

> requires only infrequent (albeit regular) maintenance to operate successfully over its design life."
>
> PIRAMID Consortium, 2003

Passive technologies include anoxic limestone drains (ALD), natural or constructed wetlands, reducing and alkalinity producing systems (RAPS) and permeable-reactive barriers (PRB). A passive remediation system at a mine site will often involve combinations of these.

Aerobic wetlands are designed primarily to treat circum-neutral *p*H mine waters such as effluents from coal mines, as the process of metal oxidation and precipitation is acid-generating. Compost wetlands and compost bioreactors, in contrast, rely on anaerobic bioprocesses, such as sulfate reduction. Sulfidogenic prokaryotes (principally *Bacteria*) reduce sulfate to sulfide which causes the precipitation of many dissolved metals as sulfides, effectively reversing the core process of AMD genesis. As sulfide precipitation is an acid-consuming process, compost systems are usually used to treat acidic waters.

Anoxic limestone drains (ALD) aim to passively add alkalinity to AMD systems. They rely on gravity to draw water through crushed limestone drains, imparting alkalinity and raising *p*H. These are usually used as an initial treatment of AMD at the front end of a passive system.

A combination of these systems was trialled at the pilot passive treatment plant at the Wheal Jane mine. While a passive system was never considered suitable for treating the mine effluent due to the high discharge volume of AMD at the site, a pilot plant was set up as a demonstration and research facility, comprising a series of aerobic cells, subsurface compost bioreactor and a final rock filter (Figure 1.3). Three plants were run in parallel, differing only in the pretreatment of the AMD. Despite not performing as had been hoped, important and invaluable lessons were learnt in terms of both wetland engineering and biological processes (Hallberg and Johnson, 2003a).

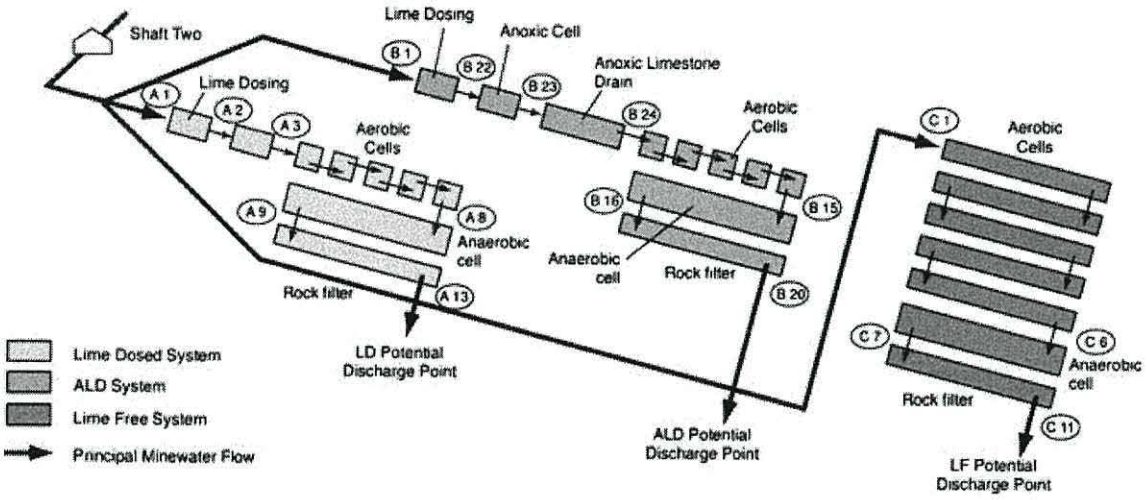

Figure 1.3 Schematic of the Wheal Jane Pilot passive treatment plant illustrating configuration of systems (after Whitehead and Prior, 2005).

Anaerobic compost bioreactors such as reducing and alkalinity producing systems (RAPS) and permeable-reactive barrier (PRB) systems have been developed and successfully implemented. RAPS aim to combine microbial sulfate reduction with chemical alkalinity derived from ALDs. They usually comprise a mixed carbon source such as manure and straw, with limestone clasts, and have been shown to be effective in treating AMD (e.g. Younger *et al.*, 2003). PRBs are similar to RAPS in that they combine mixtures of carbon sources with limestone gravel, but are usually used to treat subsurface plumes. The first U.K. application of PRB technology was in the treatment of a diffuse plume of AMD from a highly pyritic coal spoil heap (Jarvis *et al.*, 2006). The barrier was put in place to intercept the plume, and has been highly effective in reducing metal loading and generating alkalinity. It is worth noting that this system costs less than £5000 per annum to maintain, and only £60k to construct.

The choice of an AMD remediation system depends on many different aspects of the site, including location, local hydrogeology, available space and the nature of the discharge itself, and each site will be different. There must be careful consideration of the biological component of bioremedial systems, and understanding of the engineering limitations. A further problem, which applies equally to biological and non-biological systems, is how to deal with the high density sludge and other waste products of the remediation technology used. In compost-based anaerobic systems, these are potentially

reactive metal sulfide-rich sludges, while in aerobic systems these will be hydroxy- and other insoluble metal compounds. At present, most of these are dumped in landfill sites, but the implication of the 2002 E.U. landfill directive will make this increasingly difficult. The potential remobilisation of metals from compost-based wetland sludges if these materials are exposed to oxygen requires particularly careful handling and storage of these materials. A large problem for arid and semi-arid environments is the salinity of the treated effluents, as no current technology is able to reduce this potential hazard.

1.2.2 Biomining

Biomining is the utilisation of biohydrometallurgy to process metal ores. Biohydrometallurgy is essentially the application of biotechnology to processing minerals. Technically, the process is a branch of hydrometallurgy, but uniquely it involves the use of microorganisms to generate chemical oxidants, such as ferric iron and proton acidity. Biomining can be subdivided into bioleaching and biooxidation operations. Bioleaching involves the solubilisation of an insoluble metal sulfide to a soluble metal, which can be recovered from the leachate. This is most commonly used for the recovery of copper or uranium from low-grade ores. Biooxidation utilises mineral oxidation, but in this case the target metal remains in an insoluble phase. Biooxidation is often used in the pretreatment of gold concentrates, prior to conventional cyanide-extraction.

Biomining has been used, albeit unknowingly, for thousands of years. The Phoenicians and, later, the Romans recovered silver and copper from a ore deposit in the South of Spain, which was later to became the Rio Tinto mine (Rawlings, 2002), and in 166 AD a Greek naturalist and physician described *in situ* leaching of permeable copper ores in Cyprus (Brombacher *et al.*, 1997). In the 16th century, Paracelsus described copper precipitation on iron in a copper-rich water body in Hungary and, following the rediscovery of the early Rio Tinto workings by Francisco de Mendoza, the priest Diego Delgardo described to the King of Spain how iron placed in the Rio Tinto was transformed into copper (Olson *et al.*, 2003; Rawlings 2002). Such descriptions of the apparent conversion of one metal to another would likely have fuelled the alchemists' beliefs that base metals could be converted to

gold. From the19th to the mid-20th centuries, copper was recovered from water percolated through the mine void in purpose-built cementation ponds at the Mynydd Parys copper mines in North Wales, U.K. (Southwood and Bevins, 1995).

However, the modern application of biomining was only initiated in the 1960's with the construction and irrigation of heaps for the recovery of copper at the Kennecott Bingham Canyon Copper mine, Utah, U.S.A. (Brierley and Brierley, 2001). Since the 1980's, there has been a large expansion in the number of heap leaching operations for copper recovery from low-grade ores, with many operations initiated in Chile. Between 1980 and 1998, the amount of the world's copper produced from biomining operations increased from 10% to 25% (Brombacher *at al.*, 1997; Da Silva, 1998).

Biomining was originally seen a means of extracting metals from low-grade ores, tailings and other mine wastes. Initially used for the recovery of copper, since the 1960's it has also been used in the recovery of uranium, and in the mid-1980's was developed for use in the pretreatment of gold-bearing ores (Bosecker, 1997). The most important of these operations are located in developing countries, such as Chile, Indonesia, Mexico and Peru and Zambia. Many developing countries have significant mineral reserves and mining is often one of their main sources of income. Biomining, with its relatively low capital and running costs, is ideally suited to such countries (Acevedo, 2002).

Since the 1992 Earth Summit in Rio, the concept of sustainable technologies and development has become very popular (Brombacher *et al.*, 1997). Biohydrometallurgical extraction procedures find favour in this respect as they are "*almost without exception more environmentally friendly*" than physicochemical processes (Rawlings, 2002). While the environmental costs associated with mineral extraction and primary processing, such as ore crushing and to some extent mineral concentration, are comparable, the process does not require the huge amounts of energy expended during roasting or smelting and does not produce harmful gas emissions. Care must be taken with the resulting leach solution, which contains highly elevated concentrations of soluble metals and acidity, as its release into the environment could have serious consequences. However, in the long term, the waste left over from biological processing may be less chemically active.

The longer that the leaching process is continued, the lower the concentration of reactive sulfide minerals left in the resulting waste. This means that the potential for chronic pollution generation through subsequent microbial weathering is reduced. Many metals can be recovered using biomining microbes including, for example, copper from chalcocite (Cu_2S), nickel from pentlandite ($(FeNi)_9S_8$), zinc from sphalerite (ZnS), lead from galena (PbS) and gold via the dissolution of gold-bearing ores such as arsenopyrite (FeAsS), although not all are commercially processed at this time.

Biomining processes can be broadly divided into two main types: irrigation-types and stirred tank-types. Irrigation-type processes involve the irrigation of crushed rock with a leaching solution, followed by the collection and processing of the leachate or pregnant liquor solution (PLS) to recover the target metals, commonly by a solvent extraction/electro-winning (SX/EW) process. Stirred tank-type processes use continuously operating, highly aerated stirred tank bioreactors.

1.2.2.1 Irrigation-type processes

Irrigation-type processes can involve *in situ*, dump, or heap bioleaching. *In situ* bioleaching describes the process where metals are solubilised and recovered directly from the ore body itself. This process was employed to recover uranium from low-grade ore at the Denison Mine, Ontario, Canada. In this operation, blasted ore in an underground stope was flooded intermittently with AMD, and aerated. The leach liquor was removed after periods of about three weeks and the uranium recovered. During 1988, this process recovered nearly 350 tons of uranium, at a then value of US$ 25M (Rawlings, 2004; Bosecker, 1997). The suitability of such operations is entirely dependent on the local hydrogeology, which must facilitate good leachate recovery without significant loss into the surrounding environment. Loss of leachate to underlying soil, porous rock and groundwater would have a severe environmental impact due to the chemical compositions of these liquors. Dump leaching involves the recovery of metals from dumps of very low-grade mine ores and mine wastes. The Bingham Canyon biomining project in the 1950's is an example of this process; the largest of the dump leaching operations on this site comprised four billion tonnes of low-grade copper

waste (Rawlings, 2002). The Bala Ley plant, owned by Codelco in Chile runs a dump leaching operation, where huge quantities of low-grade copper ore are subjected to cycles of preconditioning, irrigation, rest, conditioning and washing. With each step taking up to a year to complete, a single cycle may run for many years.

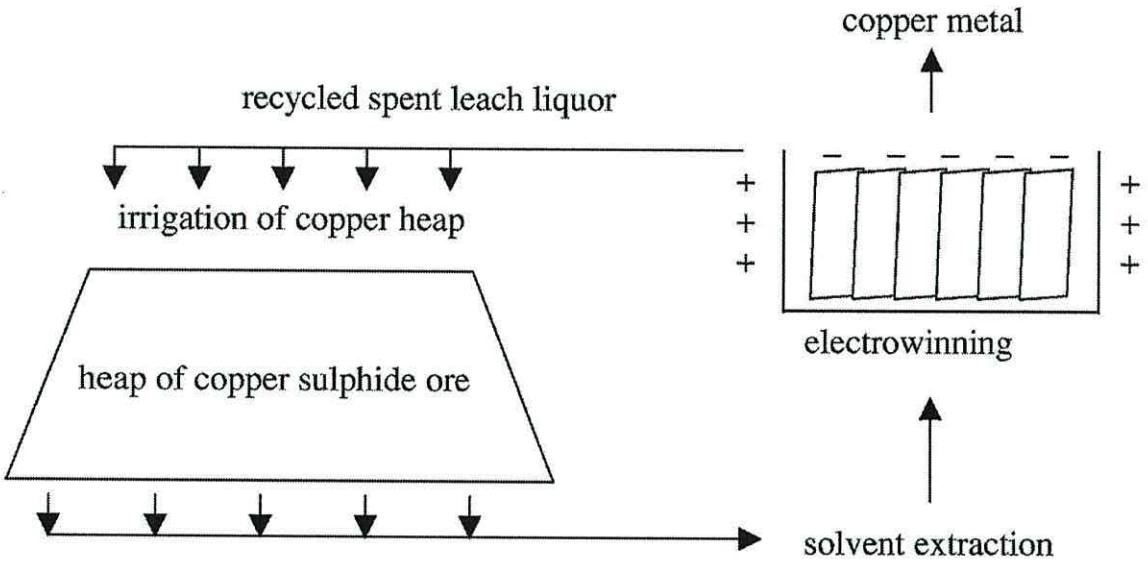

Figure 1.4 Basic schematic of a copper heap leaching operation (after Rawlings, 2002).

Heap leaching (Figure 1.4) is similar to dump leaching, but involves the construction of carefully designed heaps of ore, usually of low-grade, in specially prepared areas. The ore is first crushed and then agglomerated, usually with sulfuric acid before being stacked in heaps up to 10 m high on pads lined with an impermeable barrier, such as a high density polyethylene liner. The design of a heap operation may include aeration pipes, added during construction, to allow forced aeration of the heap. A leaching solution, often the raffinate left following metal extraction from the PLS, is used to irrigate the heap from the top. This may or may not be supplemented with inorganic nutrients and a microbial inoculum. The PLS may be recycled to the top of the heap, as an "intermediate leach solution". The heaps are designed with the optimisation of microbial activity in mind, and leaching efficiency is therefore superior to dump leaching operations. Adjustments can be made to the aeration rate, if forced aeration is employed, which may help to control temperature as well as the availability of oxygen and carbon dioxide. Irrigation

can be controlled in terms of flow rate and composition, in an attempt to ensure that sufficient nutrients are supplied to the microbial population, without saturating the heap (Brierley, 2001). Table 1.1 lists some copper heap leaching operations.

While heap leaching operations are mainly employed for the bioleaching of copper, a heap leaching operation was constructed for the biooxidation of refractory gold ore by the Newmont Mining Corporation at the Gold Quarry Mine in Nevada, U.S.A.. The process utilises a mixture of mesophilic, moderately thermophilic and thermophilic microorganisms, and allows low-grade ore containing as little as 1 g gold t^{-1} to be processed economically (Olson *et al.*, 2003; Rawlings, 2002).

Table 1.1 Some copper heap leaching operations (modified after Rawlings *et al.*, 2003; Domic, 2006).

Plant and Location	Output (Cu tonnage $year^{-1}$)
Cyprus Miami Mining Corp, Miami, U.S.A.	73,000 [a]
Cyprus Sierrita Corporation, Green Valley, U.S.A.	21,800 [a]
Burro Chief Copper Co–Phelps Dodge Tyrone, Tyrone, U.S.A.	74,980 [a]
Burro Chief Copper Co–Phelps Dodge Chino Mines Co., Santa Rita, U.S.A.	66,200 [a]
Silver Bell Mining L.L.C., Marana, AZ, USA	21,000 [a]
BHP Billiton Copper San Manuel, San Manuel, U.S.A.	22,680 [a]
BHP Billiton Copper Miami, Miami, USA	10,400 [a]
Lo Aguirre Mine, Chile	15,000
Arequipa, Peru	60,000 [a]
El Abra, Region II (Calama), Chile	225,000 [a]
Cerro Colorado, Mamina, Chile	130,000
Radimiro Tomic, Region II (Calama), Chile	180,000 [a]
Chuquicamata, Chile	12,500
Collahuasi, Chile	50,000
Dos Amigos, Chile	10,000
Hellenic Copper Mines Ltd., Nicosia, Cyprus	5,000 [a]
Compania Minera Zaldivar, Region II, Chile	131,500 [a]
Girilambone Copper Co., NSW, Australia	17,500 [a]
Mt Cuthbert Copper Co., Queensland, Australia	4,900 [a]

[a] 1998 production

Heap leaching operations are almost exclusively used to treat graded but unprocessed ores. However, GeoBiotics LLC have developed the GEOCOAT™ process, which involves coating inert, support rock with a thin layer of ore concentrate. This process offers much shorter leaching times than standard heap leaching, while avoiding the capital and running cost

associated with stirred tank operations. This process is in use at the Agnes gold mine in South Africa (Harvey and Bath, 2006).

Irrigation-type processes allow only minimal control over reaction conditions within the rock pile. These processes rely on microbial activities to produce the ferric iron lixiviant ultimately responsible for the extraction of the target metal from the ore. This requires an adequate supply of oxygen and carbon dioxide, which is difficult to achieve in a large heap. Internal temperature is difficult to measure and control, and depends on several factors, including heap height, local climate and irrigation and aeration rates. It is also intrinsically linked to the sulfide mineral content of the rock. The higher the sulfide content, the higher the temperature is likely to become. Internal temperatures between 65-80°C are not uncommon (Olson *et al.*, 2003; Esdaile *et al.*, 1999). Conversely, if the sulfide content is too low, the temperature may not be high enough to allow for sufficiently rapid mineral dissolution, rendering the heap uneconomical. The heterogenic nature of heaps, with steep *pH*, nutrient and temperature gradients creating different macro- and microenvironmental conditions adds the unpredictable performance of the heap as a whole (Acevedo, 2000).

1.2.2.2 Stirred tank-type processes

Stirred tank-type operations involve the processing of mineral concentrates in large bioreactors, and offer much more control than irrigation-based operations and therefore allow superior leaching efficiencies in terms of rates and metal recovery. Aeration, temperature and *p*H can be continually adjusted to optimise microbial activity in a stirred-tank reactor. These are usually arranged in series, with a continuous flow of material into the first, which overflows to the next, and so on (Figure 1.5). Retention time in the whole system is set to allow for sufficiently complete microbially-mediated oxidation of the target minerals. The feed usually consists of mineral concentrate, mixed with water to a set pulp density, with a microbial inoculum and additional nutrients. As with other similar forms of biotechnological application (such as bioremediation), stirred-tank reactors usually offer the most effective (though not necessarily the most economic) level of processing.

Most stirred tank operations are employed for the biooxidation of refractory gold ores; the value of the gold produced displacing the higher capital and running costs required for the implementation of these processes. Conventionally, gold is solubilised from ores and concentrates using cyanide. However, gold ores may be refractory due to the presence sulfide minerals, such as with gold-bearing arsenopyrite ores, which may occlude gold particles from the cyanide solution. In such ores, less than 50% of the gold may be recovered without pretreatment. Biooxidation is used to disrupt the sulfide mineral matrix, making the gold accessible to the lixiviant. Total gold recovery can be increased to over 95% through the use of such a biooxidation process. Table 1.2 lists some of the commercial stirred tank biooxidation plants pre-treating refractory gold concentrates.

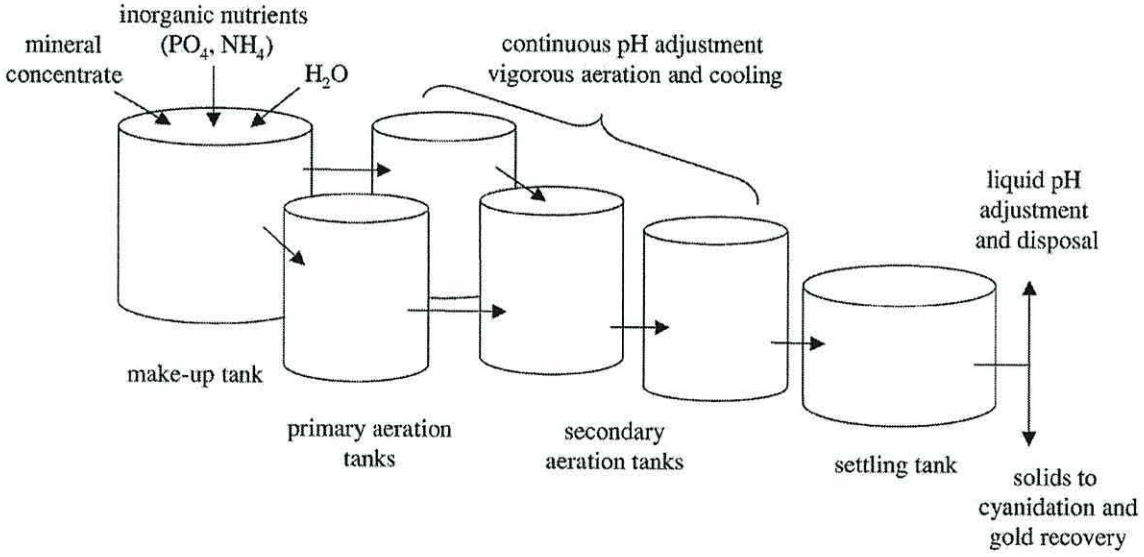

Figure 1.5 Flow diagram of a typical continuous-flow biooxidation facility for the pretreatment of gold-bearing arsenopyrite concentrate (after Rawlings, 2002).

The first biooxidation plant was commissioned in 1986 by Gencor, at the Fairview mine in South Africa. The BIOX® process developed by Gencor, operates at 40-45°C and is used by most stirred tank operations (Rawlings *et al.*, 2003). In contrast, plants utilising BacTech technology operate at moderately thermophilic temperatures between 45 and 55°C. Several more plants have been built, including a biooxidation plant at Sansu, Ghana. Commissioned in 1994, and expanded since, the plant processes 1000

tonnes of gold concentrate per day, and earns nearly half of the country's foreign exchange (Rawlings, 2002).

Table 1.2 Some stirred tank biooxidation plants pretreating refractory gold concentrates (modified after Olson *et al.*, 2003; van Aswegen *et al.*, 2006).

Plant and Location	Size (tonnes concentrate day^{-1})	Technology	Years in operation
Fairview, South Africa	62	BIOX®	1986-present
São Bento, Brazil	150	BIOX®	1990-present
Tamboraque, Peru	60	BIOX®	1990-present
Harbour Lights, Australia	40	BIOX®	1992-1994
Wiluna, Australia	158	BIOX®	1993-present
Youanmi, Australia	120	BacTech	1994-1998
Sansu, Ghana	960	BIOX®	1994-present
Beaconsfield, Australia	70	BacTech	2000-present
Laizhou, China	100	BacTech	2001-present

A commercial stirred tank operation at the Kasese Cobalt Kilembe Mine in Uganda is used to recover cobalt from a 900 Kt dump of cobaltiferous pyritic tailings stockpiled on the site during the mine's operation between 1956 and 1982. The process was developed by the Bureau de Recherches Géologiques et Minières (BRGM), France. The plant processes some 245 tonnes of tailings per day, recovering approximately 92% of the cobalt (Briggs and Millard, 1997; Morin and d'Hugues, 2006).

The BioNIC® process has been commercialised by BHP-Billiton for the extraction of nickel from low-grade ores, and is based on the BIOX process (Brierley and Brierley, 2001). Pilot-scale plants in South Africa and Australia have demonstrated the viability of the process, and Queensland Nickel have decided to proceed with a plant aimed at processing approximately 5,000 tonnes of nickel per year (Palmer and Johnson, 2005).

Biomining using highly aerated, carefully controlled stirred tank bioreactors is highly effective, with mineral decomposition occurring within days rather than weeks or months as with irrigation-type systems. However, due to the level of engineering and process control involved, these are considerably more expensive operations than irrigation-type processes. Efficient aeration is difficult to achieve, and constitutes the largest individual

running cost. Another major constraint of these systems is that only approximately 20% pulp densities can be maintained (Rawlings *et al.*, 2003). At densities greater than this, efficient aeration becomes very difficult, and shear forces due to the motion of the impellers physically damages the mineral-leaching microorganisms, affecting leaching efficiency.

1.2.2.3 The future of biomining

The earliest commercial biomining systems were not designed to specifically promote microbial activity. This has since changed, and greater effort is made to provide optimal leaching conditions for the bioleaching population, and to understand the biological processes therein.

All current commercial plants operate using mesophilic or moderately thermophilic microbial cultures. Ores such as chalcopyrite ($CuFeS_2$) and enargite (Cu_3AsS_4) are resistant to leaching at temperatures less than 50°C, and require temperatures of 70°C or higher for efficient biooxidation (Rawlings, 2002). A high-temperature chalcopyrite bioleaching pilot plant has been in operation at the Chuquicamata Mine, Chile since late 2003. This uses the BioCOP™ process, developed by BHP-Billiton in collaboration with Codelco, and is the world's first commercial demonstration of successful thermophilic bioleaching of primary copper ores (Batty and Rorke, 2005).

Other development projects involving microbial processing of ores include the processing of lateritic ores, particularly nickel; the pretreatment of double refractory gold ores i.e. those ores containing high levels of carbonaceous material, making cyanidation ineffective; and the use of iron-reducing microorganisms to reduce iron ore, producing ferrous hydroxide ($Fe(OH)_2$), meaning less coke is needed during the production of steel (Amankwah *et al.*, 2005; Valix *et al.*, 2001; Brombacher *et al.*, 1997). However, the biggest obstacle to their advancement from pilot-scale studies to commercial operations is likely to be one of economics rather than technical feasibility.

At the first International Biohydrometallurgy Symposium (IBS) in the 1970's, there was great optimism about the application of biohydrometallurgy to the mining industry. It was claimed at the time that this was a technology that could offer a greener, more efficient alternative to high temperature

pyrometallurgical processing of ore and concentrates, and would eventually replace conventional roasting operations. While heap bioleaching is firmly established as a viable, if not vital, method of copper recovery, it occupies a niche in the mining industry, allowing the processing of whole ores that could not be processed economically any other way. The use of bioreactors for treating copper concentrates can not compete with smelting due to the fact that the current price of copper does not often justify the building of new bioleaching plants, given that smelting is economically viable and that existing smelting plants are able to handle much greater capacity than they do currently (Crundwell, 2005). While biomining can fulfil certain roles extremely competently, it remains a niche technology and often cannot compete on a purely economic basis with pyrometallurgy and pressure leaching.

However, given the severe ecological impact of AMD from unmanaged, chemically active mine wastes, and in the context of increasingly stringent legislation, as described previously in sections 1.1.3-1.2.1, the future of biomining may be less to do with what the mining industry would like, and more with what it will have to do. While various remediation schemes may be effective, they can be expensive, unpredictable and often simply transfer the problem from one place to another, leaving a toxic waste that must still be dealt with. Therefore, it could be more pragmatic to remove the problem; to remove or reduce the potential for mine wastes to generate AMD. More importantly, as deposits of accessible metal ores become more scarce, and in the face of an ever-growing human population, it is going to be increasingly necessary to consider alternative sources for metal production.

The reprocessing of mine wastes is one area where biomining may play a crucial role. By accelerating the microbial decomposition of sulfidic mineral wastes, drainage can be intercepted as a contained PLS rather than released as environmentally damaging AMD. Such an operation has been undertaken at the Magma Copper Company's Pinto Valley facility, in Arizona, U.S.A.. Between 1989 and 1997 a 38 Mt tailings dump, deposited in the early 20^{th} century, was reprocessed using a tank bioleaching operation. The resultant waste was backfilled into the open cast mine void, and the amount of copper recovered led to an overall financial profit (U.S. Environmental Protection Agency, 1993). The most recent example of this is the

reprocessing of cobaltiferous tailings at the Kasese Cobalt Kilembe Mine in Uganda, as discussed in section 1.2.2.2, which is also economically viable.

The implementation of processes such as these, and the increased necessity to process minerals as efficiently as possible in the first instance along with the implementation of other remedial systems, could ensure that mines not only make the best use of the world's finite mineral resources, but also protect its equally valuable natural environment; and improve a somewhat tarnished public perception of the industry as a whole.

Table 1.3 Advancements in biomining applications based on microbiology (after Brierley, 2001).

Microbiological advancement	Potential benefit to biomining
Employ microorganisms that initiate oxidation at higher *p*H values and condition ore for conventional leaching microorganisms	Allow effective heap-leaching of high *p*H ores, expanding the types and numbers of ores amenable to leaching
Understand the adaptation or succession of leaching microorganisms to the changing character of leach solutions, high TDS situations, and possible synergistic toxic effects of multiple ions	Allow use of poor quality water for make-up of the leach solutions and closed-circuit operation of heaps, reducing capital and operating costs.
Understand the succession of thermophiles in heaps and bioreactors, interpret their function and define their effects on minerals, precipitates and reagent consumption	Extend biomining to more refractory ores; potentially enhance metal recoveries and decrease leach time, reducing costs and improving overall process economics; refine development of leaching of mineral concentrates
Select for and employ chloride tolerant iron-oxidising microbes	Expand geographical regions for heap operations by using brackish waters or sea water for leaching
Describe heterotrophic and chemolithotrophic microflora in heaps as related to nutrients and function; ascertain role, if any, in heterotrophic degradation of entrained organics in raffinate	Define conditions that could optimise performance, reducing cost

To further development in this area, greater understanding of the microbiology of biomining is needed. Microbial investigations of abandoned spoils and current biomining processes are considered necessary in order to isolate and characterise novel microorganisms with superior mineral leaching capabilities. Sought after characteristics include not only growth at elevated temperatures, but also increased tolerance to metals (such as silver), growth

in brackish and saline waters, and resistance to shear. It will therefore be necessary both to improve our ability to detect such microorganisms in the environment, and to cultivate them in the laboratory. Table 1.3 summarises how better microbiological understanding could advance biomining technology and application.

1.3 TECHNIQUES FOR DETECTING AND IDENTIFYING ACIDOPHILES

The study of acidophilic microorganisms has traditionally focused on natural extreme environments, such as acidic, sometimes thermal, springs and other water bodies, or man-made environments, such as active biomining operations and AMD waters. This may involve either classical culture techniques and biomolecular methods, or both.

Liquid media can be used to enrich populations of target microorganisms, allowing the analysis of environments with very low cell numbers. Media may be varied in terms of *p*H, energy and carbon source, oxygen status and incubation temperature. However, the use of enrichment media will impart bias on the microbial population, by favouring the growth of certain microorganisms or groups of microorganisms (Johnson and Hallberg, 2006).

The use of solid media to enumerate cultivatable acidophiles may also allow isolates to be differentiated on the basis of differences in colony morphologies. However, several difficulties are apparent in the culturing of chemolithotrophic acidophiles on solid media. Many of these prokaryotes are acutely sensitive to dissolved organic compounds, such as organic acids. Even if organic compounds are not added to media formulations, they occur as impurities in gelling agents (such as agar and agarose); pyruvate and mono/oligosaccharides also arise from hydrolysis of the polysaccharidic gels at the necessarily low *p*H of the solid media (Johnson, 1995). A major breakthrough in solid media development came with the advent of "overlay" plates. It had been noticed that obligate chemolithotrophs could be found growing in close proximity to heterotrophic organisms such as *Acidiphilium* spp. on standard agar plates, and it was thought that these heterotrophs were detoxifying the surrounding agar as they grew on the organic compounds present. Overlay plates consist of a bottom layer which has been mixed with

actively-growing heterotrophic bacteria, and a sterile top layer. As they grow, the heterotrophs metabolise small molecular weight organic materials produced by acid hydrolysis of the agarose, detoxifying the media and facilitating growth of the "hyper-sensitive" iron- and or sulfur-oxidising acidophiles, many of which were previously considered to be unculturable on solid media (Johnson, 1995).

Advances in molecular biological tools have transformed the field of microbial ecology, revealing a remarkable diversity of microorganisms in many environments (Hugenholtz *et al.*, 1998). Nucleic acid-based assays offer advantages over classical methods in so far as it is possible to study the microbial population regardless of culturability. This has proved invaluable in the detection of novel microorganisms.

The most common methods are based on analysis of the 16S rRNA gene amplified from community DNA extracts, using the polymerase chain reaction (PCR). The construction and analysis of clone libraries allows the identification of individual 16S rRNA gene sequences, and the examination of their phylogenetic relationships. The use of large enough clone libraries can enable identification of dominant microorganisms while the limit of detection is often that of the PCR itself. Molecular profiling methods, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), allow the rapid generation of community profiles. In DGGE, a portion of the 16S rRNA gene containing the highly variable regions is amplified using PCR primers that contain a "GC clamp" (Muyzer *et al.*, 1996), and resolved by denaturing gel electrophoresis. The products migrate through the gel to the point at which they are partially denatured, which is sequence-dependent. Therefore, genes with different sequences will separate from each other, producing a banding pattern. Bands can be assigned to particular organisms by band extraction and gene sequencing, and relative abundance can be inferred from the band intensity. T-RFLP utilises fluorescently-labelled PCR primers to produce a mixture of labelled 16S rRNA gene amplification products (Marsh, 1999). These products are digested using a single restriction enzyme, and the terminal restriction fragments (T-RFs) resolved by capillary gel electrophoresis. Analysis of the migration time of each T-RF, and its relative intensity produces an output consisting of peaks of

varying size and height. Each peak can be assigned to a single organism or groups of organisms, while their abundance in the original sample can be inferred from the peak height.

Another commonly used nucleic acid-based tool is fluorescent *in situ* hybridisation (FISH), a technique that depends neither on cultivation of target microorganisms nor DNA extraction and gene amplification (Amann *et al.*, 1990). This method uses fluorescently-labelled oligonucleotides to probe whole cells. These probes are designed to be specific to certain groups (ranging from domains to species) of microorganisms, and allow quantitative analysis of microbial populations. As these probes bind only to rRNA molecules, they detect only metabolically active cells. Developments in this area have come from improving existing rRNA probes and designing new ones. For example, Bond and Banfield (2001) designed seven new probes for the detection of important microbial genera found in acid mine drainage environments, based on a large amount of 16S sequence data that they had amassed.

An alternative method for molecular profiling to nucleic acid-based assays is phospholipid fatty acid (PLFA) analysis. In this process, fatty acids in the membranes of microbial cells are extracted and methylated. The resultant fatty acid methyl esters (FAMEs) are analysed and identified (Vestal and White, 1989). Individual FAMEs can be assigned to specific organisms or groups of organisms, and their relative abundances can be calculated. This produces community profiles similar in character to those obtained using T-RFLP. This has been used to examine microbial populations in AMD environments and biomining operations, and can also be used to provide an estimate of total biomass (e.g. Ben-David *et al.*, 2004; Kaksonen *et al.*, 2004; Plumb *et al.*, 2006).

The use of biomolecular methods can give a more complete picture of the broader community in a sample, and identify dominant organisms. However, it is not often possible to determine the metabolic capabilities of unknown microorganisms detected. Therefore it is useful to combine biomolecular methods with culture-based, classical techniques, as summarised in Figure 1.6. This provides information about population

complexity and biodiversity, while allowing the isolation and further study of culturable indigenous microorganisms.

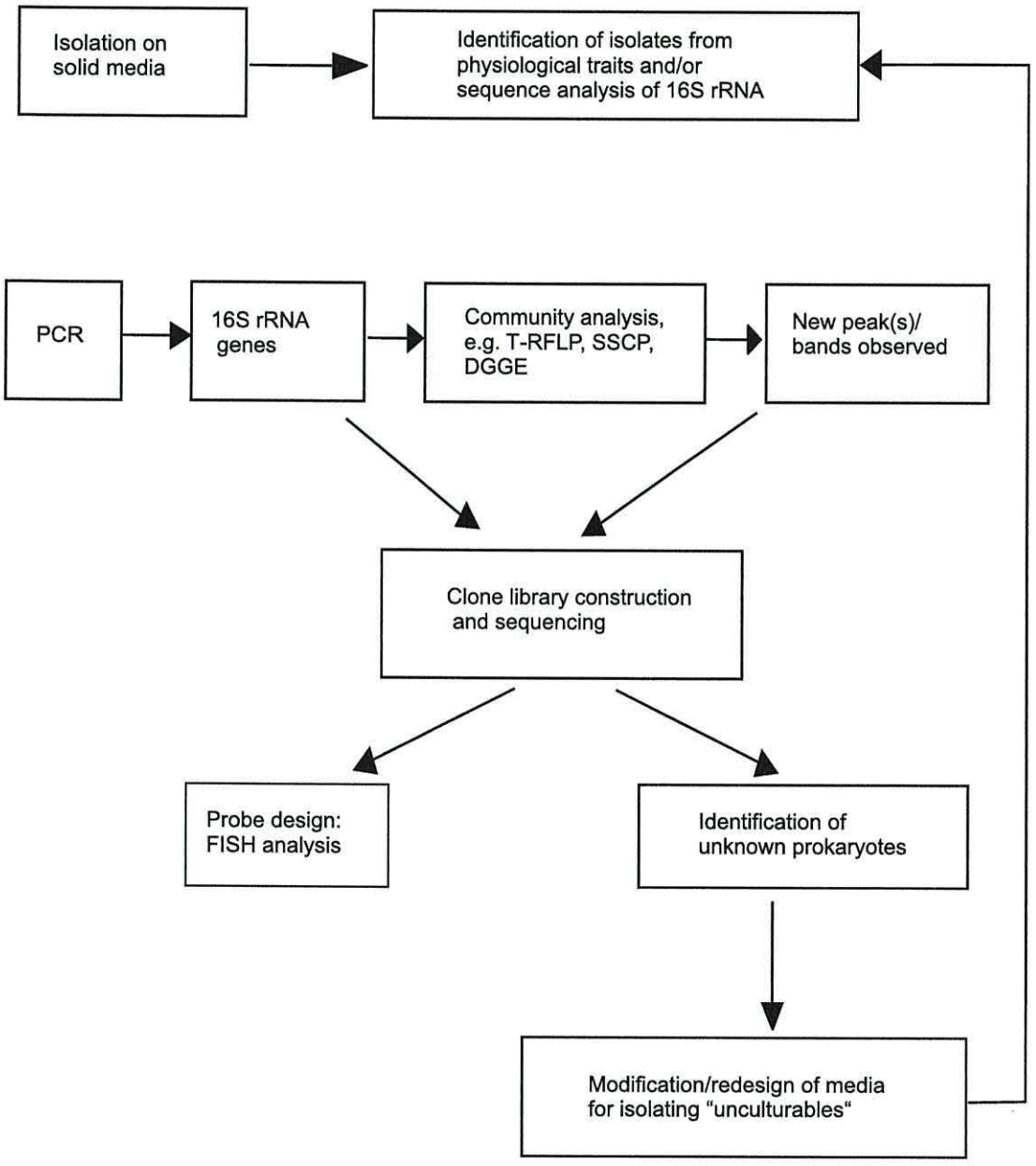

Figure 1.6 Scheme for analysis of acidophilic communities using the dual approach of cultivation and molecular microbial ecology. Enrichment cultures may be used ahead of plate isolation or PCR-based analyses, though the resulting diversity of microorganisms is likely to be greatly diminished, compared to non-enriched samples (after Johnson and Hallberg, 2006).

1.4 THE BIOLOGY OF ACID MINE ENVIRONMENTS

1.4.1 Metabolic diversity

There is a vast array of metabolic pathways utilised by the many different microorganisms that may be found in acidic, mine-impacted environments. The interactions between these organisms are complex and not fully understood. Key aspects of biological metabolism in acidic environments are described below.

1.4.1.1 Carbon sources

Essential to the growth and development of all life is a source of carbon. Heterotrophic acidophiles mostly use simple monomeric substances such as sugars and alcohols as carbon and energy sources. Autotrophs assimilate inorganic carbon such as CO_2. Such fixation requires energy derived from either sunlight (photoautotrophs) or chemical (chemoautotrophs) sources. Mixotrophic organisms have been defined as prokaryotes that use either organic or inorganic carbon as carbon source, but also as those that use organic carbon as a carbon source and gain their energy from inorganic electron donors (Johnson and Hallberg, 2006).

The availability of organic carbon is a major limiting factor for growth in many acidic environments, and so autotrophy is often the primary source of fixed carbon. While photoautotrophs such as microalgae may provide important photosynthetic sources of carbon in acid mine environments exposed to sunlight, in many acidic environments carbon fixation is thought to be primarily a prerequisite of chemoautotrophic microorganisms. Autotrophic primary producers provide organic carbon sources for heterotrophic organisms via cellular exudates, or following cell death. Heterotrophic organisms may also obtain organic carbon from other sources, such as wooden pit props and other structures (see Jenkins *et al.*, 2000) or from the detritus or decay of higher organisms that may have entered the site.

1.4.1.2 Iron metabolism

Ferrous iron provides a major source of energy in many acid mine environments, the microbial oxidation of which has been described in section

1.1.2. The ferrous/ferric iron redox couple has a standard redox potential at *p*H 2 of +770 mV which is only slightly less then that of the oxygen/dihydrogen oxide (water) couple (+820 mV). Therefore, the energy associated with ferrous iron oxidation is relatively small (-30 kJ mol^{-1} at 30°C), and large amounts of ferrous iron must be oxidised to support the growth of iron-oxidisers. However, this is often compensated for by the abundance of ferrous iron in these environments.

Due to its similarity with the O_2/H_2O redox couple, and its enhanced solubility in low *p*H liquors, ferric iron is a thermodynamically attractive alternative electron acceptor to oxygen. Some acidophiles have been shown to reduce ferric iron minerals, such as jarosites (e.g. $KFe_3(SO_4)2(OH)_6$) and schwertmannite ($Fe_{16}O_{16}(OH)_{12}(SO_4)_2$) (Bridge and Johnson, 2000; Coupland, 2005). It has been suggested that ferric iron reduction may have been the first globally significant mechanism for oxidation of carbon in Earth's history, prior to the wide abundance of oxygen (Lovley, 1991).

1.4.1.3 Sulfur metabolism

The oxidation of reduced sulfur compounds (section 1.1.2) provides more electrons per mole of substrate than that of ferrous iron. This would appear to make these compounds more attractive electron donors than ferrous iron, though the oxidation of reduced sulfur often generates significant acidity, which ultimately may affect cell viability, especially in closed systems. In mine-impacted environments, sulfur-oxidising microorganisms rely largely on the activities of iron-oxidisers to provide a source of reduced sulfur via the oxidative dissolution of sulfide minerals. While oxidation is by far the most commonly observed sulfur metabolism in acidic environments, certain prokaryotes, such as *Acidianus infernus*, a thermoacidophilic archaeon, are able to reduce elemental sulfur (e.g. Bonch-Osmolovskaya, 1994). Sulfate-reducing prokaryotes (SRP) are capable of reducing sulfate under anaerobic conditions, utilising simple carbon compounds or hydrogen as energy sources. However, all characterised SRP are inhibited by low *p*H, although acid-tolerant/acidophilic SRP have been isolated from acidic (*p*H 3.0-4.0) environments (Sen and Johnson, 1999). While sulfate reduction and concomitant removal of soluble metals as insoluble metal sulfides has been

demonstrated at low (3.0-4.0) *p*H (Kimura *et al.*, 2006; Johnson *et al.*, 2006a), systems treating acidic mine waters may often operate at neutral *p*H due to the alkalinity produced during biosulfidogenesis (e.g. Kaksonen *et al.*, 2004).

1.4.2 Biodiversity

Examples of acidophilic and acid-tolerant organisms are disseminated throughout all three kingdoms of life: the *Eukarya*, the *Bacteria* and the *Archaea*. Acidophiles have been categorised as organisms that grow optimally at *p*H values of 5.0 or less. Extreme acidophiles grow optimally below *p*H 3.0 while moderate acidophiles grow optimally between *p*H 3.0 and 5.0 (Johnson and Hallberg, 2006). Biodiversity is limited in acid mine environments, possibly due to the small number of metabolically beneficial reactions available (Baker and Banfield, 2003), though at least 11 prokaryotic phyla are known to have representatives which thrive in these environments.

Examples from the *Eukarya* are more often acid-tolerant microorganisms rather than true acidophiles. Macrobiological examples found at acid mine environments include acid-tolerant grasses and plants such as *Calluna spp* (Jenkins *et al.*, 2000). The acidic streams found running off mine sites (*p*H often <2.5) are often populated with unicellular algae such as *Euglena mutabilis*, yeasts, fungi and obligately acidophilic protozoa. Rotifers such as *Elosa worallii* and *Brachionus sericus* may also be isolated from sites with *p*H close to 3.0 (Deneke, 2000). Recent studies of the Rio Tinto in Spain have found that eukaryotes account for at least 60% of the biomass in the biome, and were more diverse than the prokaryotes (Zettler *et al.*, 2002; Zettler *et al.*, 2003). It is not thought that these organisms play a direct role in mineral dissolution, though they may be important in providing organic sources of carbon to the system, and fungal hyphae may also provide support structures for microbial "streamers" (Zettler *et al.*, 2002).

1.4.2.1 AMD-impacted environments

There has been a considerable amount of research published on the microbiology of acid mine drainage (AMD), and environments impacted by AMD, such as wetlands and receiving streams and rivers. The most commonly reported, and therefore the most well studied, organisms

associated with AMD are the acidithiobacilli and leptospirilla. A wide variety of microorganisms such as *Leptospirillum* spp., the *Thermoplasmales*, *Acidimicrobium* spp, "*Ferrimicrobium acidophilum*", uncultured *Deltaproteobacteria* and novel organisms such as the autotrophic iron-oxidising betaproteobacterium PSTR have been reported as dominating macroscopic structures within AMD (Bond *et al.*, 2000; Hallberg *et al.*, 2006). In addition, a number of moderate acidophiles, such as *Acidobacterium* spp., *Acidiphilium* spp., *Thiomonas* spp., *Propionibacterium* spp., and novel *Bacteria* such as the iron-oxidising gammaproteobacterium WJ2, have been reported in wetland environments receiving AMD (Brofft *et al.*, 2002; Hallberg and Johnson, 2003b; Hallberg and Johnson, 2005). Table 1.4 summarises the acidophilic microorganisms that have been identified in acidic mine-impacted environments.

1.4.2.2 Biomining operations: (i) Stirred tank systems

Due to the constant process monitoring and control, stirred tank biomining operations offer relatively constant and homogeneous conditions for the growth of acidophiles. As a result, populations tend to be a stable mixture of a limited number of physiologically- and phylogenetically-distinct prokaryotes, which generally includes a sulfur-oxidiser, an iron-oxidiser and a mixotrophic or heterotrophic acidophile (Norris, 2006; Okibe *et al.*, 2003).

Most commercial stirred tank systems use the BIOX process, which operates at 40-45°C. These operations tend to be dominated by the iron-oxidising *L. ferriphilum* and the sulfur-oxidising *At. caldus* (Rawlings, 2006). Analysis of a pilot-scale polymetallic sulfide stirred tank operation running at ~45°C found that the primary tank was dominated by *L. ferriphilum*, *At. caldus* and *Sulfobacillus* spp. However, in the secondary and tertiary tanks, the iron-oxidising heterotroph *Ferroplasma acidiphilum* became increasingly dominant, replacing *L. ferriphilum* completely in the final tank. Such 'heterotrophically-inclined' iron-oxidisers may tend to become increasingly dominant in secondary and tertiary reactors due to the increased availability organic carbon as a result of primary production in the initial tanks (Norris, 2006).

Foucher *et al.* (2001) reported that the culture used to inoculate the Kasese stirred tank system (described in section 1.2) mainly comprised *L.*

ferrooxidans, *At. caldus* and a *Sulfobacillus* sp.. However, the microbial population of the commercially operating system is known to be dominated by *L. ferriphilum*, *At. caldus* and a distinct *Sulfobacillus* sp., with low levels of a *Ferroplasma* sp. (Johnson, unpublished).

Improved copper extraction from chalcopyrite ore has been demonstrated in a pilot-scale stirred tank system operating at over 78°C (Batty and Rorke, 2005). At such temperatures, the microbial populations become dominated by extremely thermophilic *Archaea*, such as *Metallosphaera* spp. and *Sulfolobus* spp. (Norris, 2006).

A moderately thermophilic *Ferroplasma* sp. was found in an arsenopyrite/pyrite bioreactor when the *p*H control was removed from the system, and the operating *p*H decreased to 0.5 (Rawlings, 2002), demonstrating the importance of the *Archaea* in the most extreme conditions.

1.4.2.3 Biomining operations: (ii) Irrigation-based systems

A variety of temporal and spatial gradients, such as *p*H, temperature, O_2, CO_2 and moisture, exist within irrigation-type operations. This favours colonisation by different microorganisms in different niches, and the heterogeneity of mineral heaps means that obtaining representative samples for microbial analysis is a far more complex issue. The majority of published studies have examined the pregnant leach solution PLS draining the heaps, or have used enrichment cultures which themselves impart bias on the observed population (e.g. Goebel and Stackebrandt, 1995; Kinnunen and Puhakka, 2004). There are few descriptions in the published literature of direct approaches to study the microbiology of the mineral phase of irrigation-type operations.

The *p*H in the heaps may well be more variable than in stirred tanks, and so conditions may be similar to AMD environments. However, due to the exothermic nature of sulfide mineral oxidation, internal temperatures may be high, temperatures between 65-80°C are not uncommon (Olson *et al.*, 2003; Esdaile *et al.*, 1999), favouring the growth of moderate or extreme thermophiles (Brierley, 2001). Heterotrophic organisms may also be more important than in stirred tank systems. Goebel and Stackebrandt (1995) identified *At. ferrooxidans*, *At. thiooxidans* and *Acidiphilium cryptum* in a chalcopyrite overburden heap in Australia. Similar organisms have been

tentatively identified at the Kennecott Bingham Canyon copper ore bioleach heap in Utah, U.S.A., along with *Leptospirillum* spp and "*Ferrimicrobium acidiphilum*" (Bruhn *et al.*, 1999). Other organisms reported from copper ore heap leaching operations include *At. caldus*, *Ferroplasma* spp. and novel "*Firmicutes*" and *Crenarchaeota* (Demargasso *et al.*, 2005; Hawkes *et al.*, 2005).

1.4.2.4 Mine wastes

There is a paucity of data available on the microbial ecology of mine wastes, such as spoil and tailings. As with irrigation-type operations, variations in different conditions will create a highly heterogeneous environment. Older materials may present a higher *p*H, due to the depletion of sulfide minerals, which may favour moderate acidophiles such as *Thiomonas* spp.. Shippers *et al.* (1995) studied the microbial populations of uranium mine waste, and found the population was dominated by *At. ferrooxidans* and *At. thiooxidans*, but also detected some nitrate-reducing anaerobes. However, these heaps contained up to 7% pyrite, and were still chemically active, with internal temperatures reaching as high as 100°C in some places. Goebel and Stackebrandt (1995) studied the effluent draining a chalcopyrite heap, and found the water to be dominated by *A. cryptum*, *At. ferrooxidans* and *L. ferrooxidans*. Bruneel *et al.* (2005) detected *Thiomonas* spp., *At. ferrooxidans* and *Desulfosarcina* spp. in an aquifer contaminated with water draining a tailings impoundment. However, it is not clear how these populations relate to those of the solid materials themselves. Similarly, Kinnunen and Puhakka (2004) used enrichment cultures to study a self-heating copper mine waste heap in Indonesia. They found the population to be quite simple, dominated by "*Sulfobacillus yellowstonensis*" and *S. acidophilus*. However, this may represent the response of a more varied population to the enrichment conditions (50°C, *p*H 1.8). *Leptospirillum* spp. and *Sulfobacillus* spp. were found to dominate the oxidation front of a tailings impoundment in Chile (Diaby *et al.*, 2006). *At. ferrooxidans*, heterotrophs such as *Acidobacterium* spp. and sulfate-reducing prokaryotes were also detected.

Table 1.4 Acidophilic prokaryotic microorganisms (modified after Johnson and Hallberg, 2003).

Mineral-degrading acidophiles	Thermal classification[a]	Phylum; Class[b]
Iron-oxidisers		
Leptospirillum ferrooxidans	Meso	
L. ferriphilum	Mod Thermo	*Nitrospira*; *Nitrospirales*
L. ferrodiazotrophum		
"*Thiobacillus ferrooxidans*" m-1		*Betaproteobacteria*[c]
"Ferrimicrobium acidiphilum"	Meso	*Actinobacteria*; *Acidimicrobiales*
Ferroplasma acidiphilum		*Euryarchaeota*; *Thermoplasmata*
"Fp. acidarmanus"		
Sulfur-oxidisers		
Acidithiobacillus thiooxidans	Meso	*Proteobacteria*; "*Beta/Gammaproteobacteria*"[d]
At. caldus	Mod Thermo	
Thiomonas cuprina	Meso	*Proteobacteria*; *Betaproteobacteria*
Hydrogenobacter acidophilus	Ext Thermo	*Aquificae*; *Aquificae*[e]
Metallosphaera spp.		*Crenarchaeota*; *Thermoprotei*
Sulfolobus spp.		
Iron- and sulfur-oxidisers		
Acidianus spp.	Ext Thermo	*Crenarchaeota*; *Thermoprotei*
Sulfolobus metallicus		
Iron-reducers		
Acidiphilium spp.	Meso	*Proteobacteria*; *Alphaproteobacteria*
Acidocella spp.		
Acidobacterium spp.		"*Acidobacteria*"; *Acidobacteria*
Iron-oxidisers/reducers		
Acidimicrobium ferrooxidans	Meso	*Actinobacteria*; *Acidimicrobiales*
Iron-oxidisers/reducers and sulfur-oxidisers		
At. ferrooxidans	Meso	*Proteobacteria*; "*Beta/Gammaproteobacteria*"[d]
Sulfobacillus spp.	Meso and Mod Thermo	"*Firmicutes*"; *Bacilli*
Heterotrophic acidophiles		
Acidisphaera rubrifaciens	Meso	*Proteobacteria*; *Alphaproteobacteria*
Acidomonas methanolica		
Alicyclobacillus spp.		"*Firmicutes*"; *Bacilli*
Picrophilus spp.	Mod Thermo	*Crenarchaeota*; *Thermoprotei*
Thermoplasma spp.		
Obligate anaerobes		
Stygiolobus azoricus	Ext Thermo	*Crenarchaeota*; *Thermoprotei*
Acidilobus aceticus		

[a]Meso, mesophiles ($T_{optimum}$ < 40°C); Mod Thermo, moderate thermophiles ($T_{optimum}$ 40-60°C); Ext Thermo, extreme thermophiles ($T_{optimum}$ >60°C); [b]Classification according to Bergey's Manual of Systematic Bacteriology, Second Edition (Garrity *et al.*, 2004); [c]Stated in Johnson and Hallberg, 2003; [d]Technically members of the *Gammaproteobacteria*; [e]Inferred ability to oxidise minerals (via production of sulfuric acid).

1.5 SCOPE AND OBJECTIVES OF THE CURRENT PROJECT

The current project is concerned with gaining a better understanding of the microbial ecology of mine wastes and tailings of different ages, mineralogies and geographical locations. Microbial succession in mine wastes has implications for biomining operations, but is also important in terms of risk management. To assess the potential for AMD genesis in a waste heap and the potential longevity of this process it is necessary to understand how mineral-oxidising microbial populations change over time from first deposition.

There are relatively few published reports concerning the microbial populations of mine waste heaps and only marginally more describing those in biomining operations. Therefore, it is difficult to construct a well defined hypothesis with regard to the microbial populations that may be encountered in these situations. As the wastes age, the concentrations of sulfide minerals will, in many cases, become increasingly depleted in moist, aerobic zones, reducing the potential for acidogenesis. Therefore, it is likely that these environments will be less acidic than active biomining operations and so probably favour the growth of moderate, rather than extreme, acidophiles. The oldest heaps may be the most depleted in sulfide minerals, and may have evolved to the point where mineral-oxidising populations are essentially absent. In this case, the microbial populations would have to rely on alternate sources of energy for growth. Conversely, sulfide mineral concentrations should be highest in the fresher deposits and therefore might well present microbial populations with high relative abundances of mineral-oxidising organisms.

Geochemical data, such as *p*H and extractable concentrations of metals can indicate the potential for AMD genesis at a particular site. Low *p*H and high concentrations of readily extractable metals may imply the presence of a mineral-oxidising population. The isolation of iron- and/or sulfur-oxidising microorganisms, or the biomolecular detection of known mineral-oxidisers would confirm this.

The major objectives of the project were therefore:

- to identify a range of accessible sites that varied in terms of mineralogy, age, climate and geographic location;
- to ascertain whether the sites were still active in terms of sulfide mineral oxidation;
- to determine the biodiversity of metal-mobilising and other acidophilic microorganisms in spoil and tailings samples, using a combined cultivation-based and cultivation-independent approach;
- to examine the possibilities of accelerating sulfide mineral dissolution in spoil and tailings samples by stimulating indigenous microflora;
- to construct a working hypothesis on the evolution of acidophilic microbial populations in abandoned mine sites.

CHAPTER 2: MATERIALS AND METHODS

This chapter describes the materials and methods that were used routinely throughout the research project. Modifications or additions to these are noted in the relevant chapters. All chemicals used in this study were supplied by Sigma-Aldrich (U.S.A.), Fisher Scientific (U.S.A.), or Merck-BDH (now VWR) (U.S.A.) and were of analytical reagent grade, unless stated otherwise. Good Laboratory Practice was followed throughout, and aseptic techniques were used where appropriate.

2.1 MICROBIAL CULTIVATION-BASED TECHNIQUES

Liquid and solid media were used to cultivate extremely acidophilic (*p*H <3), moderately acidophilic (*p*H 3-6) and neutrophilic (*p*H ~7) microorganisms. Culture incubation was carried out aerobically at 20°C, 30°C or 45°C, and generally involved shaking in the case of liquid cultures. All media were heat-sterilised by autoclaving for 20 min at 121°C or filter-sterilised through sterile 0.2 µm cellulose-nitrate membranes (Whatman, U.K.). All water used for cultivation purposes was reverse osmosis (RO) grade (RiOs™, Millipore, U.K.). Yeast extract (YE), tryptone soya broth (TSB) and R2 agar (R2A) are all Oxoid (U.K.) reagents.

2.1.1 Basal salts, trace elements, ferrous sulfate and potassium tetrathionate stock solutions

The majority of liquid and solid media contained one of three basal salts formulations (Table 2.1) and a trace elements solution (TE).

The trace element solution was prepared as a 1000 x concentrate. This contained the following (g L^{-1}): $ZnSO_4 \cdot 7H_20$ (10.0); $CuSO_4 \cdot 5H_2O$ (1.0); $MnSO_4 \cdot 4H_2O$ (1.0); $CoSO_4 \cdot 7H_2O$ (1.0); $Cr_2(SO_4)_3 \cdot 15H_2O$ (0.5); H_3BO_3 (0.6); $Na_2MoO_4 \cdot 2H_2O$ (0.5); $NiSO_4 \cdot 6H_2O$ (1.0); $Na_2SeO_4 \cdot 10H_2O$ (1.0); $Na_2WO_4 \cdot 2H_2O$ (0.1); and $NaVO_3$ (0.1), dissolved in 0.01 M H_2SO_4. The *p*H was adjusted to *p*H 2.0 with H_2SO_4 and each salt was dissolved separately in water, before being combined. The solution was heat-sterilised and stored at 4°C.

The choice of basal salt solution used was dependent on the type of medium and the metabolic requirements of the microorganism being cultivated. The "plate basal salts" (BS) did not contain any phosphate and were used when tryptone soya broth (TSB) (Oxoid, U.K.) was also included, as TSB contains potassium phosphate. The "heterotrophic basal salts" (HBS) were made as a 50 x concentrate and the "plate basal salts" (BS) and modified "Umeå basal salts" (UBS) were made as 10 x concentrates. All were acidified (*p*H ~2.0), heat sterilised and stored at room temperature.

Ferrous iron was added to all media, either as a major component (e.g. when required as an electron donor) or a micro-nutrient, from a sterile 1M $FeSO_4$ stock solution. The stock solution was adjusted to *p*H 2.0 with H_2SO_4 and filter-sterilised prior to storage at 4°C.

Tetrathionate was added as a source of reduced sulfur to certain media from a sterile 100 mM stock solution of $K_2S_4O_6$. The stock solution was filter-sterilised and stored at 4°C.

Table 2.1 Composition of the basal salt concentrate solutions.

Reagent (g L^{-1})	Heterotrophic basal salts (**HBS**)	Plate basal salts (**BS**)	Modified Umeå Salts (**UBS**)
$(NH_4)_2SO_4$	22.5	12.5	30.0
$Na_2SO_4 \cdot 10H_2O$	7.5	-	-
K_2SO_4	-	-	20.0
NaCl	-	-	1.0
KCl	2.5	-	-
$MgSO_4 \cdot 7H_2O$	25.0	5.0	5.0
K_2HPO_4	-	-	0.5
KH_2PO_4	2.5	-	-
$Ca(NO_3)_2 \cdot 4H_2O$	0.7	-	0.16

2.1.2 Solid media

Gelled media were used in the enumeration, identification and routine sub-culturing of isolates and also for the purification of mixed cultures. Colonies were differentiated on basis of their appearance. Iron-oxidising colonies were identified as orange/dark brown, usually encrusted with ferric iron on iron-containing media; sulfur-oxidising colonies appeared bright white on sulfur-

containing media and heterotrophic, non-iron-/sulfur-oxidising colonies appeared as a variety of colours from clear to off-white, to yellow to pink.

All solid media except R2 Agar were solidified using 0.5% w/v Type I agarose (Sigma-Aldrich). To prevent hydrolysis of the polysaccharide at low *p*H and high temperature, agarose solutions were heat-sterilised separately from the rest of the medium. After sterilisation, the agarose and other medium component solutions were allowed to cool to ~50°C at which point they were combined. Heat-labile constituents such as $FeSO_4$ and $K_2S_4O_6$ were then added from sterile stock solutions to the appropriate concentrations, where required.

2.1.2.1 Overlay solid media

Originally devised by Johnson and M^{c}Ginness (1991), these media comprise a layer of solid medium inoculated with an acidophilic heterotroph with a sterile layer of the same medium poured over the top. The heterotroph is considered to metabolise compounds such as pyruvic acid that are produced by the hydrolysis of the agarose polysaccharides at low *p*H. Organic acids such as pyruvic acid are highly toxic to many acidophiles, especially autotrophs. Detoxification of the media in this way allows the growth of hyper-sensitive microorganisms (such as *Leptospirillum* spp.) on solid media. The variety of acidic and moderately acidic solid overlay media used, and their corresponding heterotroph inocula, are listed in Table 2.2.

The media were prepared and heat-sterilised, allowed to cool and ferrous iron and tetrathionate added where appropriate. At this point the combined medium was split; one half was inoculated with an active culture of the relevant heterotroph (0.5% v/v) while the other was kept at ~50°C. Approximately 20 mL aliquots of the inoculated medium were poured into sterile Petri dishes (the "under-layer"). Once these had set, roughly similar volumes of the sterile top layer (the "over-layer") were then poured over the top and allowed to set. Plates were matured for 24 h at room temperature and then stored at 4°C.

Table 2.2 Composition and approximate *p*H of overlay media and the species of heterotroph used as the inoculum in the under-layer.

Overlay medium (code)	Contents (final concentration)	Heterotrophic inoculum	*p*H[a]
Iron overlay (**Fe<u>o</u>**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (25 mM)	*Acidiphilium* SJH	2.5
Iron/Tetrathionate overlay (**FeS<u>o</u>**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (25 mM), $K_2S_4O_6$ (2.5 mM)	*Acidiphilium* SJH	2.5
Iron/Thiosulfate overlay (**FeT<u>o</u>**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (5 mM), $Na_2S_2O_3$ (10 mM)	*Acidiphilium* SJH	4.0[b]
Inorganic Iron overlay (**iFe<u>o</u>**)	BS, TE, $FeSO_4$ (25 mM)	*Acidiphilium* SJH	2.5
Yeast Extract overlay (**YE_3<u>o</u>/YE_4<u>o</u>**)	HBS, TE, YE (0.02% w/v), $FeSO_4$ (100 µM)	*Acidocella* PFBC	3.0 4.0

[a]Approximate values (due to the moderating effect of the agarose solution) to ± 0.5 *p*H unit,
[b]Final *p*H after addition of $FeSO_4$, medium initially adjusted to *p*H 6.5 (NaOH)

The *Acidiphilium* SJH inoculum (accession ID: AY040740) for the Fe<u>o</u> and iFe<u>o</u> media was maintained in a HBS/TE/TSB (0.025% w/v)/galactose (10 mM)/$FeSO_4$ (25 mM) liquid medium. The inoculum for the FeS<u>o</u> and FeT<u>o</u> media was maintained in the same liquid medium, amended with 2.5 mM $K_2S_4O_6$. The *Acidocella* PFBC inoculum (unpublished) was maintained in a HBS/TE/fructose (5 mM)/$FeSO_4$ (100 µM) liquid medium. All inocula were regularly sub-cultured and incubated at 30°C.

2.1.2.2 Non-overlay solid media

Where possible, isolates were cultured on non-overlay solid media. This reduced the level of competition for nutrients between the organism being cultured and the heterotroph in the under-layer and also reduced the risk of contamination due to migration of the under-layer organism to the surface of the plate. Details of the various non-overlay solid media used are shown in Table 2.3. The acidic and moderately acidic solid media were prepared in the same way as the overlay solid media, but the combined media were poured as a single sterile layer (~30 mL per plate). The R2 Agar (R2A) medium was a complete medium (Oxoid, U.K.). However, this was used at 10% strength. To allow gelling, 13.5 g L^{-1} Lab Grade agar was added so that the final agar concentration was 15 g L^{-1}. Non-overlay media were stored at 4°C.

Table 2.3 Composition and approximate *p*H of non-overlay media.

Non-overlay medium (code)	Contents (final concentration)	*p*H*
Yeast Extract (**YE_3/YE_4**)	HBS, TE, YE (0.02% w/v), $FeSO_4$ (500 µM)	3.0 4.0
TSB (**$TSB_{2.5}$/TSB_3/TSB_4**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (500 µM)	2.5 3.0 4.0
10% R2 Agar (**R2A**)	(g L^{-1}) YE (0.05), tryptone (0.025), peptone (0.075), glucose (0.05), starch (0.05), K_2HPO_4 (0.03), $MgSO_4$ (0.024), sodium pyruvate (0.03) agar (15)	7.0

*Approximate values (due to the moderating effect of the agarose solution) to ± 0.5 *p*H unit.

2.1.3 Liquid media

The various liquid media used routinely during this study are listed in Table 2.4. In general these were similar to the solid media. All liquid media were heat-sterilised and allowed to cool before the addition of heat-labile components. Fine-grain elemental sulfur (S^0) was sterilised at 110°C for 40 min and added after liquid media had cooled. Fine grain pyrite (Py) was sterilised by dry heat at 160°C for 4 h. This was added to some iron-containing media as a long-term source of ferrous iron after initial iron-oxidation by the inoculum had been observed. Additions or amendments to these standard media are detailed where relevant.

Table 2.4 Composition and *p*H of routinely used liquid media.

Liquid medium (code)	Contents (final concentration)	*p*H
Iron (**Fe**)	HBS, TE, $FeSO_4$ (25 mM)[a]	2.0
Pyrite (**P**)	HBS, TE, Py (1% w/v)	2.0
"*Ferroplasma*" medium (**Fp**)	HBS, TE, YE (0.02%), $FeSO_4$ (50 mM), K_2SO_4 (50 mM)	1.5
Iron/Yeast Extract (**FeYE**)	HBS, TE, YE (0.02% w/v), $FeSO_4$ (10 mM)	2.0
Pyrite/Yeast Extract (**PYE**)	HBS, TE, YE (0.02% w/v), Py (1% w/v)	2.0
Iron/TSB (**FeT**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (5 mM)	2.5
Iron/Tetrathionate/TSB (**FeST**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (5 mM), $K_2S_4O_6$ (2.5 mM)	2.5
Iron/thiosulfate/TSB (**FeTT**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (5 mM)[b], $Na_2S_2O_3$ (5 mM)	4.0[c]
Sulfur (**S**)	UBS, TE, Fe (500 µM), S^0 (0.25% w/v)	2.5
Yeast Extract (**YE_3**)	HBS, TE, YE (0.02% w/v), $FeSO_4$ (100 µM)	3.0 4.0
TSB (**$TSB_{2.5}$/TSB_3/TSB_4**)	BS, TE, TSB (0.025% w/v), $FeSO_4$ (100 µM)	2.5 3.0 4.0

[a]Fine-grain pyrite usually added after initial iron-oxidation observed; [b]$FeSO_4$ added immediately prior to use to prevent oxidation at this *p*H; [c]Final *p*H after addition of $FeSO_4$, medium initially adjusted to *p*H 6.5 (NaOH)

2.2 BIOMOLECULAR TECHNIQUES

All water used in reagents for biomolecular applications was ultra-pure MilliQ® A10 grade (Millipore, U.K.).

2.2.1 Extraction and amplification of genetic material

2.2.1.1 DNA extraction

DNA was extracted either from pure isolates or from whole microbial communities. In the case of pure isolates on solid media, a small amount of biomass from several colonies was suspended in 20 µL cell lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate (SDS)) and heated to 95°C for 15 min in a PCR thermocycler. The crude cell lysates were allowed to cool and 180 µL MilliQ:Tris buffer (0.01 mM Tris, *p*H 7.5) added. Biomass from liquid cultures was harvested by centrifugation (1-3 mL, 16.1 x 10^3 *g*, 15 min) and processed as above. Ferric iron-rich colonies or liquid cultures were washed first in 100 mM oxalic acid and then in sterile MilliQ water before addition of cell lysis solution. DNA from solid-phase environmental samples was extracted using the UltraClean™ Soil DNA Kit (MoBio Laboratories Inc., U.S.A.) as per the manufacturer's instructions. DNA from enrichment cultures and bioleaching cultures was extracted in a similar manner to liquid cultures. Cultures were shaken briefly to disperse any solid material (fine-grain pyrite etc.) and 3 mL removed and centrifuged at 10 x 10^3 *g* for 10 min. The supernatant was removed and the pellet resuspended in 1 mL MilliQ:Tris buffer. Larger particles were allowed to settle and 600 µL transferred to a sterile 0.6 mL microfuge tube and centrifuged again at 16.1 x 10^3 *g* for 10 min. The supernatant was removed and the pellet resuspended in 100 µL cell lysis solution and heated to 95°C for 15 min. The resultant lysate was carefully transferred to a 1.5 mL microfuge tube and 900 µL MilliQ:Tris buffer added. Extracted DNA was stored at -20°C.

2.2.1.2 Polymerase chain reaction (PCR)

PCR was used to amplify the 16S rRNA genes of *Archaea* and *Bacteria*. Bacterial 16S rRNA genes were amplified using either the 27fG:1492rG or 27fG:1387r primer pair (primers appended with the "G" suffix have an

additional guanine residue on the 5' end, see Table 2.5). Archaeal 16S rRNA genes were amplified using the 20fG:1392rG primer pair. All primers used are listed in Table 2.5. The use of these primer pairs enabled the amplification of roughly 13.5 Kb of the 16 Kb 16S rRNA gene of most prokaryotes. Primers were manufactured by MWG Biotech (Germany).

PCR reactions usually consisted of 12.5 µL PCR Master Mix (Promega, U.S.A.), 1 µL forward primer (10 *p*mol μL^{-1} stock, i.e. 10 *p*mol per reaction), 1 µL reverse primer (10 *p*mol μL^{-1} stock), 1 µL of 25 mM $MgCl_2$ (2.5 mM final concentration when combined with the PCR Master Mix), 0.5 µL ultra pure dimethylsulfoxide (DMSO) (2% v/v final concentration), 1 µL DNA template and 8 µL nuclease-free ultra-pure water (25 µL final reaction volume). Where 50 µL reactions were required, constituent volumes were doubled.

PCR reactions were carried out in a Uvigene™ (UVItec Ltd., U.K.) or a Techne® TC-312 (Midwest Scientific, U.S.A.) thermocycler. Bacterial 16S rRNA genes were amplified using "straight", as opposed to touchdown, PCR as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and polymerisation at 72°C for 90 s; followed by a final extension period at 72°C for 10 min. Archaeal 16S rRNA genes were amplified by straight PCR as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and polymerisation at 72°C for 90 s; followed by a final extension period at 72°C for 10 min. DNA inserted into the P-GEM®-T Easy vector from crude cell lysates of clones was amplified using the T7:SP6 primer pair as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 30 s and polymerisation at 72°C for 3 min; followed by a final extension period at 72°C for 10 min.

The 1492r reverse primer was used in the initial stages of this study for bacterial PCR. However, the frequent formation of primer dimers during PCR and their effect on subsequent T-RFLP analysis led to this primer being replaced by the 1397r primer. This eliminated, or at least substantially reduced, the occurrence of these dimers and improved T-RFLP analysis. The properties of this primer did lead to a decrease in ligation efficiency during the construction of clone libraries. However, this was overcome simply by picking greater numbers of colonies during blue/white screening.

Table 2.5 Priming oligonucleotides.

Primer	Sequence (5´→3´)	Target gene	Reference
27f*	AGAGTTTGATC(A/C)TGGCTCAG	Bacterial 16S rRNA	Lane *et al.*, 1991
20f*	TCCGGTTGATCC(T/C)GCC(A/G)G	Archaeal 16S rRNA	Orphan *et al.*, 2000
1100r	AGGGTTGCGCTCGTTG	Bacterial/Archaeal 16S rRNA (internal primer)	Lane *et al.*, 1991
1392r*	ACGGGCGGTGTGT(G/A)C	Bacterial/ archaeal 16S rRNA	Lane *et al.*, 1991
1387r	GGGCGG(A/T)GTGTACAAGGC	Bacterial 16S rRNA	Marchesi *et al.*, 1998
1492r*	TACGG(C/T)TACCTTGTTACGACTT	Bacterial/ archaeal 16S rRNA	Lane *et al.*, 1991
T7	TAATACGACTCACTATAGGG	Plasmid insertion site flanking region	
SP6	ATTTAGGTGACACTATAGAA	Plasmid insertion site flanking region	

f = forward; r = reverse; *Primers were also duplicated with an additional guanine on the 5' end (referred to as 'G' primers) and used to increase ligation efficiency during cloning.

2.2.1.3 Analysis of PCR products by gel electrophoresis

The success of PCR reactions was determined by agarose gel electrophoresis. The gel comprised 0.7% (w/v) electrophoresis-grade agarose in 0.5 x TBE buffer (made as a 5 x concentrate: (g L^{-1}) tris(hydroxymethyl)methylamine (54), boric acid (27.5) in 0.1 M ethylenediaminetetra-acetic acid (EDTA, stock *p*H 8.0 with NaOH)). The agarose was dissolved using a microwave oven and cooled before the addition of 0.005% v/v ethidium bromide. The gel was cast in a suitable gel tray with a comb to produce sample loading wells. PCR products were mixed in a 5:1 ratio with a 6 x concentrate DNA loading buffer (30% v/v glycerol, 0.05% w/v bromophenol blue and 60 mM EDTA). Samples were loaded into the wells and a 1 Kb DNA ladder (New England Biolabs, Inc, U.S.A. or Promega, U.S.A.) was loaded into the first well as a reference. A constant voltage, variable amperage, current was applied across the gel causing the DNA to migrate. Migration was tracked visually using the bromophenol blue dye as a reference. When sufficient migration had occurred to allow good separation of DNA fragments the DNA:ethidium bromide complex was visualised using an UV lightbox. Archaeal and bacterial 16S rRNA genes

amplified using the above primer pairs should yield a single band corresponding to roughly 13.5 Kb in length.

2.2.2 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis of 16S rRNA genes was used to group and, were possible, to tentatively identify pure environmental isolates and clones from 16S rRNA gene clone libraries (e.g. Johnson *et al.*, 2005). Groups of isolates with similar colony morphologies on specific media and identical RFLP patterns with at least two separate restriction enzymes were considered to represent just a single organism.

Restriction digestion reactions contained the following: restriction buffer, restriction enzyme(s) (5 units each per reaction) (Promega) and 10 µL PCR product, made up to 20 µL total volume with nuclease-free water. Isolates were analysed using single enzyme reactions, whereas double digests containing the enzyme *Eco*R I were used to analyse cloned genes. *Eco*R I was used to cleave the inserted gene from the plasmid insertion site-flanking region. Digestion reactions were incubated at 37°C for 2 h to allow for complete digestion. Digestion products were analysed by gel electrophoresis using a 3% agarose gel with a 100 bp DNA ladder as a reference. The gel contained: 3% w/v high-resolution agarose (Sigma-Aldrich) and 0.005% v/v ethidium bromide, dissolved in 1 x TBE. A list of the restriction enzymes used and their restriction sites are shown in Table 2.6.

Table 2.6 Restriction endonucleases and their corresponding recognition sequences and cutting sites.

Restriction Enzyme	Recognition sequence
Msp I	5′...C▼CG G...3′ 3′...G GC▲C...5′
Cfo I	5′...G CG▼C...3′ 3′...C▲GC G...5′
Alu I	5′...AG▼CT...3′ 3′...TC▲GA...5′
Rsa I	5′...GT▼AC...3′ 3′...CA▲TG...5′
*Eco*R I	5′...G▼AATT C...3′ 3′...C TTAA▲G...5′

▲ and ▼ represent the sites at which the restriction enzyme cuts double stranded DNA.

2.2.3 Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis was used to investigate microbial communities to give an overview of diversity and abundance of microorganisms in a given population, and to analyse pure cultures of isolates.

The 16S rRNA genes in a DNA extract from a community or single organism were amplified by PCR using either the 27f:1387r or 20f:1392r primer pair. However, the forward primer in each case was labelled with a blue fluorescent dye (Cy5.0., MWG Biotech, Germany) attached to the 5´ end. Three 25 µL PCR reactions were carried out and then pooled for each sample in order to minimise the effect of PCR bias (Acinas *et al.*, 2005). Pooled reactions were purified using the QIAquick® PCR Purification Kit (QIAGEN, U.K.) and digested in 10 µL restriction enzyme digestion reactions, as described in Section 2.2.2. Either 0.5 µL (pure isolates and clones) or 2.0 µL (community) digestion product was added to 30 µL sample loading solution (SLS) (Beckman-Coulter, U.S.A.) containing 0.5 µL 600 b CEQ™ DNA Size Standard (Beckman-Coulter, U.S.A.). One drop of high purity mineral oil (Beckman-Coulter, U.S.A.) was applied to the sample to prevent oxidation of the formamide in the SLS. Terminal restriction fragments (T-RFs), i.e. those restriction fragments containing the labelled primer, were resolved by capillary gel electrophoresis using a CEQ 8000 Genetic Analysis System (Beckman-Coulter, U.S.A.). Using this system it was possible to resolve the size of these fragments to +/- 1 nt. Based on the premise that each microorganism presents a single T-RF in such analysis, the abundance of that T-RF as determined by peak area gives a semi-quantitative indication of the abundance of that organism in the community analysed. T-RFs from T-RFLP analysis of a community using different restriction enzymes were compared to a database of T-RFs obtained from pure isolates and clones. In this way, inferences could be made as to the structure of a microbial community. A database was constructed specifically for this purpose using Microsoft Access. Figure 2.1 shows a screen-shot of the graphical interface. A version of the database, based on an out-dated data set can be found at http://www.coumes-spring.co.uk/Chris/Files.

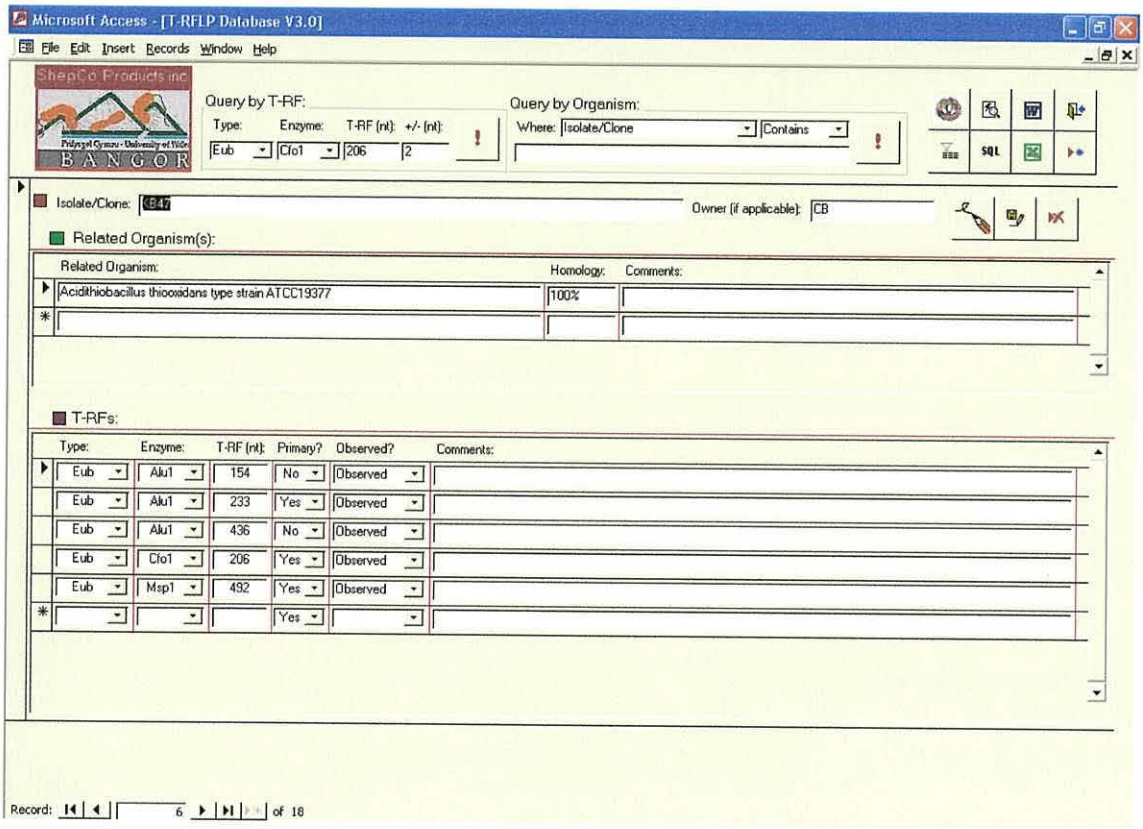

Figure 2.1 Graphical user interface of the T-RFLP Database created by the author using Microsoft Access.

Analysis of the comma-delimited files containing the T-RFLP data, produced by the CEQ software, was facilitated by a Microsoft Excel Macro written in Visual Basic by the author. This automated editing of the file and calculation of relative abundances with minimal user input. The Excel file containing this Macro can be found at http://www.coumes-spring.co.uk/Chris/Files/

2.2.4 Clone libraries from environmental DNA

2.2.4.1 PCR and ligation reactions

16S rRNA genes from environmental DNA extracts were amplified by PCR as described in section 2.2.1. Three 50 µL PCR reactions were pooled and purified. Ligation reactions were set up containing 3 µL purified PCR product, 5 µL Rapid Ligation buffer, 1 µL T4 DNA Ligase (3 Weiss units μL^{-1}) and 1 µL pGEM®-T Easy Vector (all Promega). Reactions were incubated at 4°C

overnight. The additional guanine (G) on the 5´ end of the "G" primers was used to improve ligation efficiency (Brownstein *et al.*, 1996).

2.2.4.2 Preparation of competent *Escherichia coli* DH5-α

An aliquot of pre-frozen (-70°C) stock culture of *E. coli* DH5-α was streaked onto Lauria-Bertani (LB) agar (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1.5% w/v agar, *p*H 7.0 using NaOH) and incubated at 37°C overnight. A single colony was used to inoculate 5 mL LB liquid medium (as above without agar), which was then incubated shaken (~1000 rpm) at 37°C overnight. One mL of the culture was transferred to 90 mL LB liquid medium that had been pre-warmed to 37°C. The culture was shaken (~1000 rpm) at 37°C until an optical density of 0.5 at 550 nm was reached. The culture was chilled on ice for 5 min and then centrifuged at 4°C at 2.5 x 10^3 *g* for 5 min. The supernatant was discarded and the pellet resuspended in 30 mL chilled (4°C) and filter-sterilised (through 0.2 µm cellulose-nitrate membranes, Whatman) TFB1 (which contained (g L^{-1}): potassium acetate (2.946); $MnCl_2 \cdot H_2O$ (9.9); RbCl (12.092); $CaCl_2 \cdot 2H_2O$ (1.48); 150 mL L^{-1} glycerol; in MilliQ water, *p*H adjusted to 5.8 with dilute glacial acetic acid). The cell resuspension was chilled on ice for 2 h before centrifugation at 2 x 10^3 *g* for 5 min at 4°C. The pellet was gently resuspended in 4 mL chilled and filter-sterilised TFB2 ((g L^{-1}) 3-[N-Morpholino]propanesulfonic acid (2.15); RbCl (1.21); $CaCl_2 \cdot 2H_2O$ (11); 150 mL L^{-1} glycerol; in MilliQ water, *p*H adjusted to 7.0 with NaOH) and 60 µL DMSO added. Aliquots of competent cells (100 µL) were flash-frozen using liquid nitrogen and stored at -70°C.

Alternatively, pre-prepared stocks of *E. coli* JM-109 were purchased from Promega, U.K..

2.2.4.3 Transformation of *E. coli* DH5-α/JM-109

An aliquot of competent *E. coli* was thawed on ice (5 min) and 4 µL completed ligation reaction was added. These were mixed very gently and incubated on ice for 20 min. Cells were heat-shocked for 45-50 s in a water bath at exactly 42°C and immediately returned to ice. After 2 min, 900 µL room temperature SOC medium ((g L^{-1}) tryptone (20); YE (5); NaCl (0.58); KCl (0.19);

$MgCl_2 \cdot 6H_2O$ (2.03); $MgSO_4 \cdot 7H_2O$ (2.47); glucose (3.6); *p*H adjusted to 7.0 with NaOH) was added and the cells incubated at 37°C for 1.5 h with shaking (~150 rpm). Following incubation, 50 and 100 µL transformation culture was spread onto LB agar plates containing 100 µg mL^{-1} ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 µg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Melford Laboratories Ltd., U.K.). Plates were incubated at 37°C overnight.

2.2.4.4 Blue/white screening of cloned genes

Only those cells that had been successfully transformed grew on the ampicillin-containing medium. Colonies were either blue or white in colour. White colonies contained the plasmid plus DNA insert, and were selected. Crude cell lysates of white colonies were prepared and the presence of the 16S rRNA gene insert was confirmed by PCR using the T7:SP6 primer pair as described above; clones containing the correct insert presented a PCR product of around 1.4 Kb. Clones were grouped using RFLP analysis of the PCR product using double-digestions with *Eco*R I. Representative clones from each group were transferred to 3 mL LB_{amp} broth (LB liquid medium with 100 µg mL^{-1} ampicillin) and incubated aerobically at 37°C overnight on a rapid shaker at ~1200 rpm. Plasmids were purified from active cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Hereafter, the term 'clone' is applied to the plasmid vector and the partial 16S rRNA gene insert it contained from a single *E. coli* clone from the library. Clones were further investigated by sequence and T-RFLP analysis.

2.2.5 DNA sequence analysis

DNA base sequences were determined using a CEQ 8000 capillary sequencer using the Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, U.S.A.). Purified DNA from single isolates and clones was quantified by measuring absorbance at 260 nm using a Cecil CE2292 spectrophotometer (Cecil Instruments Ltd., U.K.). Reactions containing 5.5 µL nuclease-free water and ~100 ng target DNA (i.e. it was necessary to add 300 ng clone DNA as the insert represented about 33% total DNA.), were heated

to 86°C for 3 min for isolate DNA, or 95°C for 5 min for plasmid DNA, prior to the addition of a single forward or reverse primer (0.5 µL of a 10 *p*mol μL^{-1} stock) and 4 µL DTCS. Linear amplification of target DNA was carried out by PCR using the following cycling conditions: 27fG: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s and polymerisation at 60°C for 4 min. 1387r: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 56 °C for 30 s and polymerisation at 60°C for 4 min. 20fG: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 60°C for 30 s and polymerisation at 60°C for 4 min. T7: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 48°C for 30 s and polymerisation at 60°C for 4 min. SP6: initial denaturation at 96°C for 2 min; followed by 30 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 30 s and polymerisation at 60°C for 4 min.

Amplification products were precipitated by the addition of a solution of 3 M sodium acetate (2 µL), 100 mM EDTA (2 µL) and 20 mg L^{-1} glycogen (1 µL); 30 µL ice-cold absolute ethanol (stored at -20°C) followed by centrifugation at 16.1 x 10^3 *g* for 15 min. The supernatant was removed by aspiration and the pellet washed twice using 100 µL chilled (4°C) 70% ethanol and allowed to dry completely at room temperature (30 min to 1 h). The pellet was thoroughly resuspended in 30 µL SLS and DNA base sequence determined by capillary gel electrophoresis using the long-sequence programme (Beckman-Coulter technical bulletin T-1975A) on a CEQ 8000. Good quality sequence data were obtained for between 600-800 nt target DNA. These partial 16S rRNA gene sequence data were compared with gene sequences deposited in GenBank using the basic logical alignment search tool, BLAST (Altschul *et al.*, 1997).

The DNA sequences obtained from 16S rRNA gene clones were checked manually to determine whether or not any were chimeric. The partial length of these sequences limited the usability of the Chimera-hunting programme Bellerophon (http://taxoweb.mmg.msu.edu/). The recently released Pintail (http://www.cf.ac.uk/biosi/research/biosoft/) programme seemed unable to detect Chimerae where the cross had occurred within the

first 500 nt. Isolates and clones were classified taxonomically using the 16S rRNA Classifier, provided by the Ribosomal Database Project II (RDP, http://rdp.cme.msu.edu/index.jsp).

2.4 ANALYTICAL TECHNIQUES

2.4.1 Chemical assays

2.4.1.1 Determination of ferrous iron

Soluble ferrous iron was determined using the Ferrozine colorimetric assay developed by Lovley and Phillips (1987). Fifty µL sample was added to 950 µL Ferrozine reagent ((g L^{-1}) Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (11.915) (*p*H adjusted to 7.0 with KOH) and ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (1.0)) and vortexed briefly before absorbance at 562 nm was measured using a Cecil CE1011 spectrophotometer (Cecil Instruments Ltd., U.K.). Samples were diluted in acidified RO water (*p*H 1.8 with H_2SO_4) prior to mixing with Ferrozine reagent if necessary. Ferrous iron concentrations were determined by comparing absorbance readings to a standard curve produced using 0 to 1 mM dilutions of ferrous sulfate.

2.4.1.2 Determination of sulfate

Sulfate concentrations were determined turbidimetrically by the formation of insoluble barium sulfate, as described by Kolmert *et al.* (2000). A 5 mL sample (diluted in RO water if necessary) was added to 400 µL conditioning reagent (100 mL glycerol, 60 mL concentrated HCl, 200 mL 95% ethanol; and 150 g NaCl, made up to 1 L total volume with RO water) and vortexed for 10 s. Crushed barium chloride crystals (~60 mg) were added in excess and vortexed for 10 s. After 5 min, the absorbance at 420 nm against a sulfate-free blank was recorded using a Hydrocheck system (WPA Ltd., U.K.). The reading from the Hydrocheck meter was compared to a standard curve produced using 0 to 1.5 mM dilutions of potassium sulfate.

2.4.2 Atomic absorption spectrophotometry (AAS)

Atomic absorption spectrophotometry (AAS) was used to quantify concentrations of soluble transition metals.

Concentrations of dissolved iron, copper, zinc and manganese were determined against metal-free blanks using either a Pye Unicam SP2900 (Pye Unicam, England) double beam atomic absorption spectrophotometer fitted with a SP9-10 gas controller unit (Pye Unicam) using a fuel-lean air/acetylene flame or a Varian SpectrAA Duo atomic absorption spectrophotometer operated in flame mode (Varian, U.K.). Metal standards were made in the same matrix as the sample.

2.4.3 Ion chromatography (IC)

As an alternative to AAS, concentrations of soluble transition metals were determined on some occasions by ion chromatography (IC) using a Dionex DX-320 ion chromatograph with an Ion Pac® CS5A column, an AD25 absorbance detector (520 nm) and an IP25 isocratic pump (Dionex, U.S.A.). The eluent comprised 7 mM pyridine-2,6-dicarboxylic acid (PDCA), 66 mM KOH, 5.6 mM K_2SO_4, and 74 mM formic acid at *p*H 4.2 ± 0.1. The post column reagent consisted of 0.12 g 4-(2-pyridylazo)resorcinol (PAR) dissolved in 1 L diluent (comprising 1 M 2-dimethylaminoethanol, 0.5 M ammonium hydroxide, and 0.3 M sodium bicarbonate at *p*H 10.4 ± 0.2). All reagents used for IC were prepared with MilliQ water, and samples, standards and all reagents were filtered (0.2 µm cellulose-nitrate membranes) or centrifuged (16.1×10^3 *g* for 3 min) before analysis. The flow rate was set at 1.2 mL min^{-1}; column pressure *ca.* 1,600 p.s.i., and sample injection volume of 250 µL. Data were analysed using Chromeleon software, version 6.40 SP2 Build 731 (Dionex, U.S.A.).

2.5 SAMPLING PROTOCOLS

2.5.1 Sample collection and analysis

Solid-phase samples were collected in sterile containers and processed as quickly as possible.

To measure *p*H, 1 g samples of moist material were added to 2.5 mL RO water and left standing at room temperature for 1 h. The *p*H of the liquor was then measured using an Accumet *p*H meter 50 coupled to a BDH pHase Rapid Renew combination *p*H electrode.

Concentrations of readily extractable metals were used as an index of bioavailable metals. Readily extractable metal concentrations were determined by shaking 5 g solid material in 100 mL 0.1 M H_2SO_4 using a reciprocal shaker at 120 cycles min^{-1} at room temperature, for 1 h. The extracts were filtered through nitrocellulose filters (0.2 µm pore size) and metal concentrations measured by AAS or IC. Total extractable metals were determined using the sequential nitric acid oxidation method described by Dacey and Colbourn (1979). Triplicate 0.2 g samples were refluxed at 105°C with 50 mL of 2M HCl for 1 h. Solutions were allowed to cool and filtered through Whatman No. 42 filter papers (Whatman, U.K.). The residues were washed thoroughly with RO water and the filtrate and washings kept. Residues plus filter papers were refluxed again in 50 mL 2M HNO_3, cooled, filtered and washed. Combined filtrates and washings were made up to 500 mL with RO water and metal concentrations determined by AAS or IC analysis.

Where material was to be used in bioleaching experiments, a quantity was mixed, dried, crushed (with a geological hammer) and screened to select particles between 63 µm and 630 µm diameter.

Dry weights, and from these θm (%) values, were determined by drying 5 g wet samples in an oven at 105°C overnight. Differences in weight before and after drying represented the amount of water the samples contained. θm, expressed as a percentage value, is given by dividing the mass of water by the mass of dry spoil, multiplied by 100.

To detach microorganisms from the sample matrix, 5 g sample was added to 100 mL HBS solution and shaken using a reciprocal shaker at 120 cycles min^{-1} at room temperature, for 30 min. The *p*H of the HBS solution was adjusted to a similar *p*H to that of the sample. Once the solid phase had settled, the resultant liquor was serially diluted and plated onto a variety of solid media.

2.5.2 Bioleaching experiment sampling

Bioleaching experiments were set up to examine the ability of an organism or mixed population to bioleach mineral substrates. Carried out in shake flasks, the specific details of these experiments are given in the relevant sections.

At t = 0, the flasks were swirled to evenly disperse the solid phase and 2 x 1 mL samples were taken. Each 1 mL sample was centrifuged at 16.1 x 10^3 *g* for 3 min. With one of these, 750 µL was carefully removed and mixed 1:1 with 6 M HCl (to maintain metals in solution) and total soluble metals analysed by AAS or IC. The other centrifuged sample was analysed for ferrous iron and sulfate concentrations, and *p*H. Flasks were weighed and incubated. Before each subsequent sampling, flasks were re-weighed and sterile RO water added to compensate for water lost through evaporation during incubation.

2.6 MICROSCOPY

2.6.1 Phase-contrast microscopy

Organisms were visualised by phase-contrast microscopy using a Labolux light microscope fitted with a Phaco 2 objective lens (40x; total magnification 400 x) and a phase-contrast condenser (Leitz, Germany).

2.6.2 Fluorescence microscopy

Fluorescence microscopy was used to enumerate microbial populations.

2.6.2.1 Sample fixation

750 µL aliquots of cell suspensions obtained from solid-phase spoil and tailings samples were centrifuged and resuspended in one volume of ice-cold filter-sterilised phosphate buffered saline (PBS, (g L^{-1}): NaCl (7.6); $Na_2HPO_4 \cdot 12H_2O$ (3.58); $NaH_2PO_4 \cdot H_2O$ (0.46); *p*H adjusted to 7.2 with NaOH or HCl) then mixed with 250 µL 12% (w/v) paraformaldehyde (PFA) fixative in PBS (made by adding 6 g PFA to 33 mL heated MilliQ, adding 10M NaOH until dissolved, making up to 50 mL with 3 x concentrate PBS, and finally adjusting *p*H to 7.2 with NaOH or HCl) and incubated at 4°C for 1 to 3 h. The fixed suspension was centrifuged at 16.1 x 10^3 *g* for 5 min, the supernatant

discarded and the pellet washed twice in PBS. The samples were then washed with PBS, as described above. Fixed suspensions were finally resuspended in 1:1 PBS/ice-cold absolute ethanol, and stored at -20°C.

2.6.2.2 DAPI staining

The DNA-staining dye, 4',6-diamidino-2-phenylindole (DAPI) facilitates the visualisation of microbes present in a sample to obtain total cell counts. DAPI staining was used to obtain total numbers of microorganisms within samples.

Fixed samples were drawn onto black 25 mm polycarbonate filters (0.2 µm pore size, Millipore) under vacuum using a multi-filtration manifold (1225 Sampling manifold, Millipore). Iron-rich samples were washed twice by drawing filtered acidified (*p*H 1.8 with H_2SO_4) RO water through the filter then rinsing with MilliQ water. Other samples were washed twice with MilliQ water. Rinsed fixes were stained with a DAPI solution (1 µg mL^{-1}) for 10 min at room temperature then again washed twice with MilliQ water. The membranes were allowed to dry and placed onto a drop of non-fluorescent immersion oil (CITIFLUORTM AF87; Citifluor Ltd., U.K.) on a glass slide. A 'mounting medium' (comprising 90% glycerol, 50 mM Tris *p*H 9.5, and 23.3 mg mL^{-1} 1,4-diazabicyclo-[2,2,2]-octane (DABCO)) was applied directly to the filter to retard fading of the DAPI. Cells were visualised under UV light using an ECLIPSE E600 (Nikon, Japan) fluorescence microscope, and at least 500 cells were counted per sample from randomly selected fields of view.

CHAPTER 3: MICROBIAL POPULATIONS IN SURFACE SPOIL AT THE ABANDONED MYNYDD PARYS COPPER MINES, NORTH WALES

3.1 INTRODUCTION

Mynydd Parys, located in the northern part of the Isle of Anglesey, North Wales is an extremely old mine site with evidence of Bronze Age and Roman workings (Jenkins *et al.*, 2000). However, large-scale operations did not begin until the discovery of a huge sulfidic copper ore body in the 1760's, which turned it into one of the most important copper mines in Europe (Southwood and Bevins 1995). This ore body was worked by two companies, the Mona Mining company and the Parys company. The open pit exploitation of the Parys company created the "Great Opencast", a huge void in the west of the site while Mona Mining worked underground to the east. These underground workings collapsed shortly after they were abandoned in 1785 when Mona Mining's lease was not renewed.

The main copper ore body was depleted by the 1790's. More lodes were discovered northwards, but these had to be worked underground until it became uneconomical to do so; by the 1890's most large-scale operations had ceased. As a result of these activities, there are extensive dumps of sulfidic mine waste, totalling several million tonnes. This waste is entirely mine spoil and overburden. No tailings were ever deposited on the site as much of the mining activity at Mynydd Parys predates the advent of concentration methods such as floatation. Although significant quantities of copper were recovered by leaching and sedimentation (cement copper production) well into the 1950's, the most recent deposition of spoil was about 120 years ago.

The microbial community of a mine site is implicit in its overall environmental impact. If mineral-oxidising microorganisms are not present then there will be relatively little mobilisation of exposed sulfide minerals (Gleisner and Herbert Jr., 2002). Thus, Mynydd Parys presents an interesting opportunity to study the microflora of unmanaged spoil that still contains significant amounts of reactive minerals. Elucidation of the indigenous microbial population will enable the assessment of the mineral-oxidising potential of the spoil, and therefore its potential for continued pollution of the

surrounding environment. An absence of microorganisms capable of facilitating mineral-oxidation may suggest that in the hundreds of years since it was first deposited, the ability of Mynydd Parys spoil to generate AMD has been attenuated.

3.2 MATERIALS AND METHODS

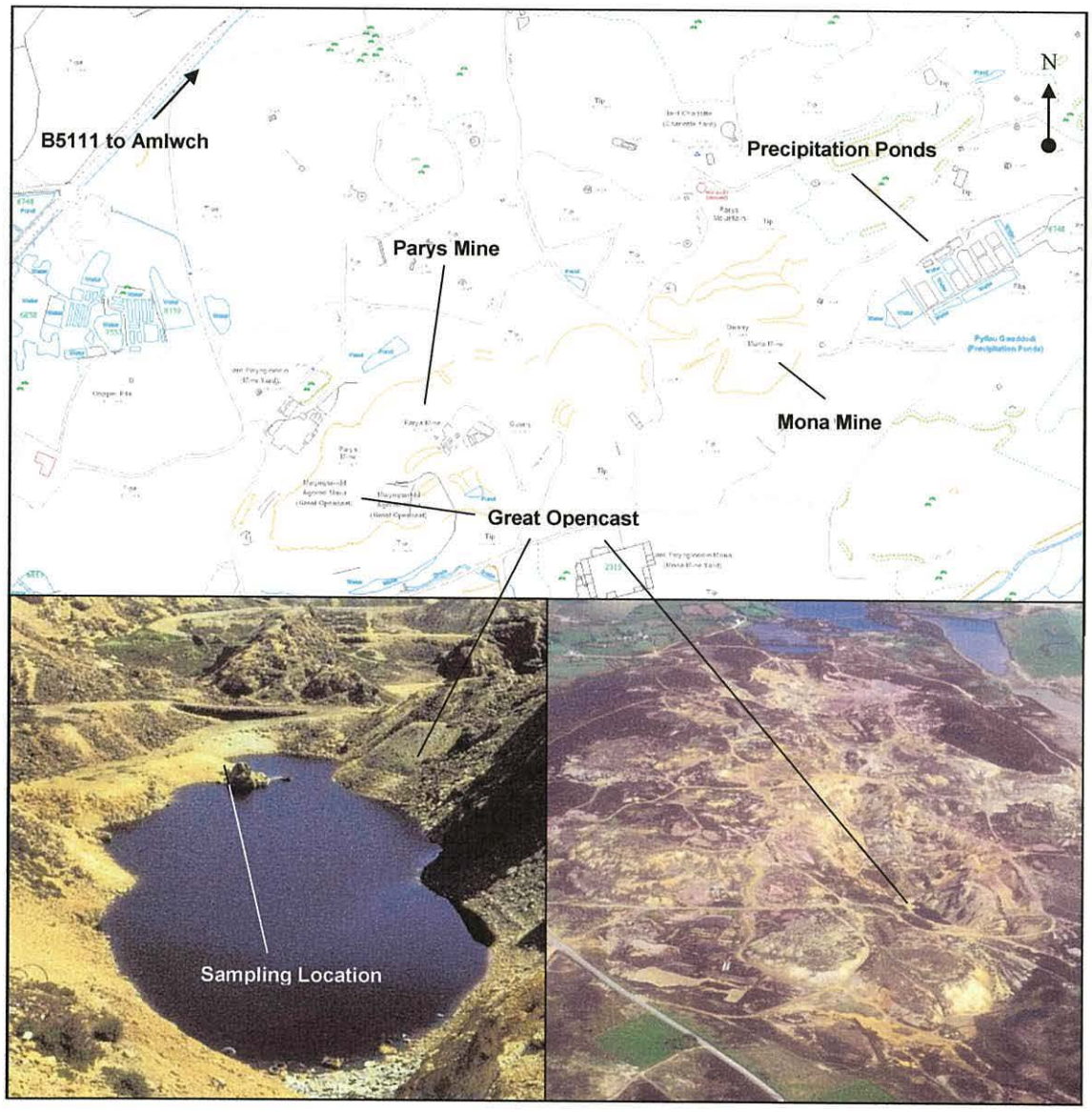

Figure 3.1 Location of the Mynydd Parys former copper mines and details of the site, including the sampling location. (Source: Digimap.)

Mine spoil samples were collected from one location within the Great Opencast, a massive void in the west of the site (Figure 3.1). Approximately 300 g of surface material was collected in sterile bags and transported back to

the laboratory. Samples were homogenised by hand, and stored at 4°C or processed within 24 hours. The *p*H and readily extractable metals for all samples were determined as described in section 2.5.1. Sample 1 was collected in November 2002, sample 2 was collected in February 2003, sample 3 was collected in June 2005 and sample 4 was collected in April 2005.

3.2.1 Indigenous microbial populations

Cells were detached from the spoil matrix by shaking in HBS at *p*H 2.5 for 30 min, as described in section 2.5.1. The cells were serially diluted and plated onto a variety of solid media and incubated at 20°C for three to four weeks, under aerobic conditions. Sample 1 was plated onto Fe$\underline{o}$, FeS$\underline{o}$ and FeT$\underline{o}$ solid media, sample 2 onto Fe$\underline{o}$, FeS$\underline{o}$, FeT$\underline{o}$ and YE$_4\underline{o}$ solid media and sample 3 onto Fe$\underline{o}$, FeS$\underline{o}$, FeT$\underline{o}$ and YE$_4\underline{o}$ solid media. Isolates were categorised by colony morphology, and identity and classification inferred by 16S rRNA gene RFLP and sequence analysis, as described in section 2.2. Cell counts of the liquor used in the plating exercise were determined for sample 2 using the DNA-staining dye DAPI, as described in section 2.6.2.

DNA was extracted from 1 g spoil from samples 1, 2 and 4 using the MoBio Soil DNA extraction kit. Microbial 16S rRNA genes were amplified by either bacterial or archaeal PCR, as described in section 2.2.1. Bacterial PCR using DNA from sample 1 used the 27f(G):1492rG primer pair. Bacterial PCR on DNA from samples 2 and 4 used the 27f(G):1387r primer pair.

The indigenous microbial population was examined by T-RFLP analysis using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I, and the construction and analysis of 16S rRNA gene clone libraries, using amplified DNA (sections 2.2.2-2.2.3).

3.3.2 Isolate Pa33

Isolate Pa33 was obtained from sample 3, and subsequently identified as a novel iron-oxidising actinobacterium within the *Rubrobacteraceae* family. The isolate was partially characterised, as described below.

The *p*H and temperature optima for growth were determined using double strength (0.05% w/v TSB, BS, TE, 500 µM $FeSO_4$) TSB liquid media,

in a 2 L Electrolab P350 bioreactor (Electrolab, U.K.) with temperature and *p*H control. Samples were removed every hour and ferric iron precipitates were dissolved by the addition of 20 µL 1 M H_2SO_4 to 1 mL sample. Sample plus acid was vortexed briefly and left to stand for 1 min before culture biomass was assessed by measuring optical density at 600 nm. Culture doubling times were determined for the *p*H range 2.0 to 4.0, inclusive, in 0.5 unit increments, incubated at 30°C, and for the temperature range 20°C to 40°C, inclusive, in 5°C increments at *p*H 3.0. Each temperature or *p*H variation was performed in triplicate.

Partial 16S rRNA gene sequences were determined using the 27fG, 1100r and 1387r bacterial primers. Reverse complement strands for the reverse primer sequences were created using Chromas (Technelysium Pty Ltd., Australia) and sequence data aligned and concatenated using Microsoft Word. The partial sequence has been deposited in the GenBank sequence repository, and has been allocated the accession ID DQ533685.

Iron oxidation by isolate Pa33 was assessed in TSB_3 liquid media containing different concentrations of ferrous iron, in triplicate 50 mL flasks, incubated shaken at 120 rpm at 30°C. Samples were taken at regular intervals and ferrous and total iron concentrations were assessed colorimetrically using the ferrozine assay. Changes in ferrous and total iron concentrations over time were compared to those observed with uninoculated controls. Iron oxidation by the type strain of the autotroph *Acidithiobacillus ferrooxidans* was assessed, as above, using Fe liquid medium. Iron oxidation by the heterotrophic iron-oxidiser "*Ferrimicrobium acidiphilum*" (strain T23, the proposed type strain) was assessed, as above, using FeYE liquid medium. Viable cell counts (as cfu mL^{-1}) of isolate Pa33 after 289 h of the iron-oxidation experiment were determined using the Miles-Misra plating method on $TSB_{2.5}$ solid media (Miles and Misra, 1938).

The effect of pre-exposure of isolate Pa33 to different iron concentrations on the subsequent rate of iron oxidation was assessed. Pa33 was grown in TSB_3 liquid medium containing either 100 µM or 25 mM $FeSO_4$. Cells were counted using a Thoma counting chamber (Weber, Teddington, U.K.) and triplicate flasks of TSB_3 liquid media containing 25 mM $FeSO_4$ were

inoculated with the same number of cells from either the 100 µM or 25 mM $FeSO_4$ cultures. Iron-oxidation was determined as described above.

The specific iron oxidation rate for isolate Pa33 (as µg iron oxidised per mg protein per minute) was determined. Cells were harvested by centrifugation and washed once in HBS (*p*H 3.0) and finally resuspended in a small volume of HBS (*p*H 3.0). The oxidation of ferrous iron was monitored in universal bottles containing 5 mL reaction mixture, aerated and maintained at 30°C in a water bath. The reaction mixture comprised a volume of cell suspension, 3 mM $FeSO_4$ and 0.1 M β-alanine-sulfuric acid buffer at *p*H 3.0. The β-alanine-sulfuric acid buffer was allowed to equilibrate in the water bath prior to the addition of the cell suspension, and the ferrous iron substrate. Iron-oxidation over time was monitored colorimetrically, using the ferrozine assay (section 2.4.1). Protein concentrations were determined using the Bradford assay, as follows. Cells were harvested from the cell suspension used above by centrifugation and resuspended in 0.5 mL 0.5 mM NaOH. This mixture was incubated at room temperature for 15 min, before 100 µL extract (diluted in 0.5 M NaOH as necessary) was added to 1 mL Bradford reagent (which contained 100 mg Coomassie Brilliant Blue G-250 in 1 L 5% v/v ethanol in 10% v/v phosphoric acid) and incubated at room temperature in the dark for 2 min. Absorbance was measured at 595 nm and compared to a bovine serum albumin (BSA) standard.

To test the ability of cell-free liquor to oxidise iron, an active culture of Pa33 grown in TSB_3 liquid medium containing 100 µM $FeSO_4$, and an uninoculated control were filtered through a 0.2 µm nitrocellulose filter (Whatman, U.K.). $FeSO_4$ was added to 10 mL of each of these to a concentration of 10 mM and the solutions incubated at 30°C in a shaking incubator. Soluble ferrous iron concentrations were determined at regular intervals over a period of 30 days using the ferrozine method, as described in section 2.4.1.1.

The effect of organic carbon on the growth of isolate Pa33 was assessed. Isolate Pa33 was grown in TSB_3 medium containing 100 µM $FeSO_4$. Cells were harvested by centrifugation and cell pellets washed twice in HBS (*p*H 3.0). The resulting cell suspension was used to inoculate 10 mM

ferrous sulfate/HBS-TE liquid medium at *p*H 3.0 to which no organic carbon was added, and TSB_3 liquid medium supplemented with 100 μM $FeSO_4$. The higher ferrous iron concentration was necessary to provide an adequate source of energy for detectable cell growth. Cell growth was assessed qualitatively by examining the cultures with a phase-contrast microscope over a two-week period.

3.3 RESULTS

The average values from the basic geochemical measurements and their standard deviations are summarised in Table 3.1. The spoil samples were consistently acidic and metal-rich; of the readily-extractable metals analysed, the highest concentrations were those of iron, though these were highly variable.

Table 3.1 Basic geochemical data from Mynydd Parys spoil (mean values from all samples).

*p*H*	Readily Extractable Metals (μg g^{-1})				
	Fe	Cu	Zn	Mn	Al
3.04	369	8.86	3.07	1.23	41.8
(0.46)	(167)	(5.76)	(2.01)	(0.79)	(25.35)

Bracketed figures are standard deviation; $n = 6$; *1:2.5 spoil to RO water.

3.3.1 Sample 1

The culture data suggested that one iron-oxidising isolate, denoted Pa1, dominated the indigenous microbial population of this spoil sample. This isolate was shown to have approximately 99% 16S rRNA gene sequence identity with a moderately acidophilic iron-oxidising heterotroph, isolate WJ2, a member of the *Gammaproteobacteria* class. Isolate WJ2 was obtained from a wetland treating drainage from the former Wheal Jane tin mine, Cornwall, U.K. (Hallberg and Johnson, 2003b) and very similar microorganisms have since been isolated from mine-impacted environments from other locations (Kimura *et al.*, 2005; Johnson, unpublished data).

3.3.2 Sample 2

Culture data obtained from sample 2 (Table 3.2) indicated that the microbial diversity was much greater than had been suggested by sample 1. Isolates Pa11 and Pa17 were numerically dominant, representing more than 50% of the total isolates; both of these were found to be closely related to gammaproteobacterium WJ2. These isolates were initially differentiated on the basis of their colony morphologies, but were found subsequently to share greater than 99.8% pairwise sequence identity. Both isolates shared between 99.7% and 100% 16S rRNA gene sequence identity with isolate Pa1. No molecular data were obtained for just over 25% of the isolates so no reliable identification could be established, although roughly 20% of these appeared to be iron- or sulfur-oxidisers based on their colony morphologies.

Two further isolates (Pa22 and Pa24) accounted for nearly 20% of the colony forming units (cfu) obtained. These were two closely related *Actinobacteria*, which shared greater than 99.8% pairwise 16S rRNA gene sequence identity, but could be differentiated by RFLP analysis. Interestingly, they were closely related to the moderately thermophilic iron-oxidising bacterium *Acidimicrobium* sp. Y0018 (Johnson *et al.*, 2003), though they were most closely related to a clone obtained from Hawaiian volcanic deposits. Other heterotrophic isolates included: (i) two *Acidobacteria* (isolates Pa10 and Pa18), which accounted for just under 1% of the total cultured population; (ii) an *Alphaproteobacterium*, isolate Pa20, which accounted for nearly 1.5% of the total cultured population, and (iii) an isolate, (Pa19), which belonged to the class *Bacilli*, within the "*Firmicutes*" phylum, and accounted for 1.25% of the total cultured population.

The *Gammaproteobacteria* isolates (isolates Pa11 and Pa17) were capable of oxidising iron and sulfur on solid media, growing as either ferric iron-encrusted colonies on FeTo or Feo plates, or as white, very gelatinous colonies on FeTo solid medium. The latter showed 'clearing' zones of the normally orange, incubated FeTo plates, indicating acid production due to thiosulfate oxidation and subsequent decrease in *p*H. Isolates Pa19, Pa22 and Pa24 were not shown to oxidise iron or sulfur in solid or liquid media, despite being related to sulfur- and/or iron-oxidising species.

Two chemolithotrophic *Bacteria* were isolated from this sample. Isolate Pa9 was closely related to *Leptospirillum ferrooxidans,* and represented just over 1.5% of the cultured population and isolate Pa13 was closely related to the sulfur-oxidising bacterium *Acidithiobacillus thiooxidans*, and accounted for about 1% of the total isolates.

Plate count data are summarised in Figure 3.2. Cell counts using DAPI were calculated as 5.0×10^6 cells g^{-1} dry spoil, compared to plate counts of 2.1×10^5 cfu g^{-1} dry spoil, which represented a plating efficiency of approximately 4%.

The bacterial T-RFLP profiles (Figure 3.3) suggested greater biodiversity than had been found by cultivation. The only isolates whose T-RFs were found consistently throughout the different restriction enzyme profiles were the *Actinobacteria* isolates, Pa22 and Pa24, and the *Acidobacteria* isolates, Pa10 and Pa18. Interestingly, the abundant *Gammaproteobacteria* isolates were not represented in any of the restriction enzyme profiles. The T-RFs were evenly distributed in terms of relative abundance, with no single T-RF dominant. This implied that there was no single numerically dominant bacterium in the population.

Table 3.2 Descriptions of isolates from sample 2 and their closest relatives, based on 16S rRNA gene identity.

Isolate	Description	Nearest organism(s) (accession ID)	Identity
Pa9	Iron-oxidising colony from Feo plate	*Leptospirillum ferrooxidans* CF12 (AF356834)	99.6%
Pa10	Large pink colony from Feo plate	Uncultured bacterium clone RCP2-4 (AF523897)	98.2%
		Acidobacterium sp. WJ7 (AY096034)	95.2%
Pa11	Small, off-white colony from Feo plate	Gammaproteobacterium WJ2 (AY096032)	99.5%
Pa13	Large white colony from FeSo plate	*Acidithiobacillus thiooxidans* ATCC19377 (Y11596)	98.5%
Pa17	Concentric circular colony from FeTo plate	Gammaproteobacterium WJ2 (AY096032)	99.7%
Pa18	Ruby-red colony from YE_4o	*Acidobacterium* sp. WJ7 (AY096034)	100.0%
Pa19	Yellow cone-shaped colony from YE_4o	Uncultured bacterium clone F2-36 (AY096169)	94.4%
		Acidophilic iron-oxidizing "*Firmicutes*" bacterium Y0010 (AY140235)	89.1%
Pa20	Yellow colony from YE_4o	*Acidiphilium acidophilum* (D30769)	97.3%
Pa22*	Small translucent colony from YE_4o plate	Uncultured bacterium clone 1959a-22 (AY917655)	97.0%
		Acidimicrobium sp. Y0018 (AY140240)	94.0%
Pa24*	Small gelatinous colonies from YE_4o plate	Uncultured bacterium clone 1959a-22 (AY917655)	96.9%
		Acidimicrobium sp. Y0018 (AY140240)	94.3%

*These isolates were differentiated on the basis of 16S rRNA gene RFLP analysis, despite sharing greater than 99.8% 16S rRNA gene sequence identity.

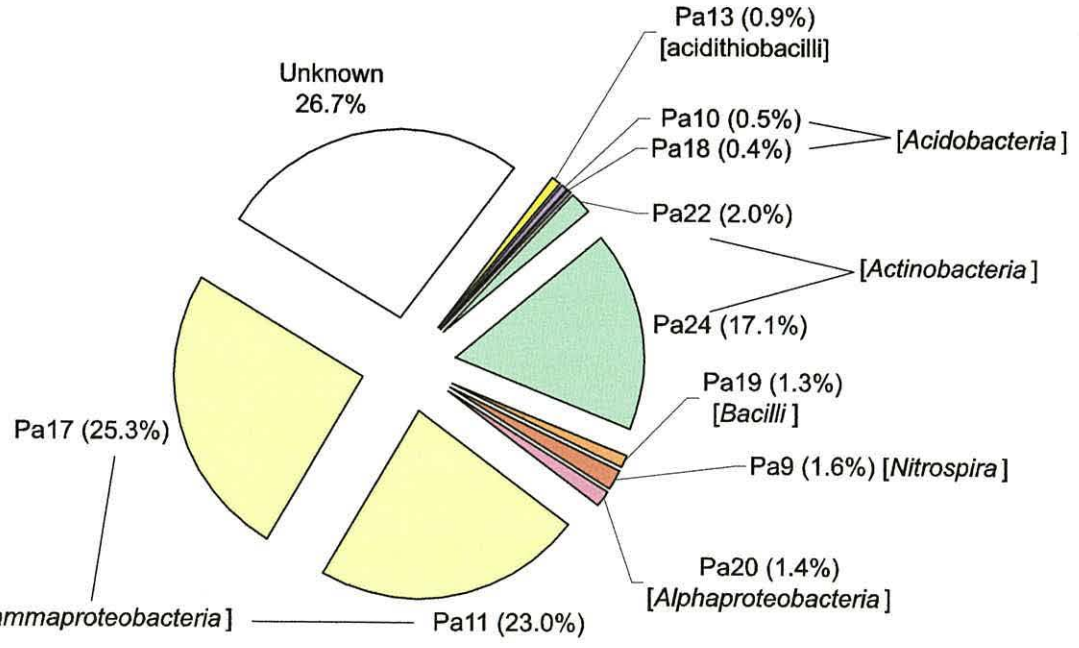

Figure 3.2 The relative abundance of isolates, by taxonomic class, from sample 2, based on plate count data.

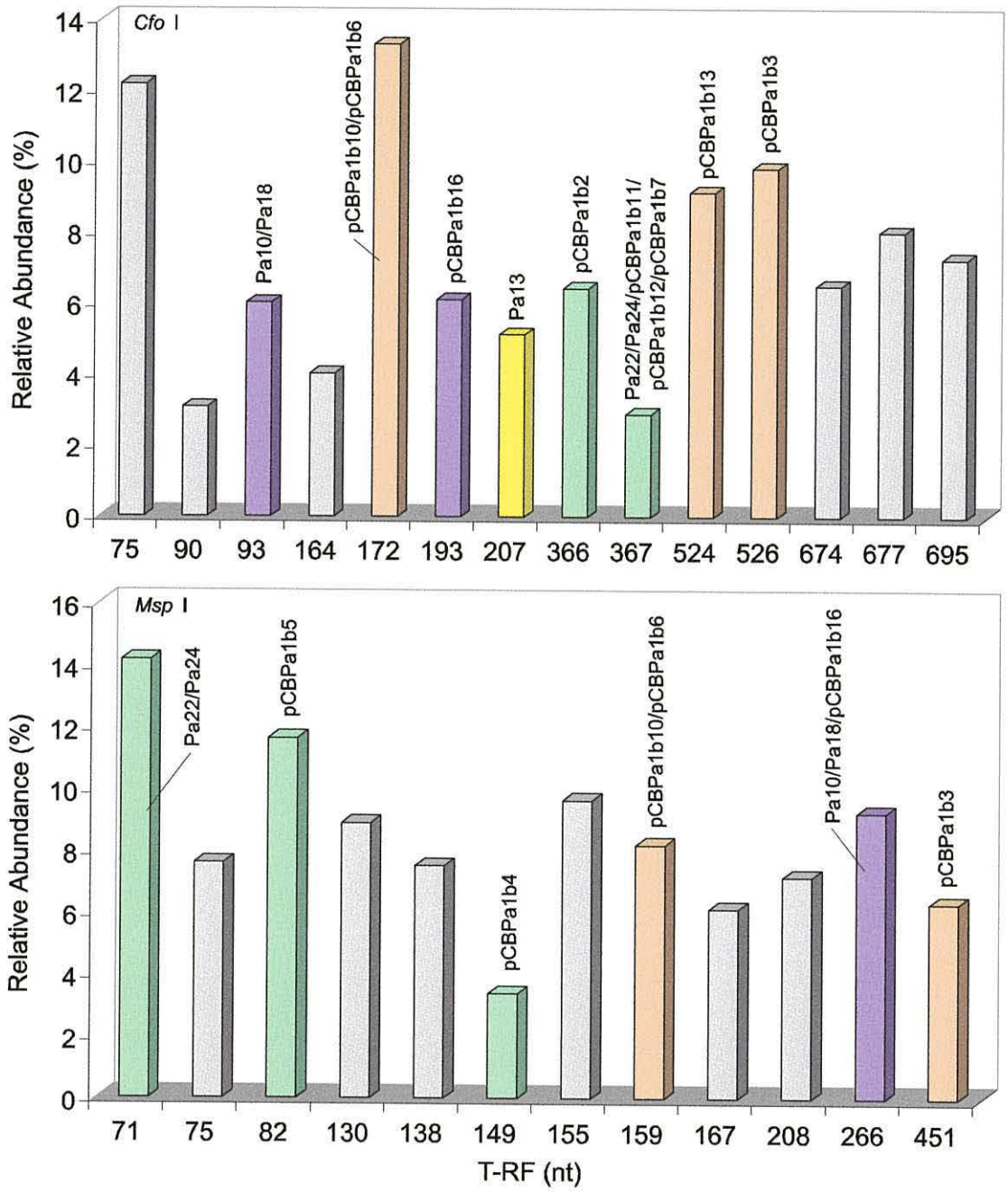

Figure 3.3 T-RFLP profile of sample 2, using the 27f:1387r primer pair and the restriction enzymes *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , Unclassified; , *Acidobacteria*; , *Acidithiobacillus* spp..)

Sequence and Classifier data from the bacterial 16S rRNA gene clone library are summarised in Table 3.2. Of 20 colonies picked, 19 had the correct inserts. The T-RF of each clone was determined using either *Cfo* I or *Msp I* and compared to the community T-RFLP profiles (Figure 3.3).

Table 3.3 Abundance and nearest relatives of 19 clones obtained from a 16S rRNA gene clone library of sample 2, grouped by taxonomic class.

Clone	Nearest relative (accession ID)	Identity	No. of clones
Acidobacteria			
pCBPa1b16	Uncultured bacterium clone MCS36 (AJ830726)	98.4%	2
			2
Actinobacteria			
pCBPa1b2	Uncultured bacterium clone fb11 (DQ303258)	95.5%	1
pCBPa1b11		95.3%	1
pCBPa1b4	Uncultured forest soil bacterium clone DUNssu275 (AY913475)	96.1%	1
pCBPa1b7	Uncultured *Actinomycetales* clone TM208 (X92703)	94.4%	3
pCBPa1b12	Uncultured bacterium clone HSM-SS-004 (AB238767)	95.8%	1
pCBPa1b15	Uncultured bacterium clone RCP2-68 (AF523918)	99.6%	1
pCBPa1b17		99.6%	2
			10
Betaproteobacteria			
pCBPa1b1	*Ralstonia pickettii* ATCC 27511 (AY741342)	99.6%	1
			1
Unclassified			
pCBPa1b3	Uncultured bacterium clone 1894a-24 (AY917553)[V]	88.2%	1
pCBPa1b6		89.0%	1
pCBPa1b10		88.7%	1
pCBPa1b13		88.4%	1
			4
Chimerae			
pCBPa1b5	-		2
			2

[V]Clone from Hawaiian volcanic sediment

Clone library data indicated that different *Actinobacteria* made up half the population, although none of these clones shared more than 94% sequence identity with the *Actinobacteria* isolates. A group of four clones that could not be classified were all distantly related to a clone obtained from volcanic deposits on Hawaii (Gomez-Alvarez and Nuesslein, unpublished). A clone belonging to the *Acidobacteria* class was found, but shared less than 95% identity with the two *Acidobacteria* isolates. No clones belonging to the *Gammaproteobacteria* class were identified, indicating that they were not as abundant as cultivation analysis had suggested. The *Betaproteobacteria* were represented by a single clone, closely related to *Ralstonia pickettii*. One clone was found to be a chimera of two *Actinobacteria*. None of the clones could be

identified as known mineral-oxidising microorganisms, as none of their closest matches in GenBank have been cultivated.

Some of the T-RFs derived from the clones could be matched to T-RFs in the community profile. To some extent this suggested that the unclassified group might have the greatest abundance. However, many of the community T-RFs could not be identified and equally, several of the clones were not represented in the community profile, indicating that the population was probably far more complex than suggested by either method.

Archaeal T-RFLP analysis of DNA extracted from the spoil sample suggested that only one archaeon species was present in the sample (data not shown). This result was supported by clone library analysis, as all ten clones picked from the archaeal 16S rRNA gene clone library were identical, generated from a single archaeon. Sequence analysis found that this was related to an uncultured *Crenarchaeote* from the Kalahari Shield, South Africa (accession ID: DQ223192). The exact gene identity was difficult to assess. This was because the sequence appeared to have a 47 nt insertion relative to sequences obtained using BLAST. Omitting this putative insertion, gene sequence identity was 96.6%.

3.3.3 Sample 3

Analysis of sample 3 focused on culturable acidophiles, with specific emphasis on the isolation heterotrophic organisms. However, the only organisms that were successfully isolated were isolate Pa29, which had 99.1%, sequence identity with *Acidithiobacillus ferrooxidans* NO37 and isolate Pa33, a novel actinobacterium that appeared to oxidise iron on Feo solid media. Rather than appearing as ferric iron-encrusted colonies, typical of iron-oxidising microorganisms, this isolate appeared to oxidise the iron within the medium, producing an orange 'halo' diffusing into the solid phase. The closest cultured relative of Pa33 is the recently described actinobacterium, *Conexibacter woesei* (Monciardini *et al.*, 2003), with which it shares 94.7% sequence identity.

3.3.4 Sample 4

No archaeal PCR product was obtained using DNA extracted from this sample. T-RFLP analysis of amplified bacterial 16S rRNA genes (Figure 3.4) showed that a single T-RF was dominant in terms of abundance in each restriction enzyme profile. However, neither this T-RF nor most of the others could be linked to a previous isolate or clone. The only isolates consistently represented with each enzyme were the *Actinobacteria* isolates, Pa22 and Pa24; the *Acidobacteria* isolates, Pa10 and Pa18 and the *Alphaproteobacteria* isolate, Pa20. T-RFs corresponding to some of the *Actinobacteria* and *Acidobacteria* clones were also identified, though not in every restriction enzyme digest.

The 16S rRNA gene clone library data are summarised in Table 3.4. No sequence data were obtained for two of the clones and two further clones were found to be chimeric. Of the 41 remaining clones, 23 could not be classified taxonomically and of these, 19 were most closely related to clones obtained from volcanic deposits from Hawaii and the rest were most closely related to clones obtained from a forested wetland impacted by AMD (Brofft *et al.*, 2002). The most abundant classified clones were those belonging to the *Actinobacteria* class, although these shared less than 90% sequence identity with the *Actinobacteria* isolates Pa22 and Pa24.

The *Acidobacteria* represented roughly the same proportion of the library as they had in the library from sample 2, with 4 clones. Clone pCBPa0405-1 shared 98% sequence identity with isolate Pa10, while clone pCBPa0405-21 shared 96% identity with isolate Pa18. Again the *Gammaproteobacteria* were not represented. Two *Alphaproteobacteria* clones were identified and three *Deltaproteobacteria* clones. Neither of the two *Alphaproteobacteria* clones shared greater than 91% sequence identity with isolate Pa20. None of the clones could be identified as known mineral-oxidising microorganisms, as none of their closest matches in GenBank have been cultivated.

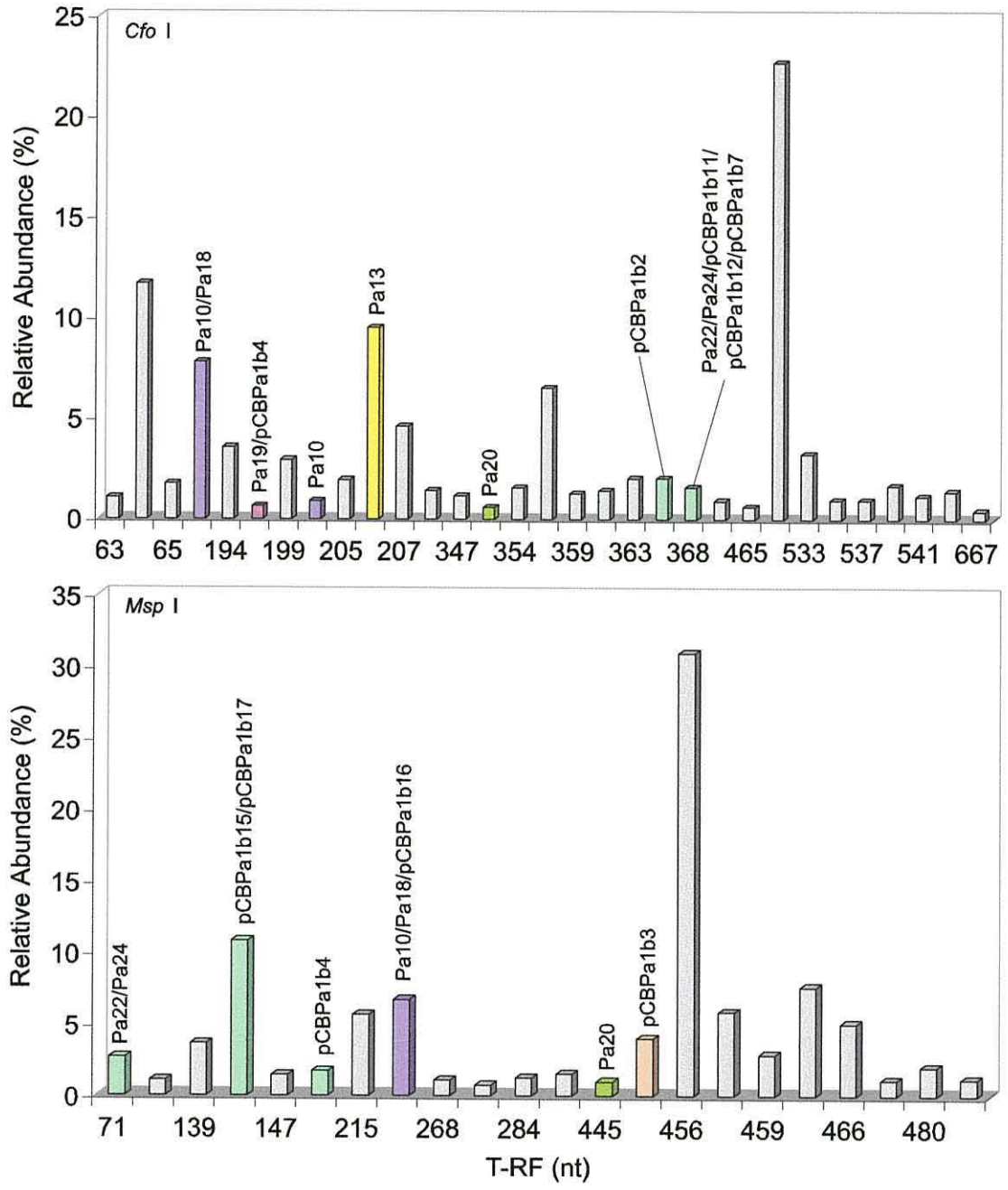

Figure 3.4 T-RFLP profile of sample 4, using the 27f:1387r primer pair and the restriction enzymes *Cfo* I and *Msp I*. (Key: , *Actinobacteria*; , Unclassified; , *Acidobacteria*; , *Acidithiobacillus* spp.; , *Bacilli*; , *Alphaproteobacteria*.)

Table 3.4 Abundance and nearest relatives of 43 clones obtained from a 16S rRNA gene clone library of sample 4, grouped by taxonomic class.

Clone	Nearest relative (accession ID)	Identity	No. of clones
Acidobacteria			
pCBPa0405-1	Uncultured forest soil bacterium clone DUNssu368 (AY913541)	99.4%	1
pCBPa0405-21		98.2%	2
pCBPa0405-37		93.3%	1
			4
Actinobacteria			
pCBPa0405-4	Bacterium Ellin504 (AY960767)	97.5%	1
pCBPa0405-15	Bacterium Ellin301 (AF498683)	97.0%	1
pCBPa0405-18	Uncultured forest soil bacterium clone DUNssu196 (AY913402)	98.2%	1
pCBPa0405-19		98.1%	1
pCBPa0405-26	Uncultured bacterium clone 8F42 (AY387339)	94.6%	1
pCBPa0405-29	Bacterium Ellin504 (AY960767)	97.0%	1
pCBPa0405-36	Uncultured bacterium clone 1974a-28 (AY917856)[V]	97.2%	3
			9
Alphaproteobacteria			
pCBPa0405-30	Uncultured eubacterium WD248 (AJ292598)	95.5%	1
pCBPa0405-35	Uncultured bacterium clone AKIW397 (DQ129264)	95.9%	1
			2
Deltaproteobacteria			
pCBPa0405-6	Uncultured bacterium clone WIM-Mm-29 (AY309180)	94.7%	2
pCBPa0405-22	Uncultured bacterium clone 21BSF20 (AJ863290)	94.5%	1
			3
Unclassified			
pCBPa0405-2	Uncultured bacterium clone 1894a-17 (AY917549)[V]	97.5%	1
pCBPa0405-3		97.8%	1
pCBPa0405-5		97.5%	1
pCBPa0405-7		97.8%	1
pCBPa0405-11		97.9%	1
pCBPa0405-39		94.2%	1
pCBPa0405-43		97.6%	1
pCBPa0405-44		97.6%	1
pCBPa0405-9	Uncultured bacterium clone 1790g2-02 (AY917343)[V]	91.4%	1
pCBPa0405-40		92.0%	1
pCBPa0405-10	Soil clone MC25 (X64384)	96.2%	1
pCBPa0405-12	Uncultured forest soil bacterium clone DUNssu193 (AY913399)	97.8%	1
pCBPa0405-16	Uncultured forest soil bacterium clone DUNssu259 (AY913460)	96.1%	1
pCBPa0405-17	Uncultured bacterium clone 1982b-09 (AY917973)[V]	98.5%	1
pCBPa0405-28		96.6%	1
pCBPa0405-31		98.3%	1
pCBPa0405-23	Uncultured bacterium clone 1959-8 AY425792)[V]	95.4%	1
pCBPa0405-32		95.4%	1

Table 3.4 Cont..

Clone	Nearest relative (accession ID)	Identity	No. of Clones
pCBPa0405-33	Uncultured bacterium clone 1969b-39 (AY917756)[V]	96.9%	1
pCBPa0405-24	Uncultured bacterium clone 1974a-30 (AY917857)[V]	95.4%	1
pCBPa0405-38	Uncultured forest soil bacterium clone DUNssu259 (AY913460)	95.4%	1
pCBPa0405-41	Uncultured bacterium clone 1790a1-17 (AY917315)[V]	94.8%	1
pCBPa0405-42		94.6%	1
			23
Chimerae			
pCBPa0405-20	-		1
pCBPa0405-45	-		1
			2

[V]Clone from Hawaiian volcanic sediment.

3.3.5 Characterisation of isolate Pa33

Culture doubling times of isolate Pa33 at different temperatures and *p*H are shown in Figure 3.5. The optimum temperature for growth was found to be between 30°C and 35°C, and the optimum *p*H for growth was found to be around *p*H 3.0. Cells appeared as non-filamentous, non-motile cocco-bacilli that did not form endospores, when viewed under a phase-contrast microscope. Isolate Pa33 was not shown to grow in the absence of an organic carbon source.

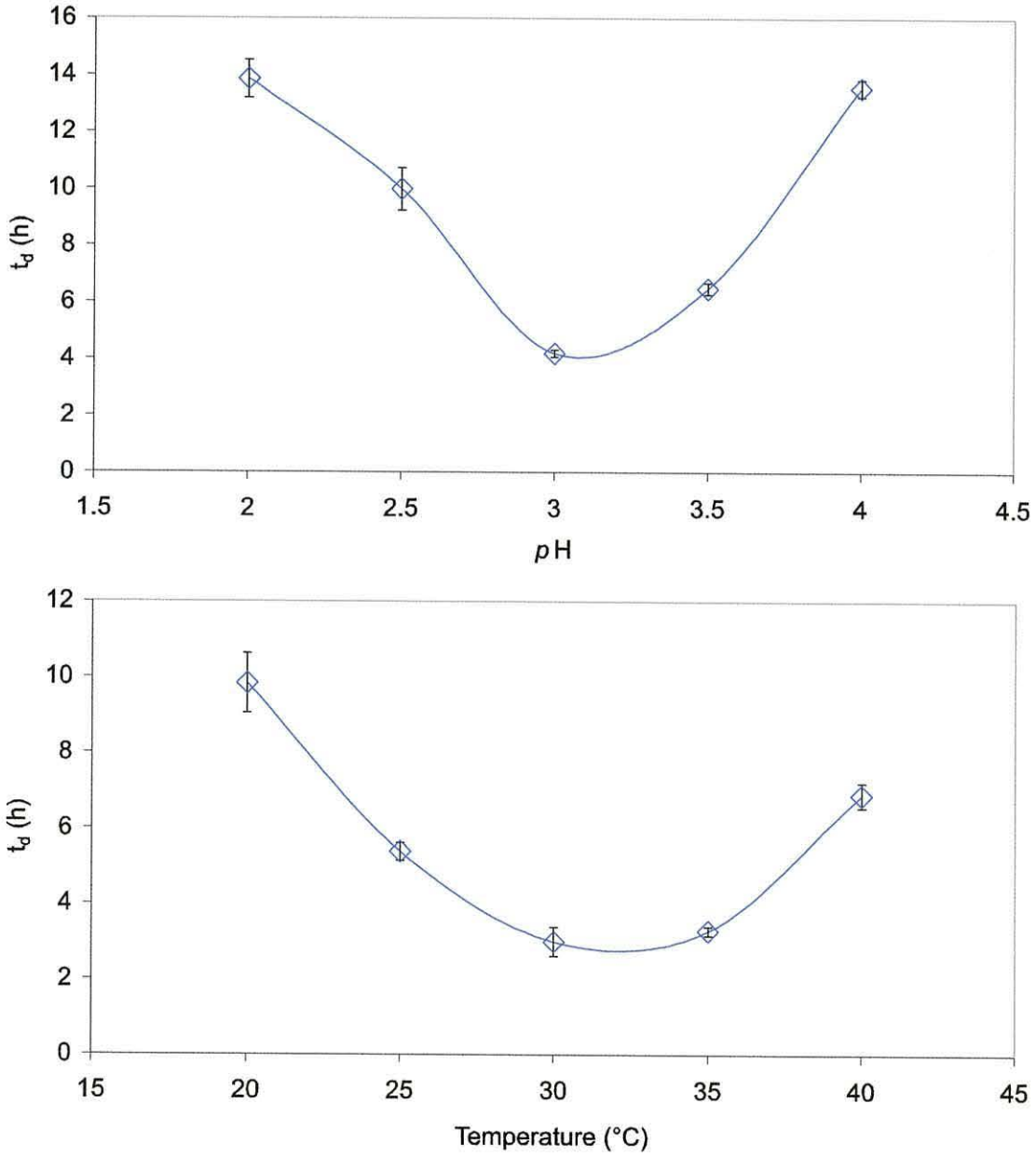

Figure 3.5 Doubling times of isolate Pa33 in TSB media with varying *p*H or temperature. (*n* = 3 for each data point; Error bars show standard error.)

Iron oxidation data for isolate Pa33 are shown in Figure 3.6. In media containing 1 mM iron or less, all of the iron was oxidised within 72 h. In medium containing 5 mM iron, oxidation occurred at a steady rate over 144 h, after which point all the iron had been oxidised. The rate of iron oxidation in medium containing 10 mM iron was initially quite slow, but increased suddenly after 96 hours; almost all of the iron had been oxidised by 144 h. A similar acceleration phase for the first 96 hours was observed in medium containing 25 mM iron medium, after which iron oxidation progressed steadily up to 289 h incubation, by which time most of the iron had been oxidised. Rates of iron oxidation by isolate Pa33 are in contrast to those of *At. ferrooxidans* and "*Fm. acidiphilum*". These two organisms showed similar trends in iron oxidation rates to each other, and were both much faster than isolate Pa33. The initial rate of iron oxidation by *At. ferrooxidans* and "*Fm. acidiphilum*" was quite slow, until after approximately 20 h, at which point a very rapid rate of oxidation was observed, with almost all the iron oxidised within 62 h.

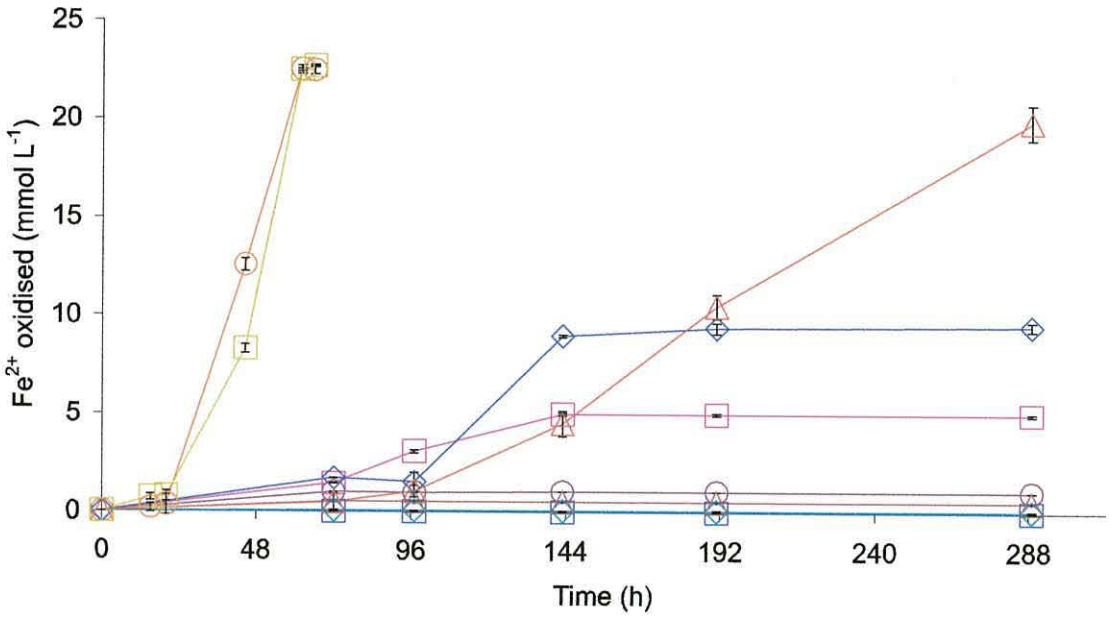

Figure 3.6 Iron oxidation by isolate Pa33 in media containing different concentrations of ferrous iron compared to the type strains of *At. ferrooxidans* and "*Fm. acidiphilum*". (Key: ○, *At. ferrooxidans* in medium containing 25 mM ferrous iron; □, "*Fm. acidiphilum* in medium containing 25 mM ferrous iron + 0.02% yeast extract; Isolate Pa33 in TSB medium containing: △, 25 mM; ◇, 10 mM; □, 5 mM; ○ , 1 mM; △, 0.5 mM; ◇, 0.1 mM or □, 0 mM ferrous iron; Error bars show standard error; n = 3 for each data point.)

Total soluble iron concentrations are shown in Figure 3.7. Total soluble iron showed a gradual decrease over time, due to the precipitation of ferric iron at *ca. p*H 3.0. Total soluble iron levels remained unchanged throughout the experiment with *At. ferrooxidans* and "*Fm acidiphilum*", presumably due to the lower *p*H of the media (data not shown). No changes in ferrous or total iron concentrations were observed with the uninoculated controls (data not shown).

The pattern of iron oxidation by Pa33 on solid media suggested that an extracellular product could be responsible for iron oxidation. To test this, cell-free liquor from an active culture of Pa33 grown in iron-containing liquid medium was assessed for its ability to oxidise iron, compared to sterile TSB. However, no differences in iron oxidation were observed, even after four weeks. Therefore, iron oxidation seems to be dependent on the presence of Pa33 cells.

Iron oxidation by isolate Pa33 pre-exposed to either 100 µM or 25 mM iron is shown in Figure 3.8. Oxidation rates were identical over the first 147 h. The rate then decreased in flasks inoculated with the culture pre-exposed to 25 mM iron, while remaining steady in those inoculated with Pa33 pre-exposed to 100 µM iron. Total soluble iron concentrations were consistently higher in the flasks inoculated with culture pre-exposed to 25 mM iron. This was presumably due to the fact that a much larger volume of 25 mM Fe inoculum was needed to provide the same number of cells as the 100 µM Fe medium (data not shown).

Viable cell counts of isolate Pa33, grown in different initial concentrations of ferrous iron a shown in Figure 3.9. The data show that the culture grew to the highest cell density in medium initially containing 100 µM ferrous iron, and that above this concentration viable cell numbers after 289 h incubation were progressively less.

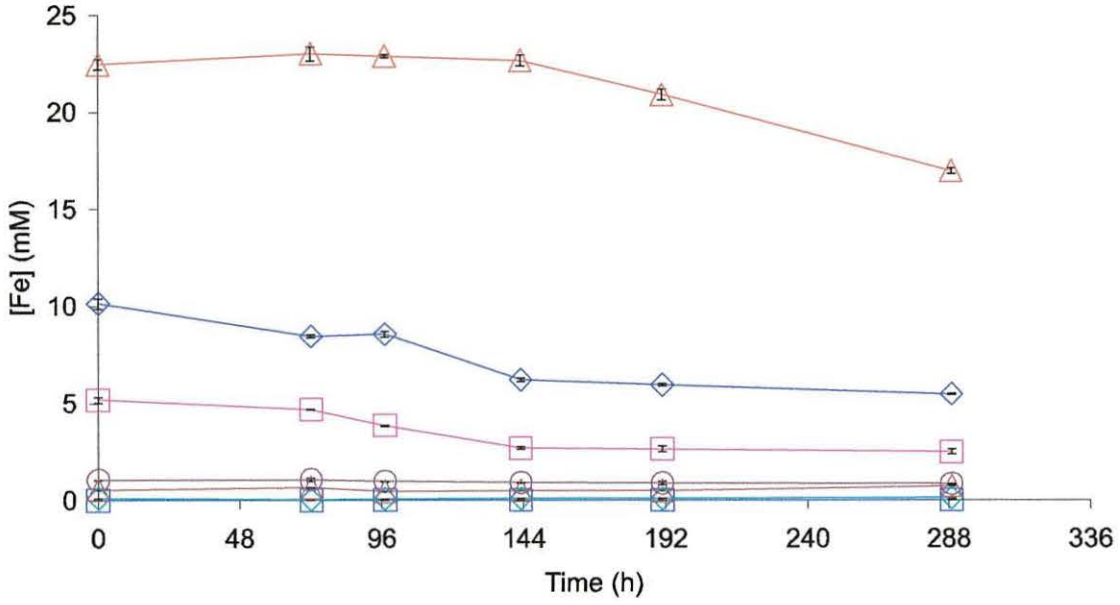

Figure 3.7 Changes in total soluble iron concentrations in TSB media containing different concentrations of iron, inoculated with isolate Pa33. (Key: △, 25 mM Fe; ◇, 10 mM Fe; □, 5 mM Fe; ○, 1 mM Fe; △, 0.5 mM Fe; ◇, 0.1 mM Fe; □, 0 mM Fe; Error bars show standard error; *n* = 3 for each data point.)

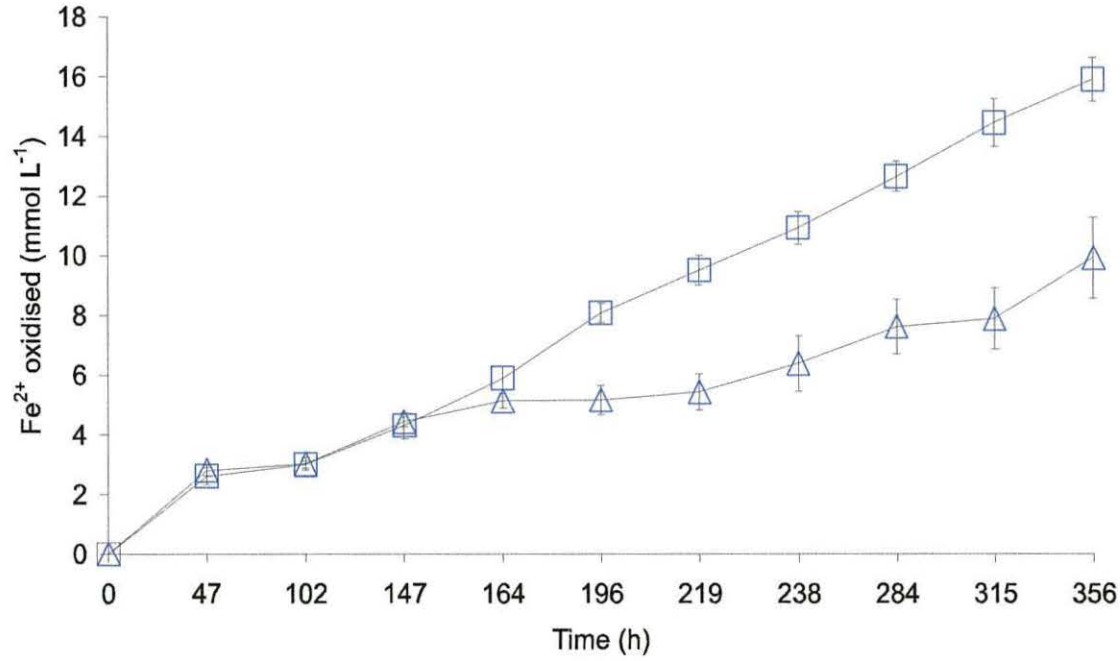

Figure 3.8 Iron oxidation by isolate Pa33 pre-exposed to different concentrations of iron. (Key: □, pre-exposed to 100 µM Fe; △, pre-exposed to 25 mM Fe; Error bars show standard error; *n* = 3 for each data point.)

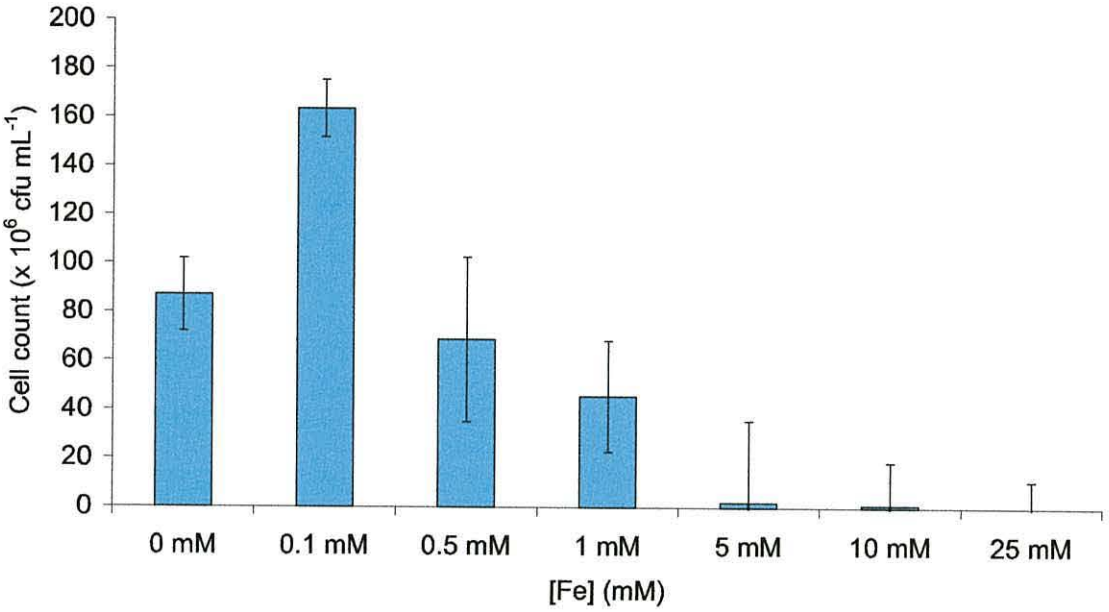

Figure 3.9 Viable cell counts of isolate Pa33 grown in medium containing different initial concentrations of ferrous iron, determined after 289 h. (Error bars show standard error; n = 3 for each data point.)

The specific rates of iron oxidation for isolate Pa33, compared to other microorganisms, are shown in Table 3.5. The specific iron oxidation rate of isolate Pa33 was around an order of magnitude lower than those of the autotrophic iron-oxidising bacteria, *At. ferrooxidans* and *L. ferrooxidans*, and only about 25% of that of the iron-oxidising heterotroph, "*Fm. acidiphilum*", though it was comparable to that of the iron-oxidising heterotrophs, T25 (an actinobacterium) and the firmicute SLC2.

Table 3.5 Specific iron oxidation rates of isolate Pa33 and some iron-oxidising *Bacteria*.

Organism	Specific iron oxidation rate (μg iron oxidised minute^{-1} mg protein^{-1})
Isolate Pa33 (Heterotroph)	62.4
At. ferrooxidans (Autotroph)	560*
L. ferrooxidans (Autotroph)	422*
"*Fm acidiphilum*" (Heterotroph)	215*
Actinobacterium T25 (Heterotroph)	77.7*
Firmicute SLC2 (Heterotroph)	58.2*

*Data taken from Bacelar-Nicolau (1997).

3.4 DISCUSSION

3.4.1 Microbial communities in Mynydd Parys mine spoil samples

The low *p*H and high soluble metal concentrations of the Mynydd Parys spoil suggest that, despite the many years since it was first deposited, it is still contributing to acidic, metal-rich run-off waters that drain the site. The initial isolation of a mineral-oxidising bacterium confirmed that the potential for mineral dissolution exists within the microbial population. More detailed cultivation work showed this was not limited to a single organism.

Despite the presence of obligate chemoautotrophs, the numerically dominant microorganisms, in terms of plate-counts, were related to the moderately acidophilic iron- and sulfur-oxidising heterotrophic bacterium WJ2. It may be possible that, due to the extensive weathering of the spoil, the microbial population has become more heterotrophically inclined, with conditions favouring microorganisms with more adaptable metabolic capabilities; there may not be a high enough concentration of available sulfides to sustain a large population of mineral-oxidising autotrophs. This notion is exemplified by isolates Pa19, Pa22 and Pa24, which are related to mineral-oxidising heterotrophs, but did not appear to facilitate the dissolution of sulfide minerals, via iron- or sulfur-oxidation, themselves.

Of the cells dispersed from the spoil matrix, DAPI-stained cell counts suggested that up to 4% were successfully cultured. This may explain why an isolate that accounted for about 50% of the total cultured isolates did not appear at all in the molecular analyses. Based on these data, the iron-oxidising *Gammaproteobacteria* would account for approximately 2% of the total population. This level is at or around the detection limit for T-RFLP, and it would only be expected to occur once or twice in a clone library consisting of 100 clones. The largest clone library used in this study comprised half this number, so it is entirely plausible the organism was missed. Substantially larger clone libraries may have helped resolve this.

This is testament to the caution that must be taken when applying quantitative culture-based and PCR-based data to an entire population. Whilst examination of the culture data allows inferences to be drawn about the metabolic capacity of the population, the media used are selective for

acidophiles, and iron- and sulfur-based media specifically aimed at the isolation of mineral-oxidising microorganisms. Additionally, the isolation technique is dependant on the detachment of viable microbial cells from the spoil matrix whereas the DNA extraction procedure is not. However, the effects of PCR-bias (e.g. von Wintzingerode *et al.*, 1997) may lead to a single 16S rRNA gene being over-represented in the final product, thereby artificially enhancing its relative abundance. The sensitivity of PCR, and thus that of T-RFLP analysis, must also be considered. While culture media can, in theory detect the presence of just a single bacterium in the plated inoculum, single-round PCR is certainly less sensitive. For example, while de Wulf-Durand *et al.* (1997) could detect the numerically dominant *L. ferrooxidans* in a bioleaching culture with standard, single-round PCR, organisms that formed a minor fraction of the population could only be detected by nested PCR. Although the use of nested PCR may increase the over-all sensitivity, this technique is difficult to optimise, and prone to contamination.

There were differences in the molecular data obtained from the two spoil samples analysed, collected from the same location, but on different dates. The T-RFLP profiles were different in terms of the T-RFs detected and their relative abundances. This may be due to seasonal differences or simply due to heterogeneity within the site. These effects may be accounted for by sampling a transect of the site and/or repeated sampling at frequent intervals. However, constraints of time, and the broader scope of this research project, restrict the viability of this approach. Although the T-RFLP profile of sample 4 suggested a single organism might represent approximately 20% of the population, a group of eight closely related, but non-identical clones from the same sample represented a similar proportion. These clones may share the same T-RFs, thus their total abundance would be represented as a single T-RF in the profile. Analysis of the sequence data indeed suggested this to be the case. The terminal restriction site for each enzyme used appeared to occur at the same location for all eight of these clones. This demonstrates the limitations of T-RFLP, if used in isolation, to elucidate the diversity of a microbial population. Different organisms may share one or more T-RFs. Conversely, closely related organisms may present different T-RFs. Despite

this, the profile did provide a very useful insight into population complexity and dynamics.

The clone library data, together with the T-RFLP data, show that, while the specific composition of the population is quite dynamic, it is more important to consider groups of organisms rather than individual species, or even genera. Both libraries suggest that *Actinobacteria* form a large proportion of the microbial community. This is interesting as *Actinobacteria* are more often found in soil communities and, apart from the very few described *Actinobacteria* isolates such as "*Ferrimicrobium*" spp. and *Acidimicrobium* spp., have rarely been associated with acid mine environments, certainly not as a major section of the community. While a large proportion of the clones from each library could not be classified, the majority of these were most closely related to clones isolated from volcanic sediment on a Hawaiian island. These clones represented a substantial section of the microbial community, probably more so than the *Actinobacteria*.

Known mineral-oxidising microorganisms, such as *At. ferrooxidans* and *Leptospirillum* spp., were conspicuous by their absence from the molecular data. A premature conclusion based on this may be that all the mineral oxidation that occurs within the spoil is due to the activities of a mineral-oxidising population whose numbers are below the detection limits of the molecular techniques. However, it is not possible to comment with any certainty on the metabolic capabilities of uncultured clones. The isolation of the iron-oxidising *Rubrobacteraceae* isolate, Pa33, is indicative that iron-oxidation may be more widespread in the *Actinobacteria* class than previously thought. Additionally the Hawaiian island study examined microbial colonisation and succession of another highly disturbed environment in which, presumably, mineral oxidation also has a large influence.

It is proposed that the facilitation of mineral mobilisation by the oxidation of ferrous iron and sulfur is not the preserve of a small group of highly specialised organisms, but is in fact much more ubiquitous. Given the hypothesis that lithotrophy, specifically the oxidation of ferrous sulfide (FeS) and hydrogen sulfide (H_2S or HS^-) was the primary source of energy for carbon fixation utilised by emerging life on this planet (quoted in Rawlings and

Johnson, 2002), such a wide distribution of mineral oxidising ability is not surprising.

The Mynydd Parys spoil is very interesting in that it may represent the gradual transition of an extremophilic, mineral-oxidation based population to a more soil-like microbial community. The site has yet to progress to a stage where it can support plants and other macroscopic life, and, although the population is becoming more heterotrophically inclined, there may still be an underlying reliance on lithotrophy as a primary energy source. While this lithotrophic capacity remains high, the spoil will continue to generate AMD and so pose a considerable threat to the environment. The abundance and diversity of known mineral-oxidising microorganisms may be diminished, but the process is clearly still occurring.

3.4.2 Isolate Pa33

Isolate Pa33 represents a novel genus of the order *Rubrobacterales* in the *Actinobacteria* class of the *Bacteria*. This is the first description of an iron-oxidising bacterium in this order, and one of very few within the *Actinobacteria* class. The specific iron oxidation rate of this organism is comparable with some other iron-oxidising heterotrophic prokaryotes, though, it is much lower than those of the obligate chemolithotrophs tested and the highly effective iron-oxidising heterotroph, "*Fm. acidiphilum*". The absence of growth in organic carbon-free medium suggests that Pa33 is an obligate heterotroph. The distribution of cell counts when grown in varying iron concentrations implies that increasing iron concentrations are toxic, and putatively suggests that the optimal concentration for growth is around 100 µM. The decrease in cell numbers may have been partially due to cellular adhesion to ferric iron precipitates. However, no ferric precipitate was obvious in flasks with less than 5 mM iron, yet cell numbers decreased above 100 µM iron.

Iron oxidation could not be stimulated by exposure to increased ferrous iron concentrations; rather it appeared to be dependent on cell numbers. This explains the variable initial rates of iron oxidation with differing iron concentrations. Higher iron concentrations slowed the growth rate, thereby depressing the rate of iron oxidation. This may also explain why the iron oxidation rates in the pre-exposure experiment began to diverge after 147 h.

More ferric iron precipitate was carried over with the inoculum grown in 25 mM iron. This would potentially keep a greater concentration of ferric iron in solution due to its effects on the hydrolysis equilibrium (section 1.1.3). Indeed, there was a higher concentration of total iron throughout the experiment in flasks inoculated with Pa33 pre-exposed to the higher iron concentration.

These data suggest that Pa33 utilises iron oxidation as a protection mechanism from the presumed toxicity of soluble iron. No evidence was obtained to suggest that this microorganism acquires energy from this process. The hypothesis is that, by oxidising soluble ferrous iron to the far less soluble (at *p*H > 2.5) ferric state, the bioavailability (and hence toxicity) of iron is reduced. The irony in the case of isolate Pa33 is that the continued oxidation of ferrous iron in an acidic environment would perpetuate the mineral dissolution cycle in the spoil. While most of the ferric iron produced would indeed precipitate at this *p*H, some would still remain in solution. This would attack any residual sulfidic minerals, including pyrite, bringing more ferrous iron into solution in the process. Additionally, the very process of ferric iron precipitation would depress *p*H, in the process increasing its solubility.

However, this gives further support to the idea that the population of the Mynydd Parys spoil is an adaptive one, still influenced by the processes of mineral dissolution, but becoming less dependant on lithotrophy and more reliant on heterotrophy for growth.

CHAPTER 4: CHARACTERISATION OF THE MICROBIAL POPULATIONS OF TAILINGS AND SPOIL AT THE ABANDONED SÃO DOMINGOS COPPER MINE, AND THEIR ABILITIES TO ACCELERATE MINERAL DISSOLUTION

4.1 INTRODUCTION

São Domingos is an abandoned copper mine located several kilometres from the town of Mértola in the Beja district of southern Portugal. It is part of the Iberian pyritic belt (IPB), and was first exploited in Roman times. The IPB is a massive sulfide body covering much of South Western Spain and Southern Portugal, and includes other important mines such as the Rio Tinto. Commercial-scale mining, principally of copper and sulfur, began at São Domingos in 1859, and continued until 1966. The site was formally closed in 1968 and remains abandoned. Both opencast and underground operations were employed, resulting in the extraction of approximately 25 Mt of ore during the lifetime of the mine (Matos *et al.*, 2003). Much of the ore processing was carried out on site and it is estimated that more than 5 Mt of tailings waste are stored in the area, in addition to the large opencast void and associated mine spoil. There are several large tailings dumps at São Domingos, one of which lies next to the former sulfur factory in the Achada do Gamo region of the site. The wastes contain various heavy metals including copper, zinc, lead, antimony, silver, mercury and cadmium (Matos *et al.*, 2003).

Currently only limited measures are in place to protect the local environment from contamination emanating from the abandoned mine site, and local watercourses and surrounding soils are heavily contaminated with a variety of soft metals, including lead, arsenic, antimony and cadmium. A system of channels, dams and ponds has been built to prevent the spread of pollution by retaining the AMD on site. However, during periods of heavy rainfall, flash floods wash contaminated soil and AMD into the River Chança, which then flows into the River Guadiana (Gerhardt *et al.*, 2004). A small reservoir just outside the village of Mina do São Domingos receives storm water and is particularly affected by surface runoff and deposition of

atmospheric arsenic from the site. Part of the mine is inside the Vale do Guadiana Natural Park and future plans for the site include a heritage museum (Matos *et al.*, 2003). Limiting the environmental impact of the São Domingos mine wastes is therefore desirable, and to this end a better understanding of the processes involved in generation of pollution at this site is needed.

The São Domingos site is interesting in that conditions can change radically from season to season. During the summer months there is, usually, virtually no rainfall, to the extent that a large red pond at the foot of the Achada do Gamo tailings heap becomes thixotropic and eventually tar-like in consistency, and the tailings themselves become very dry. How these constant changes affect the microbiology of the site, and ultimately the rate at which mineral leaching occurs, is currently unresolved. As a first step to address this question, the microbial populations of São Domingos tailings from the Achada do Gamo and spoil from the opencast void were examined using cultivation-dependant and cultivation-independent methods. Additionally, the ability of microorganisms isolated from the site to catalyse the dissolution of sulfide minerals under laboratory conditions was investigated.

4.2 MATERIALS AND METHODS

Mineral waste samples were collected from a deposit of sulfide mineral-rich tailings near in the Achada do Gamo region of the site and from mine spoil near to the large open cast void in the North East of the site. Run-off from the tailings drained into a large (*ca.* 50 m diameter) pool, which was highly acidic (*p*H 2.5) and deep red in colour. A small quantity of tailings surface material was collected in July 2003. Further samples were collected from the tailings and spoil from three depths, 0-10 cm, 20-30 cm and 50-60 cm in November 2003 and March 2004. Spoil and tailings samples were transported in sterile plastic bags to the UK, and processed immediately upon return. The *p*H and readily extractable and total extractable metal concentrations were determined, as described in section 2.5.1. Figure 4.2 shows the mine location and details of the site.

A quantity of the tailings or spoil collected from different depths in November 2003 was mixed, dried, crushed (with a geological hammer) and

screened to select particles between 63 and 630 µm diameter. This material was used subsequently in bioleaching experiments.

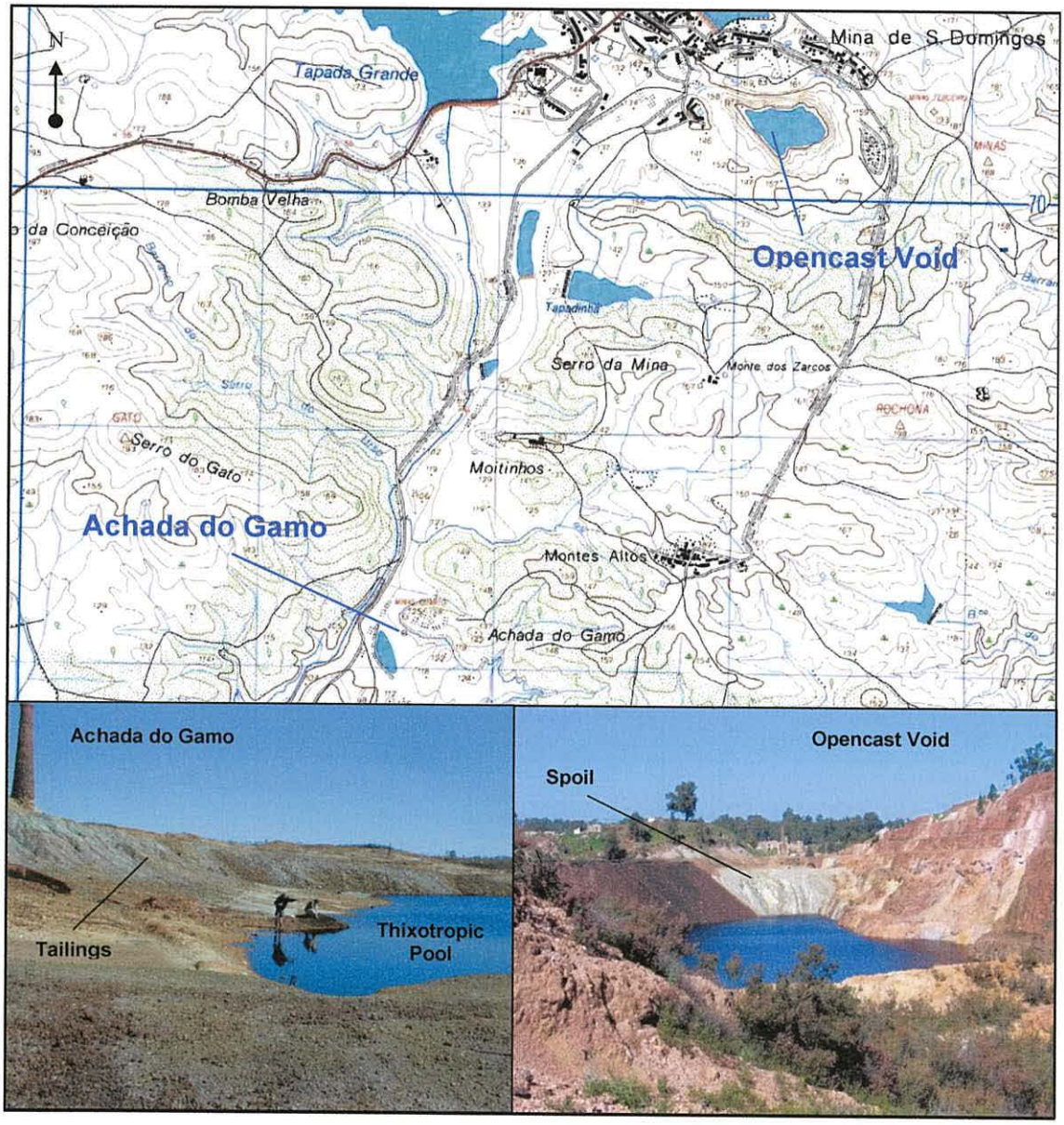

Figure 4.1 Location of the opencast void spoil and Achada do Gamo tailings heaps within the former São Domingos copper mine site. (Map courtesy of Daniel de Oliveira, INETI Geological Data Centre, Portugal.)

4.2.1 Isolation of indigenous microorganisms

Microorganisms were detached from the mineral matrix, as described in Chapter 2, using HBS at *p*H 2.0. The resulting liquor was serially diluted, plated onto Fe$\underline{o}$, FeS$\underline{o}$, FeT$\underline{o}$ and YE3$\underline{o}$ solid media (section 2.2.1.2) and incubated aerobically at 30°C for 2-3 weeks.

Iron (Fe), pyrite (P), iron-yeast extract (FeYE), pyrite-yeast extract (PYE) and sulfur (S) liquid media (section 2.1.3) were used to enrich for acidophilic microorganisms in the mine spoil and tailings. The media (single replicates of 50 mL in 100 mL flasks) were inoculated with small quantities (*ca.* <0.25 g total) of spoil or tailings, from either 0-10 cm or 50-60 cm depth, and incubated aerobically, shaken at 120 rpm, at either 30°C or 45°C. Enrichment cultures were viewed periodically using a phase contrast microscope. When microbial growth was evident they were streaked onto Feo and FeSo solid media. Plates were incubated aerobically at 30°C or 45°C, as appropriate, and examined regularly for colony growth.

The dominant colony types from each plate were sub-cultured on appropriate solid media prior to further identification, as described in sections 2.1-2.6. Isolates were transferred to iron-tetrathionate-TSB (FeST), sulfur (S) or "*Ferroplasma*" (Fp) liquid media, as appropriate, for use in defined population bioleaching experiments.

The ability of isolate SDE4 to grow in conditions of elevated osmotic potential was compared to that of *At. thiooxidans* type strain (ATCC 19377) and *At. caldus* type strain (DSM 8584). Sodium chloride was used to increase the osmotic potential of liquid medium. Universal bottles containing 5 mL of tetrathionate medium (comprising UBS (*p*H 2.5), 100 µM $FeSO_4$, 2.5 mM $K_2S_4O_6$) with or without 500 mM NaCl were inoculated and incubated at 30°C. Growth was assesed qualitatively by microscopy.

4.2.2 Bioleaching experiments

Bioleaching of spoil or tailings from the São Domingos mine was carried out in triplicate 100 mL shake flasks containing 1% (w/v) of ground sieved spoil or tailings and 50 mL of either RO water or a basal salts solution (BS+TE), adjusted to *p*H 2.0. Flasks were inoculated with 0.5 mL aliquots from the pyrite (30°C) enrichment cultures that had been inoculated with spoil or tailings from the surface (0-10 cm) and 50-60 cm depth. These were incubated in a shaking incubator (120 rpm) at 30°C and sampled at regular intervals, as described in section 2.5.2. Concentrations of ferrous iron, total soluble iron, copper and zinc, and sulfate were measured. Total soluble metals were measured by AAS using a Varian AA Duo system operated in flame mode.

Subsequent tailings bioleaching experiments used either pure cultures or a defined population of isolates. In these experiments, the liquid media were either the basal salts solution, or basal salts supplemented with 0.02% (w/v) yeast extract (*p*H 2.0).

The inocula used were either the pyrite enrichments from the spoil or tailings, pure cultures of SDE2, SDE4, SDE29 or SDKH with *Acidiphilium* sp. SJH, or defined mixed cultures of SDE2, SDE4, SDE13 and SDE17 (the 'SDEfour' consortium) or SDE2, SDE4, SDE13, SDE17 and SDE29 (the 'SDEfive' consortium). SDE2 was grown in FeT liquid medium aerobically at 30°C. SDE4 was grown in S liquid medium aerobically at 30°C. SDE29 was grown in Fp liquid medium microaerobically at 37°C. SDKH/*Acidiphilium* sp. SJH were grown in Fe liquid medium aerobically at 30°C. The SDEfour and SDEfive consortia were created by inoculating FeT liquid medium with SDE2, SDE4 and SDE29 from liquid media and with SDE13 and SDE17 colonies from solid media. It was necessary to inoculate the consortia with isolates SDE13 and SDE17 from solid media as these organisms would not grow in any of the liquid media tried. All defined liquid cultures were incubated at 30°C for 5 days prior to their use as inocula.

4.2.3 Biomolecular analysis

Spoil and tailings materials were pre-treated to increase *p*H and remove soluble ions prior to DNA extraction. Samples (0.25-1.0 g) were suspended in 100 µM - 1M concentrations of TRIS buffer with or without 50 mM EDTA. These were then centrifuged for 2 min at different speeds and the *p*H of the supernatant measured. The process was repeated as necessary until the *p*H was raised (if possible) to around *p*H 7.0. Pellets were then transferred to the MoBio DNA extraction kit and processed as described in section 2.2.1.1.

Crude lysates of both the enrichment cultures and the bioleaching cultures were prepared as described in section 2.2.1.1. PCR and T-RFLP analysis were carried out on community DNA from the enrichments, bioleaching experiments and on individual isolates using the 27f(G):1387r primer pair. T-RFs in community samples were compared to a list of those obtained from individual isolates.

4.3 RESULTS

The *p*H and concentrations of extractable metals in the spoil and tailings samples collected in March 2004 are shown in Table 4.1. Both samples were highly acidic, and contained high concentrations of readily extractable metals. The spoil was the more acidic, and had the highest concentrations of readily extractable zinc, but had the lowest concentrations of readily extractable iron and copper. Most of the copper in the tailings was in a readily extractable form. On the surface of the tailings there were abundant greenish-blue crystals, tentatively identified as melanterite ($FeSO_4 \cdot 7H_2O$) and copper sulfate minerals, such as bonattite and chalcanthite ($CuSO_4 \cdot nH_2O$).

Table 4.1 Basic geochemical data for the São Domingos spoil and tailings (mean values from materials from different depths).

Sample	*p*H*	Readily extractable (mg g^{-1})			Total extractable (mg g^{-1})		
		Fe	Cu	Zn	Fe	Cu	Zn
Spoil[a]	2.06	15.19	0.45	0.69	104.0	1.24	2.17
	(0.06)	(1.38)	(0.09)	(0.05)	(1.55)	(0.09)	(0.27)
Tailings[b]	2.29	26.27	5.65	0.24	192.4	8.82	1.13
	(0.03)	(4.39)	(2.9)	(0.06)	(1.76)	(0.14)	(0.56)

Bracketed figures are standard deviation; $n = 3$; [a]Spoil from the opencast void, [b]Tailings from the deposit in the Achada do Gamo region; *1:2.5 spoil or tailings to RO water.

The run-off from the tailings is retained within a deep-red thixotropic pool, which dries up almost completely in the hot summer months. Extremely elevated metal concentrations and conductivities have been recorded in this pool at different times of the year, as shown in Table 4.2.

Table 4.2 Microbial and geochemical data for the Achada do Gamo pool, São Domingos copper mine, Portugal (modified after Johnson, unpublished).

Sampling date	*p*H	Conductivity (mS cm^{-1})	$[Fe^{2+}]$ (mg L^{-1})	$[Fe_{total}]$ (mg L^{-1})	$[SO_4^{2-}]$ (mg L^{-1})	Viable microorganisms (cfu mL^{-1})
July 2003	2.1	630	38,000	180,000	350,650	<10
Nov 2003	1.7	26	10,000	31,000	14,850	3.5×10^6
March 2004	2.55	15	2,100	6,400	38,015	5.0×10^7
Feb 2005*	1.13	88.5	18,000	66,000	180,000	3.5×10^3
	0.19	1,567	33,000	176,000	585,600	<10

*On this sampling date, the pond was mostly dry, following a long period without rainfall. Data are from two small pools within the pond basin that still retained some water.

4.3.1 Isolation of indigenous microorganisms

The initial cultivation exercise resulted in the isolation of two "*Firmicutes*" from the Achada do Gamo tailings. Isolate SD4 was most closely related to the acidophilic iron-oxidising moderate thermophile *Sulfobacillus* sp. KOZ02 (accession ID: DQ350778), which was isolated from a geothermal spring in the Yellowstone National Park, U.S.A., sharing 97.5% 16S rRNA gene sequence identity. Isolate SD5 was most closely related to another thermotolerant iron-oxidising acidophilic firmicute, Y0010 (accession ID: AY140235), also isolated from Yellowstone, sharing 96.5% 16S rRNA gene sequence identity. Despite pretreatment, it was not possible to amplify DNA from this sample with the DNA extraction method used.

No isolates were obtained directly from the spoil or tailings collected in November 2003. Again, DNA was not successfully amplified from the spoil or tailings, despite a numerous attempts involving a variety of pretreatments.

No DNA was amplified from the spoil or tailings collected in March 2004. However, on this occasion, microorganisms were successfully isolated from enrichment cultures inoculated with either spoil or tailings. Enrichment cultures maintained at 45°C showed no signs of microbial growth after six weeks of incubation, even though temperatures at the mine site can often exceed 40°C in the summer months, suggesting that moderate thermophiles were not present in either sample. In contrast, the 30°C enrichment cultures showed signs of microbial growth after two to three weeks incubation. In total, 13 bacterial isolates showing different colony morphologies were obtained from the spoil and 13 from the tailings. Together these were found to represent 5 distinct RFLP groups. In addition, a single archaeon was isolated from the spoil. Table 4.3 summarises the colony morphologies of these isolates, and their closest known relatives based on 16S rRNA gene sequence identity. Table 4.4 details the two dominant organisms on the Feo and FeSo plates inoculated with each of the different enrichment cultures.

Isolate SDE2 is most closely related to an iron-oxidising firmicute (G1) isolated from Montserrat, W.I. (Johnson, unpublished), although the relatively low 16S rRNA gene identity (94.5%) indicates that the two organisms are only distantly related. 16S rRNA gene identity suggests that isolate SDE4 is

identical to the type strain of *At. thiooxidans*. However, this is based on the analysis of a partial length of the gene, and so this isolate can only be described as *At. thiooxidans*-like, in the absence of further characterisation. Isolates SDE9, SDE13 and SDE17 are closely related to uncultured "*Firmicutes*", tentatively assigned to the family *Alicyclobacillaceae*. The latter two isolates shared greater than 99% pairwise 16S rRNA gene identity to each other, but differed slightly in their RFLP patterns. Partial (~1.3kb) 16S rRNA gene DNA sequences of isolates SDE2 and SDE17 have been deposited in the GenBank sequence repository and have accession IDs DQ533683 and DQ533684, respectively. SDE29 is closely related to *Ferroplasma acidiphilum*, an iron-oxidising archaeon, which is now thought to be obligately heterotrophic (Dopson *et al.*, 2004).

Table 4.3 Colony morphologies of São Domingos isolates and their closest known relatives, based on 16S rRNA gene identity.

Isolate	Description	Nearest organism(s) (accession ID)	Identity
SDE2	Iron/sulfur-oxidising colony on FeSo plate	Iron-oxidizing acidophilic firmicute G1 (AY529492)	94.5%
		Alicyclobacillus pomorum (AB089840)	89.4%
SDE4	Large sulfur-oxidising colony on FeSo plate	*Acidithiobacillus thiooxidans* strain OGCS3 (AY830898)	99.4%
SDE9	Large iron-oxidising colony on FeSo plate	*Uncultured Sulfobacillus* sp. K55 (AF460984)	97.2%
		Sulfobacillus thermosulfidooxidans strain G2 (AY140233)	96.8%
SDE13	Large iron-oxidising colony on FeSo plate	Uncultured *Sulfobacillus* sp. (AY262719)	98.9%
		Sulfobacillus thermosulfidooxidans strain G2 (AY140233)	95.4%
SDE17	Iron-oxidising colony on FeSo plate	Uncultured *Sulfobacillus* sp. (AY262719)	99.8%
		Sulfobacillus thermosulfidooxidans strain G2 (AY140233)	97.0%
SDE29	Small, subsurface translucent colonies on FeSo plate	*Ferroplasma acidiphilum* strain DR1 (AY222042)	99.5%

*Isolates differentiated on the basis of 16S rRNA gene RFLP patterns, despite sharing greater than 99% 16S rRNA gene sequence identity.

While none of the bacterial isolates were able to grow at 45°C on FeSo plates, isolate SDE4 survived for several days at this temperature, and was able to grow when the plate was subsequently incubated at 30°C. Isolate SDE4, and the type strains of both *At. thiooxidans* and *At. caldus* were able to grow in medium containing 500 mM NaCl. This suggests that both of these sulfur-oxidising acidithiobacilli are tolerant of osmotic stress caused by high solute concentrations, and are more tolerant of chloride than *At. ferrooxidans*.

The "*Firmicutes*" more commonly dominated the FeSo plates from the enrichment cultures; the *Alicyclobacillaceae* isolates were seemingly more common in the enrichments inoculated with spoil and both these and the G1-like isolate were common in the enrichments inoculated with tailings.

Table 4.4 Dominant isolates obtained from Feo and FeSo solid media inoculated with different enrichment cultures.

Isolate	Enrichments inoculated with spoil from the surface and from 50-60 cm depth						Enrichments inoculated with tailings from the surface and from 50-60 cm depth					
	P	PYE	Fe	FeYE	S	Total	P	PYE	Fe	FeYE	S	Total
SDE2						**0**	+		+	+		**3**
SDE4	+		++			**3**	++					**2**
SDE9				+		**1**						**0**
SDE13	++		+		++	**5**			+		++	**3**
SDE17	+	+			+	**3**	++		+	++		**5**
SDE29				+		**1**						**0**

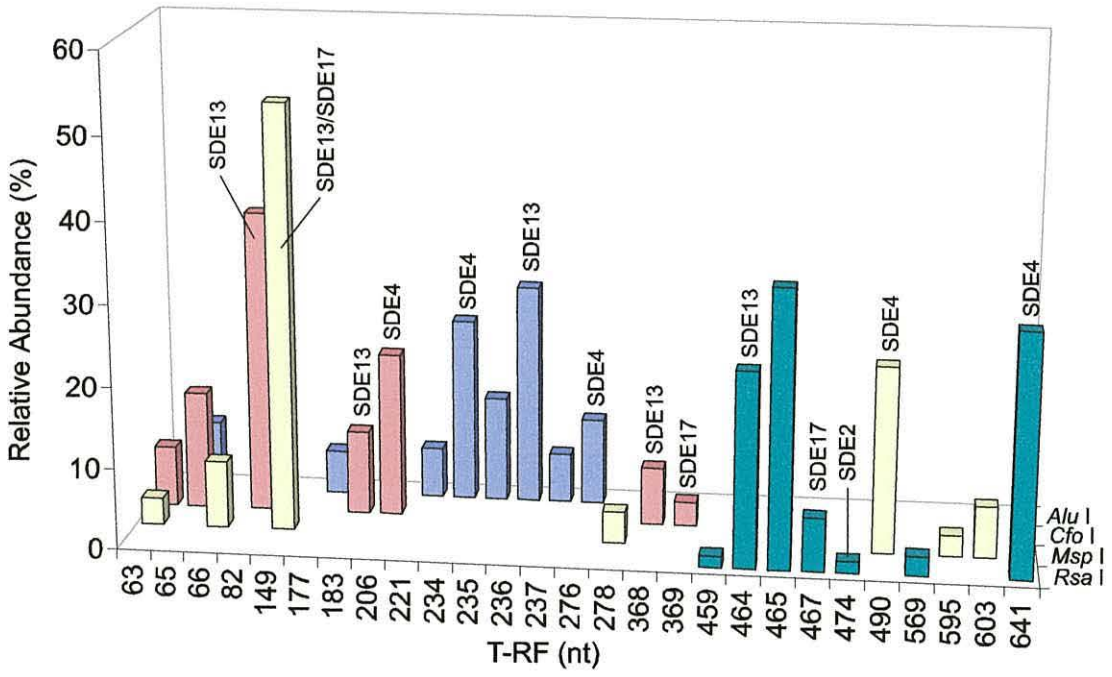

Figure 4.2 Community T-RFLP profile of the pyrite enrichment culture inoculated with São Domingos tailings, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I, *Msp* I and *Rsa* I. (Key: , *Alu* I; , *Cfo* I; , *Msp* I; , *Rsa* I.)

No DNA was amplified from cell lysates made from the spoil enrichments, and only bacterial DNA was amplified from lysates made from the pyrite enrichments inoculated with the tailings. T-RFLP profiles

constructed using the restrictions enzymes *Alu* I, *Cfo* I, *Msp* I and *Rsa* I are shown in Figure 4.2. T-RFs could be matched with those obtained from the enrichment isolates. Some isolates presented multiple T-RFs, and in some cases sequence analysis appeared to demonstrate that these were due to different alleles of the 16S rRNA gene. The enzyme *Rsa* I was needed to distinguish isolate SDE4 from isolate SDKH (an *At. ferrooxidans*-like organism isolated from the Achada do Gamo pool) in the community as these two isolates had identical *Alu* I, *Cfo* I and *Msp* I T-RFs.

T-RFLP data indicated that the *Alicyclobacillaceae* isolates SDE13 and, to a lesser extent, SDE17 and the *At. thiooxidans* isolate SDE4 dominated the population of the pyrite enrichment inoculated with tailings. In contrast, the plates from this enrichment were dominated by isolates SDE4 and SDE2.

4.3.2 Bioleaching experiments

The results of bioleaching of São Domingos spoil and tailings by the pyrite enrichment cultures inoculated with either spoil or tailings, respectively, are described below. The effects of inorganic nutrient amendment, in the form of HBS, were investigated.

4.3.2.1 Spoil

The pyrite enrichment culture obtained from the spoil was largely ineffective in leaching metals from spoil material. The addition of inorganic nutrients made little or no difference in the concentrations of solubilised analytes.

Although ferrous iron concentrations did appear to have dropped slightly by day 70 in nutrient-amended cultures, concentrations remained similar to those of uninoculated controls, and followed the same trends. This was true for all analytes. These data are summarised in Figure 4.3 and Figure 4.4.

No DNA was amplified from lysates made from the spoil bioleaching cultures, and therefore, biomolecular analysis of the bioleaching cultures was not possible.

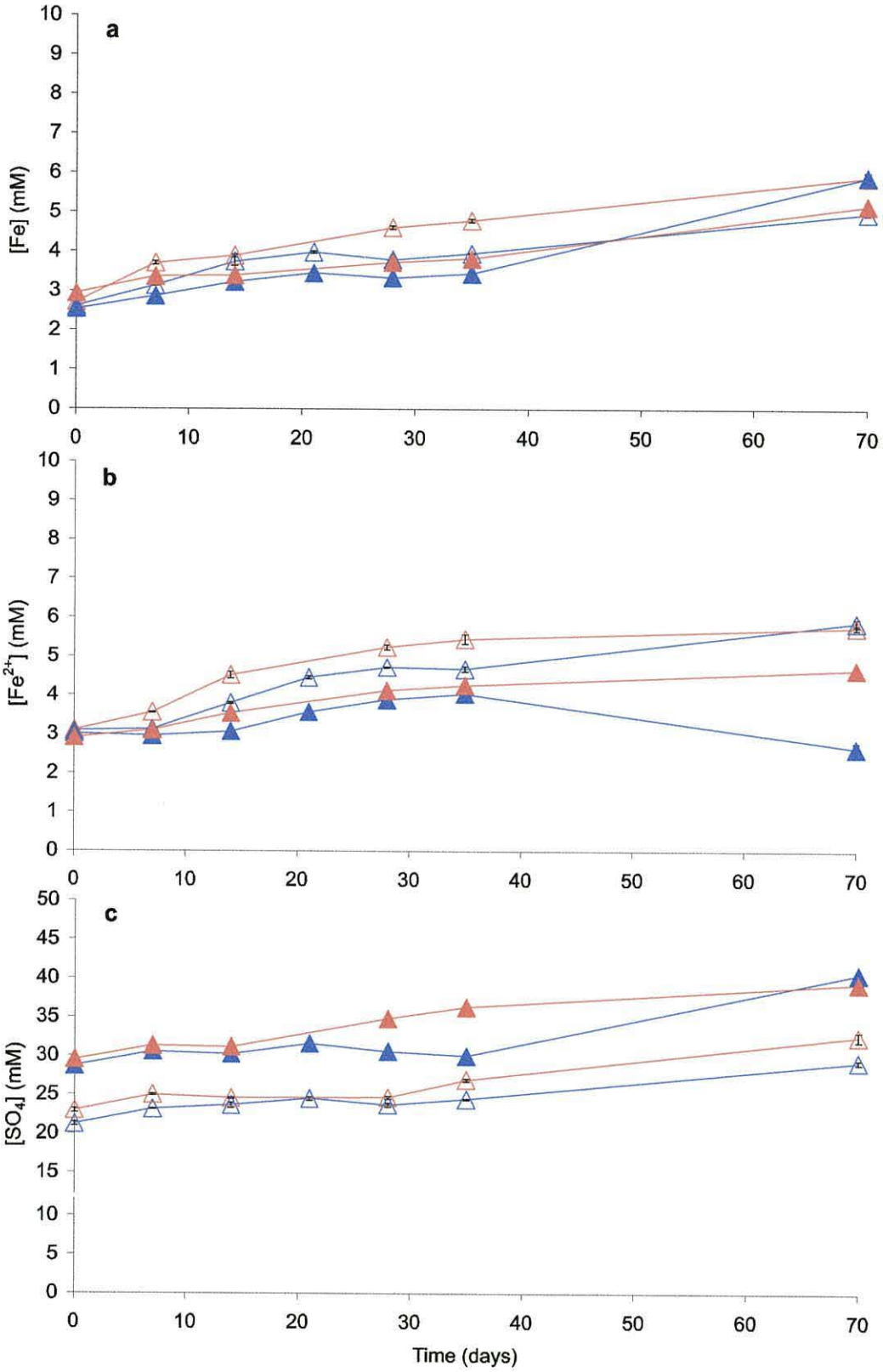

Figure 4.3 Changes in soluble (a) total iron, (b) ferrous iron and (c) sulfate, during bioleaching of São Domingos spoil by the pyrite enrichment culture. (Key: △▲, inoculated with pyrite enrichment culture; △▲, uninoculated controls; Closed symbols, nutrient-amended; Open symbols, nutrient-free; Error bars, where visible, show standard errors; n = 3 for each data point.)

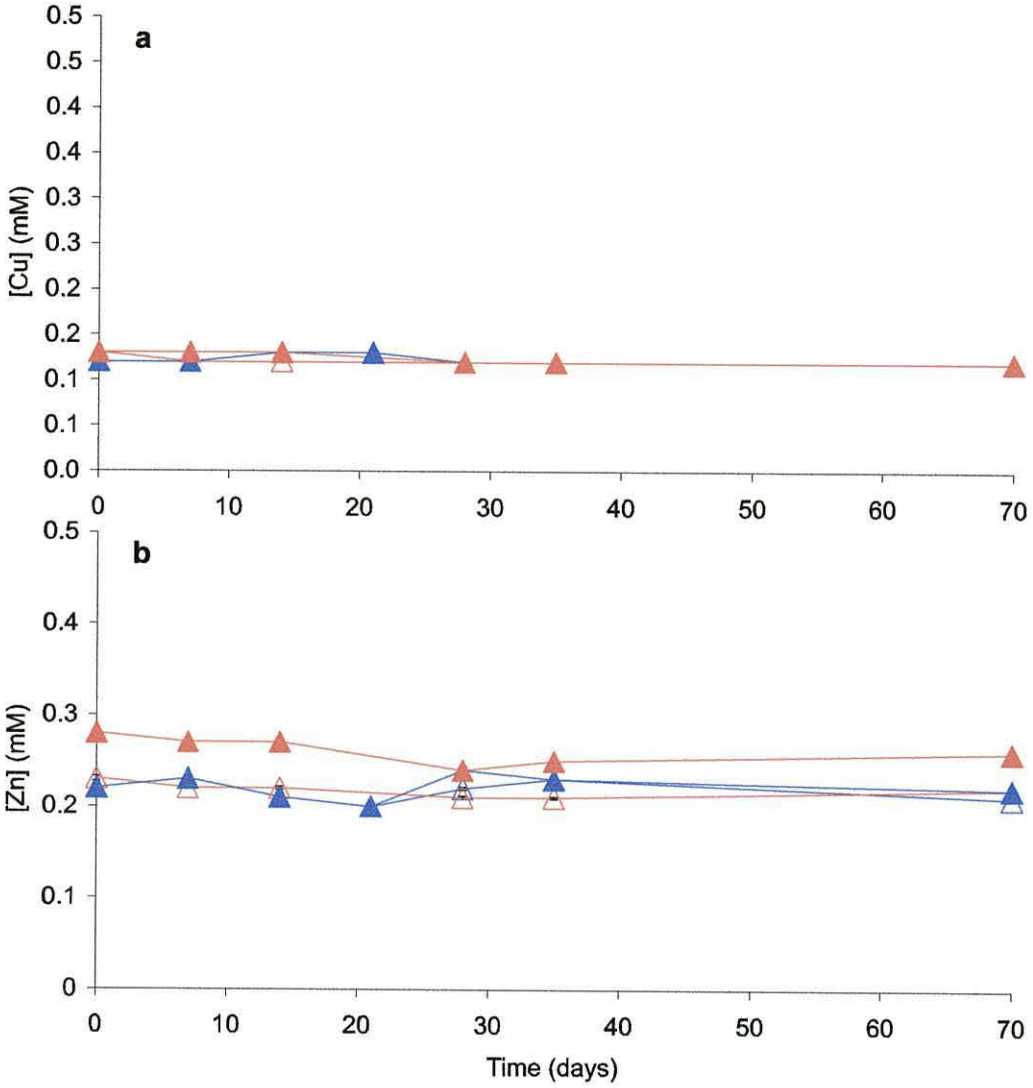

Figure 4.4 Changes in concentrations of soluble (a) copper and (b) zinc, during bioleaching of São Domingos spoil by the pyrite enrichment culture. (Key: △▲, inoculated with pyrite enrichment culture; △▲, uninoculated controls; Closed symbols, nutrient-amended; Open symbols, nutrient-free; Error bars, where visible, show standard errors; n = 3 for each data point.)

4.3.2.2 Tailings

The pyrite enrichment culture obtained from the tailings was capable of extensive mineral mobilisation from sterilised tailings, but only when additional inorganic nutrients were supplied.

Total soluble iron and sulfate concentrations in both cultures increased slightly over the first 21 days. Concentrations then rapidly increased in nutrient-amended cultures compared with the uninoculated controls, whereas nutrient-free cultures showed only a slight increase over time. By day 70, 97%

of the total extractable iron had been leached from the tailings in the nutrient-amended bioleaching cultures.

Soluble ferrous iron concentrations in the nutrient-amended bioleaching cultures decreased steadily to day 21 and remained at <1 mM for the rest of the experiment. In nutrient-free cultures, concentrations decreased initially but then increased slightly and over all differed little from the uninoculated controls (Figure 4.5).

Soluble copper concentrations increased steadily in both nutrient-amended and unamended cultures until around day 21, after which point concentrations in the amended cultures increased more rapidly. Concentrations in both uninoculated controls remained relatively constant. Changes in soluble zinc concentrations showed an apparent acceleration phase of about 21 days, followed by a more pronounced increase in concentrations in nutrient-amended cultures compared with both nutrient-free cultures and uninoculated controls. By day 70, 74% of the total soluble copper and 52% of the total soluble zinc had been leached from the tailings (Figure 4.6).

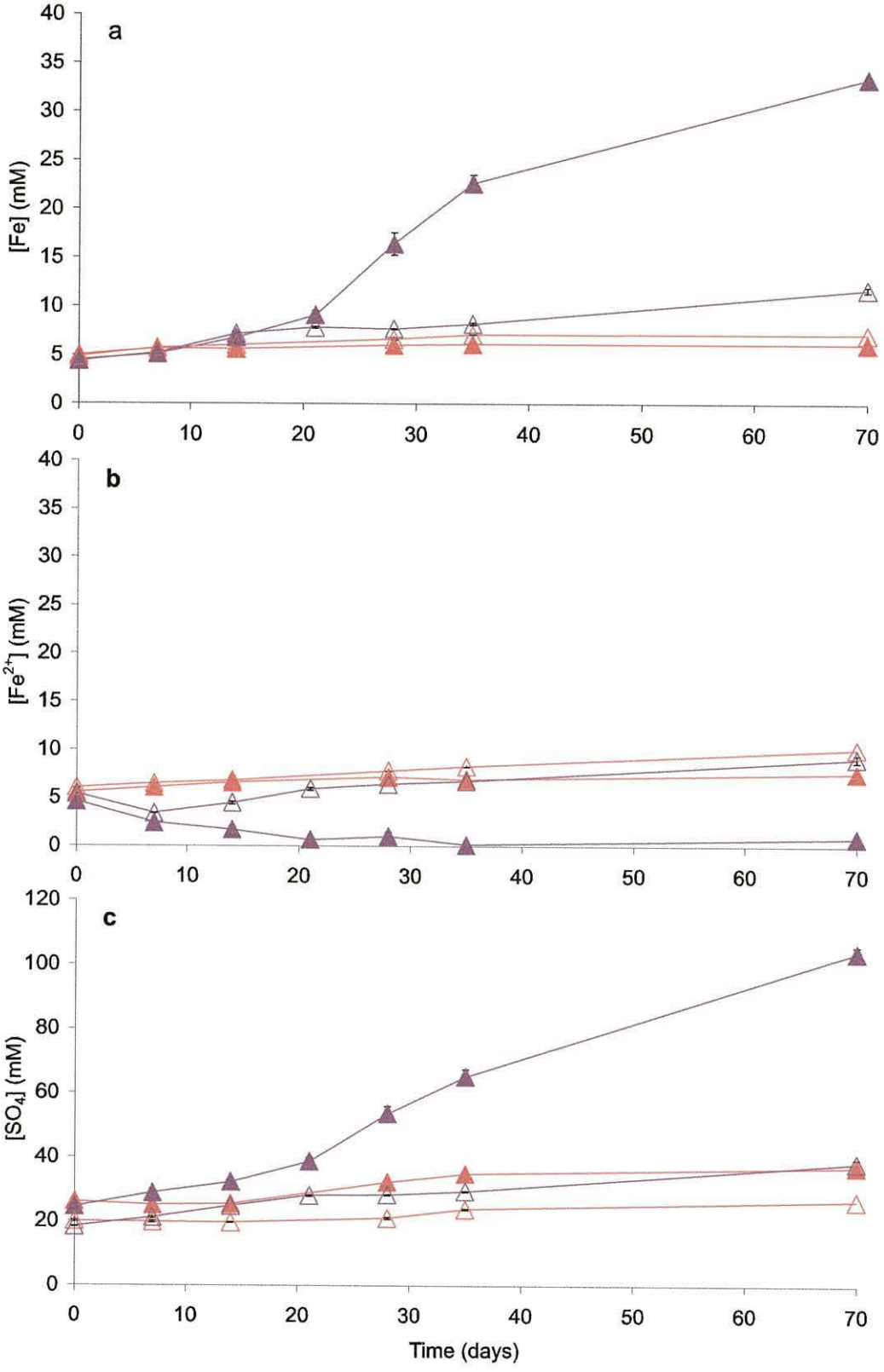

Figure 4.5 Changes in concentrations of soluble (a) total iron, (b) ferrous iron and (c) sulfate, during bioleaching of São Domingos tailings by the pyrite enrichment culture. (Key: △▲, inoculated with pyrite enrichment culture; △▲, uninoculated controls; Closed symbols, nutrient-amended; Open symbols, nutrient-free; Error bars, where visible, show standard errors; *n* = 3 for each data point.)

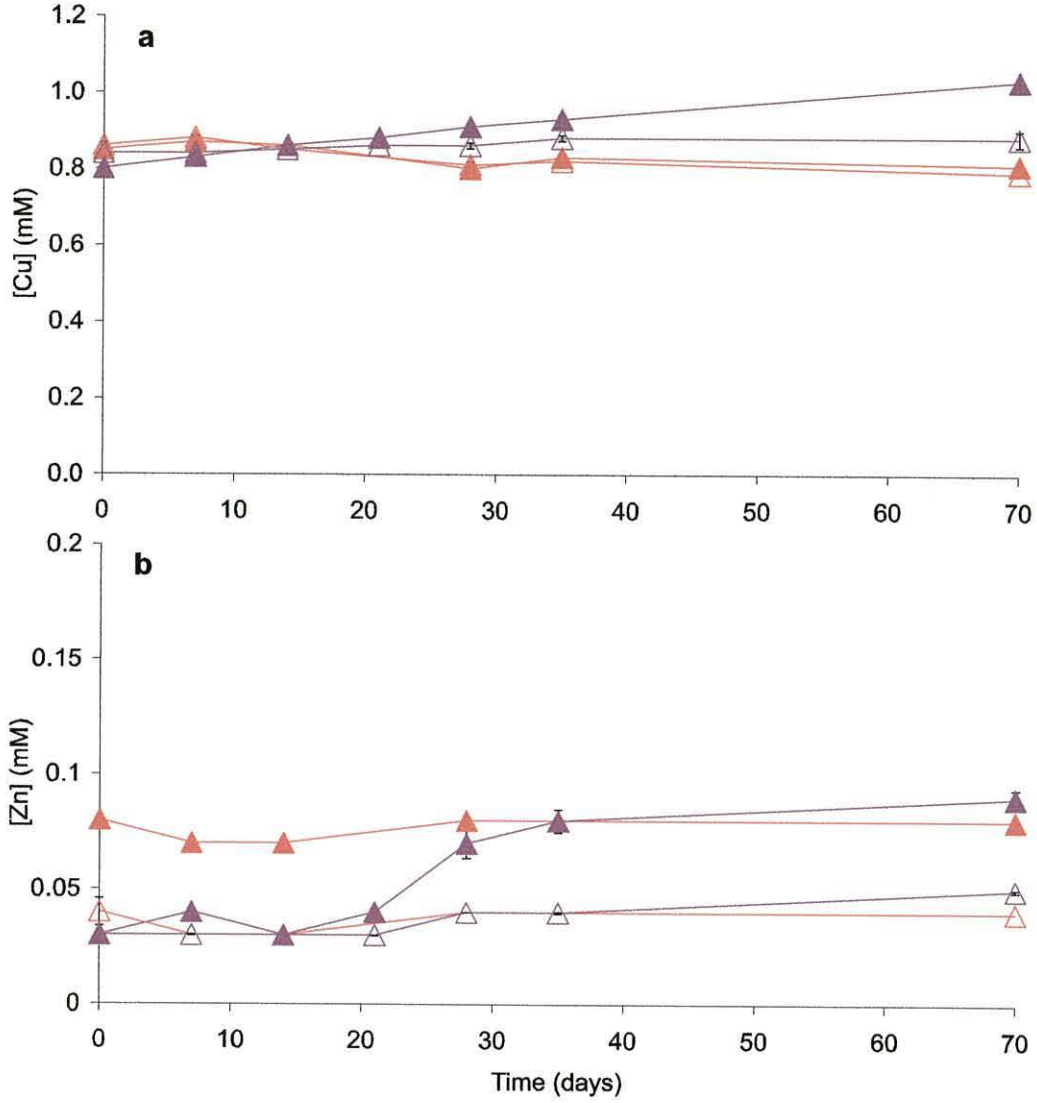

Figure 4.6 Changes in concentrations of soluble (a) copper and (b) zinc, during bioleaching of São Domingos tailings by the pyrite enrichment culture. (Key: △▲, inoculated with pyrite enrichment culture; △▲, uninoculated controls; Closed symbols, nutrient-amended; Open symbols, nutrient-free; Error bars, where visible, show standard errors; n = 3 for each data point.)

Bacterial DNA could be amplified only from crude lysates prepared from the nutrient-amended bioleaching cultures. The T-RFLP profile using the restriction enzymes *Alu* I, *Cfo* I, *Msp* I and *Rsa* I is shown in Figure 4.7. This shows that the population was dominated by the *Alicyclobacillaceae* isolates SDE13 and, to a lesser extent, SDE17 and the *At. thiooxidans* isolate SDE4. In contrast, the only two isolates to grow on FeSo plates at the end of the bioleaching exercise were SDE2 and SDE4. When compared to the population profiles of the pyrite enrichment used as the inoculum, these data imply that the population composition was essentially the same at the start

and at the end of the bioleaching process. However, while each method suggested two or more isolates were the numerically dominant members of the community before and after bioleaching, the culture data imply that these were isolates SDE2 and SDE4, whereas the T-RFLP data imply that these were isolates SDE4, SDE13 and SDE17.

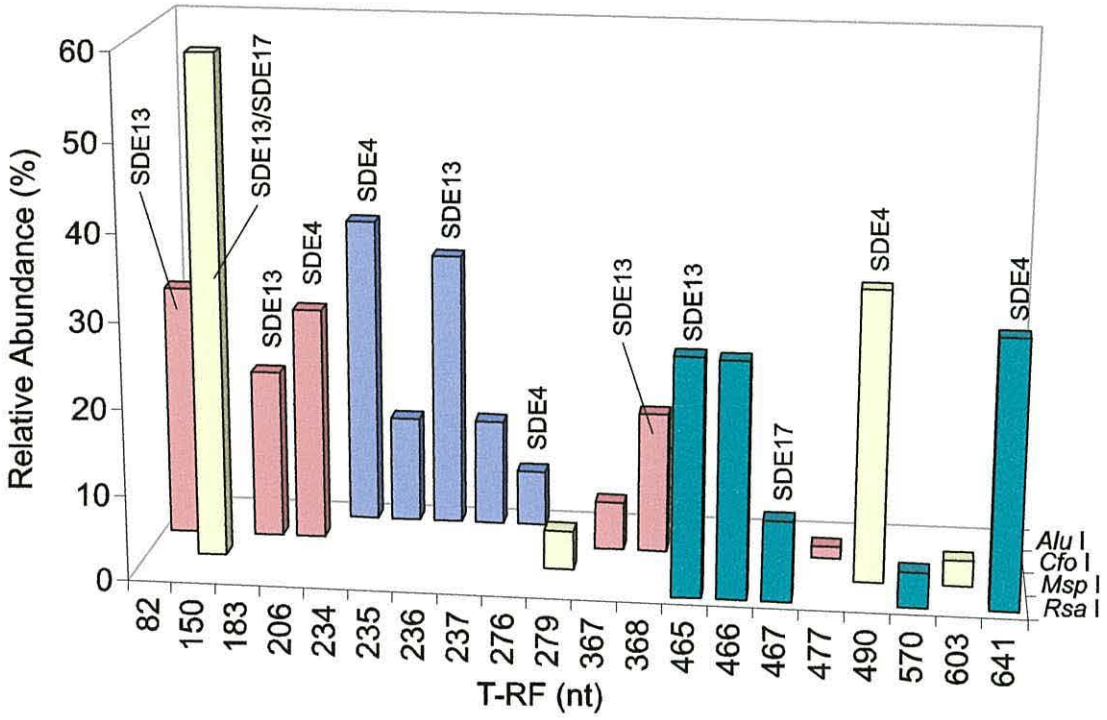

Figure 4.7 Community T-RFLP profile of the nutrient-amended tailings bioleaching culture after 70 days, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I, *Msp* I and *Rsa* I. (Key: , *Alu* I; , *Cfo* I; , *Msp* I; , *Rsa* I.)

4.3.2.3 Pure and defined mixed cultures

Results of bioleaching of tailings by pure or mixed cultures of São Domingos isolates with or without additional organic carbon, in the form of yeast extract (YE), are described below.

4.3.2.3.1 Isolate SDE2

Pure cultures of isolate SDE2, an iron-oxidising firmicute, were able only to effect increased mineral dissolution when amended with an organic carbon source, supplied as yeast extract (YE).

Total soluble iron and sulfate concentrations in YE-amended cultures followed similar trends. Following an initial acceleration phase of 7 days, concentrations rose steadily. Sulfate concentrations appeared to plateau after day 21, but increased again after a second addition of YE (equivalent to 0.02% w/v) at day 63, whereas total iron concentrations continued to increase steadily. Concentrations of both total iron and sulfate were very similar to those in the controls and the cultures that did not contain YE.

Soluble ferrous iron concentrations in YE-amended cultures decreased initially, but began to increase after day 7 and by day 35 were at similar levels to those of the uninoculated controls. Concentrations decreased again following the addition of more YE at day 63. In YE-free cultures, ferrous iron concentrations dropped initially, but by day 14 had risen to the same levels as the controls and paralleled these for the rest of the experiment. These data are summarised in Figure 4.8

Copper concentrations were very similar to those of the controls in both YE-amended and YE-free cultures. The same was true for soluble zinc concentrations, although these did appear to increase slightly in both YE-free and amended cultures after day 21, relative to the controls. The *p*H of the inoculated cultures appeared to decrease relative to the controls, though only slightly. These data are summarised in Figure 4.9.

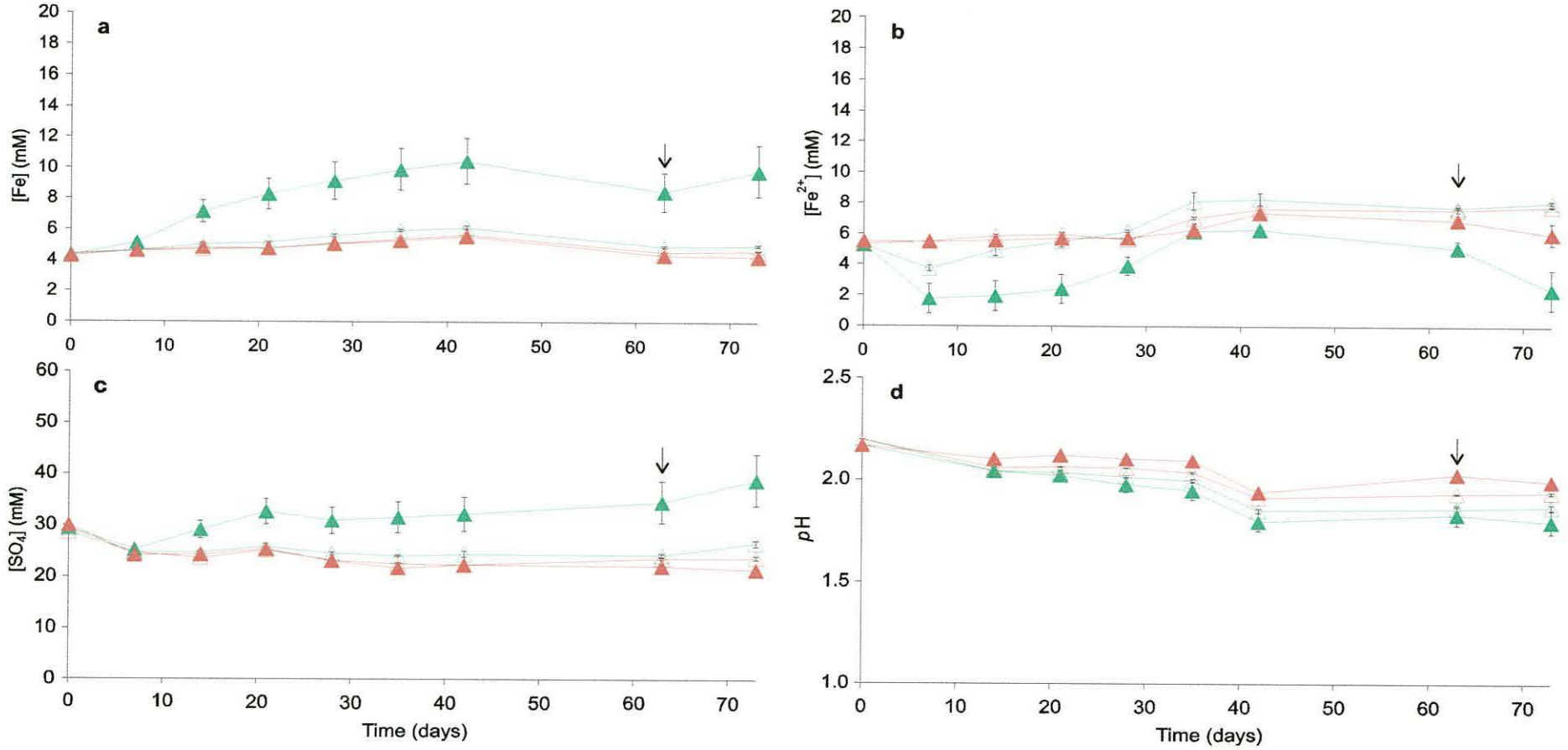

Figure 4.8 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and of (d) *p*H, during bioleaching of São Domingos tailings by a pure culture of isolate SDE2. (Key: △▲, inoculated with isolate SDE2; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; n = 3 for each data point.)

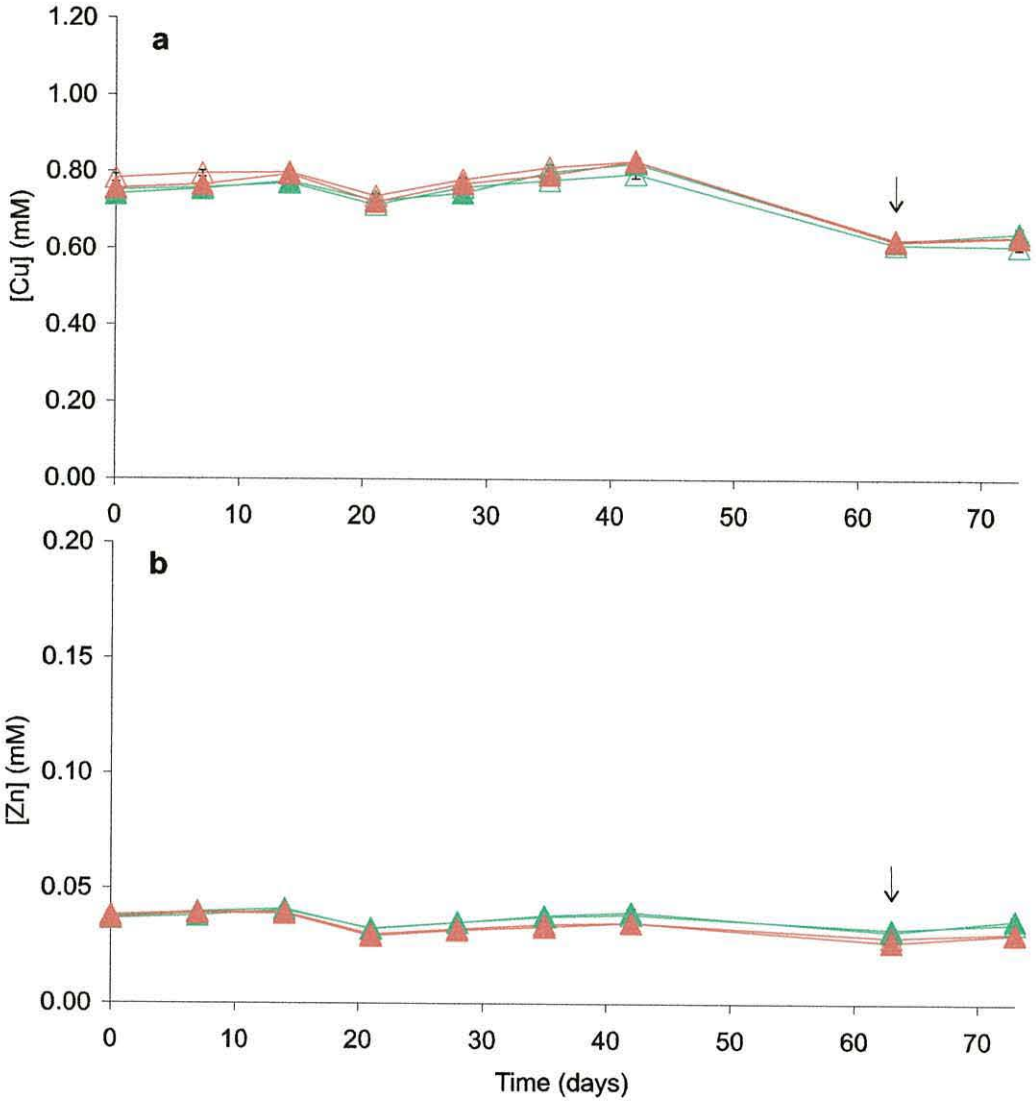

Figure 4.9 Changes in concentrations of soluble (a) copper and (b) zinc, during bioleaching of São Domingos tailings by a pure culture of isolate SDE2. (Key: △▲, inoculated with isolate SDE2; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which additional YE was added to YE-amended cultures; Error bars, where visible, show standard errors; n = 3 for each data point.)

4.3.2.3.2 Isolate SDE4

The *At. thiooxidans*-like isolate SDE4 was unable to accelerate the oxidative dissolution of the São Domingos tailings in pure culture. Concentrations of soluble total iron, ferrous iron, copper and zinc in both YE-amended and YE-free cultures were essentially the same as those of the controls for the duration of the experiment. Sulfate concentrations in the controls decreased slightly between day 0 and 7 and then remained fairly constant. There was no *p*H decrease in either the YE-amended or YE-free cultures and sulfate

concentrations remained constant, though the *p*H was consistently lower in inoculated cultures compared to that of the controls. This may be due to initial oxidation of reduced sulfur compounds associated with the tailings. These data are summarised in Figure 4.10 and Figure 4.11.

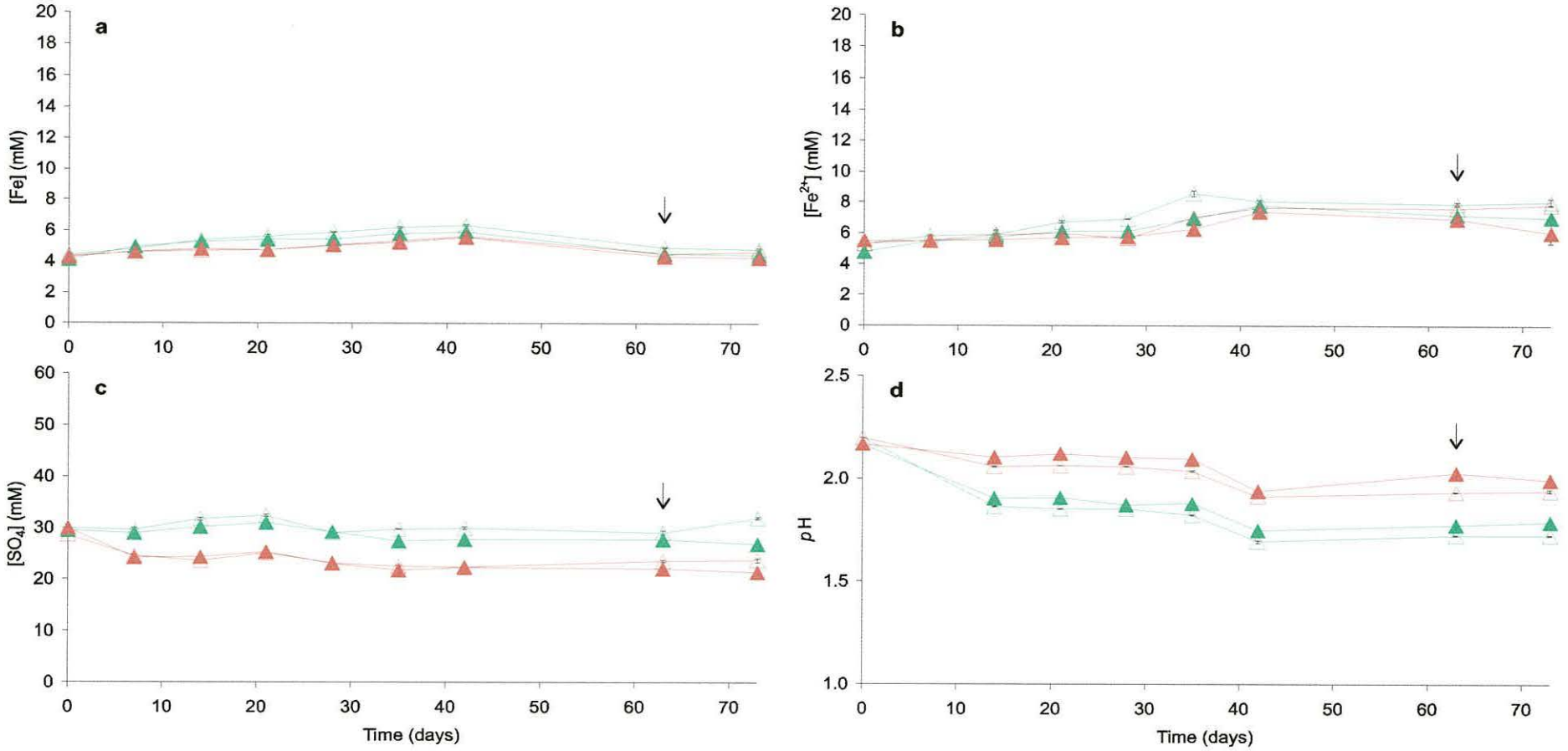

Figure 4.10 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and of (d) *p*H, during bioleaching of São Domingos tailings by a pure culture of isolate SDE4. (Key: △▲, inoculated with isolate SDE4; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; *n* = 3 for each data point.)

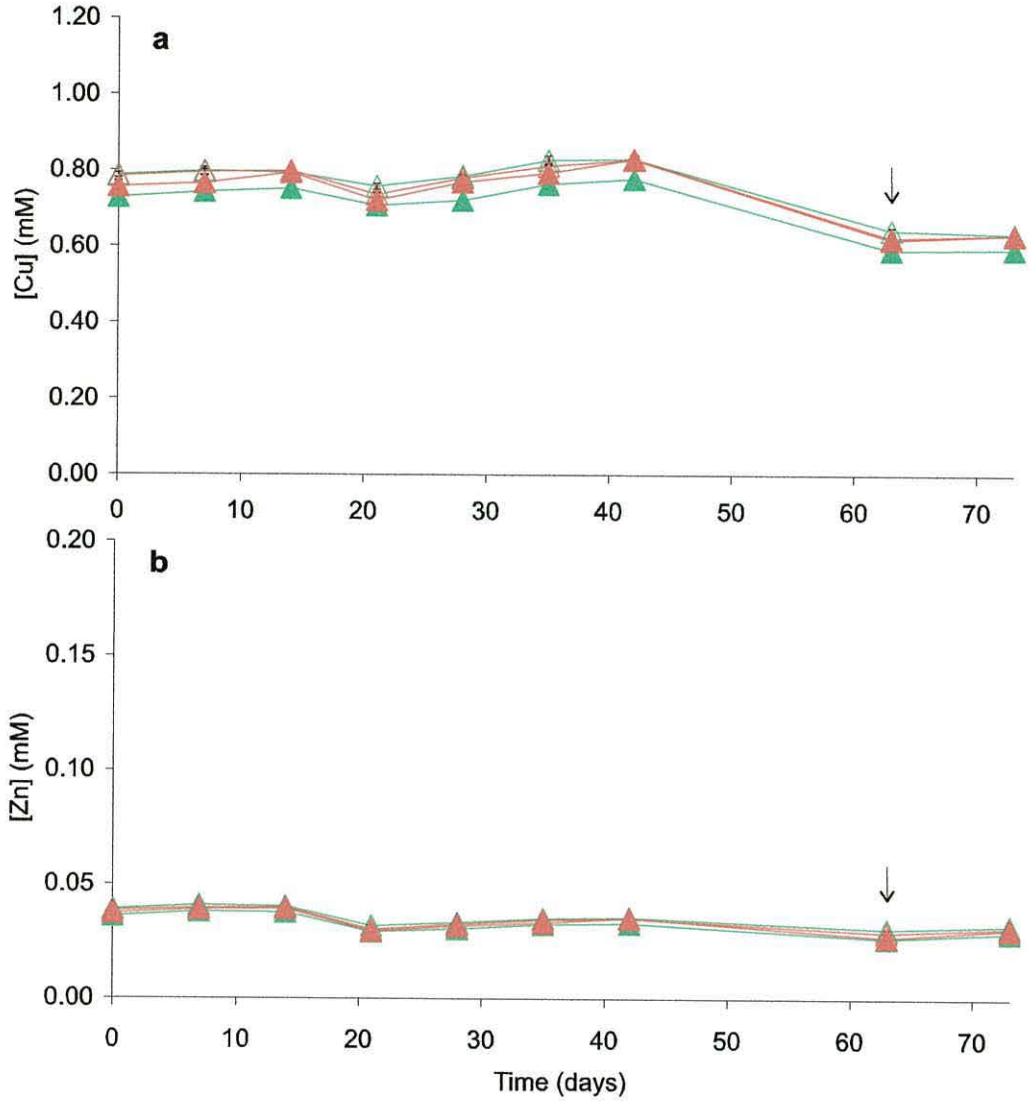

Figure 4.11 Changes in concentrations of soluble (a) copper and (b) zinc, during bioleaching of São Domingos tailings by a pure culture of isolate SDE4. (Key: △▲, inoculated with isolate SDE4; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; *n* = 3 for each data point.)

4.3.2.3.3 Isolate SDE29

The archaeal isolate SDE29 appeared unable to accelerate the oxidative dissolution of the São Domingos tailings in pure culture. Despite the apparent iron oxidation in YE-amended cultures, total soluble iron and sulfate concentrations remained unchanged for the duration of the experiment. Soluble ferrous iron concentrations in YE-amended cultures decreased steadily over time, falling to 0.25 mM by day 35. Despite decreasing in a similar fashion initially, concentrations in YE-free cultures ultimately differed

little from those of the controls. Culture *p*H decreased slightly after day 21 in the inoculated cultures, while remaining fairly constant in the uninoculated controls. The addition of YE appeared to have little effect. These data are summarised in Figure 4.12.

Soluble copper concentrations remained mostly unchanged in inoculated and uninoculated cultures until around day 21. After this point, concentrations in the inoculated controls decreased slightly, while those of the uninoculated controls remained constant. The addition of YE made little or no difference. Concentrations of soluble zinc remained fairly constant in each culture for the duration of the experiment. These data are summarised in Figure 4.13.

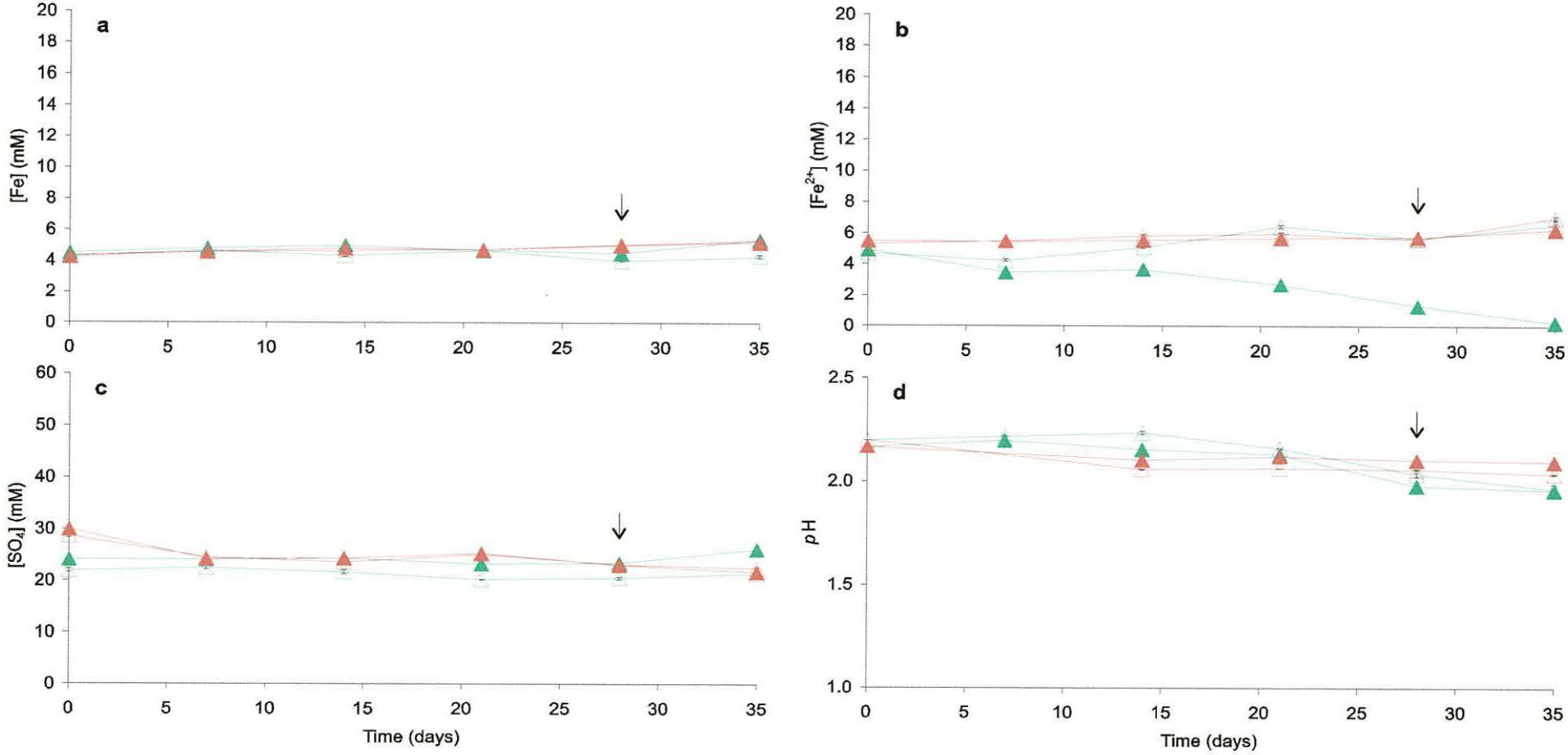

Figure 4.12 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and of (d) *p*H, during bioleaching of São Domingos tailings by a pure culture of isolate SDE29. (Key: △▲, inoculated with isolate SDE29; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard error; n = 3 for each data point.)

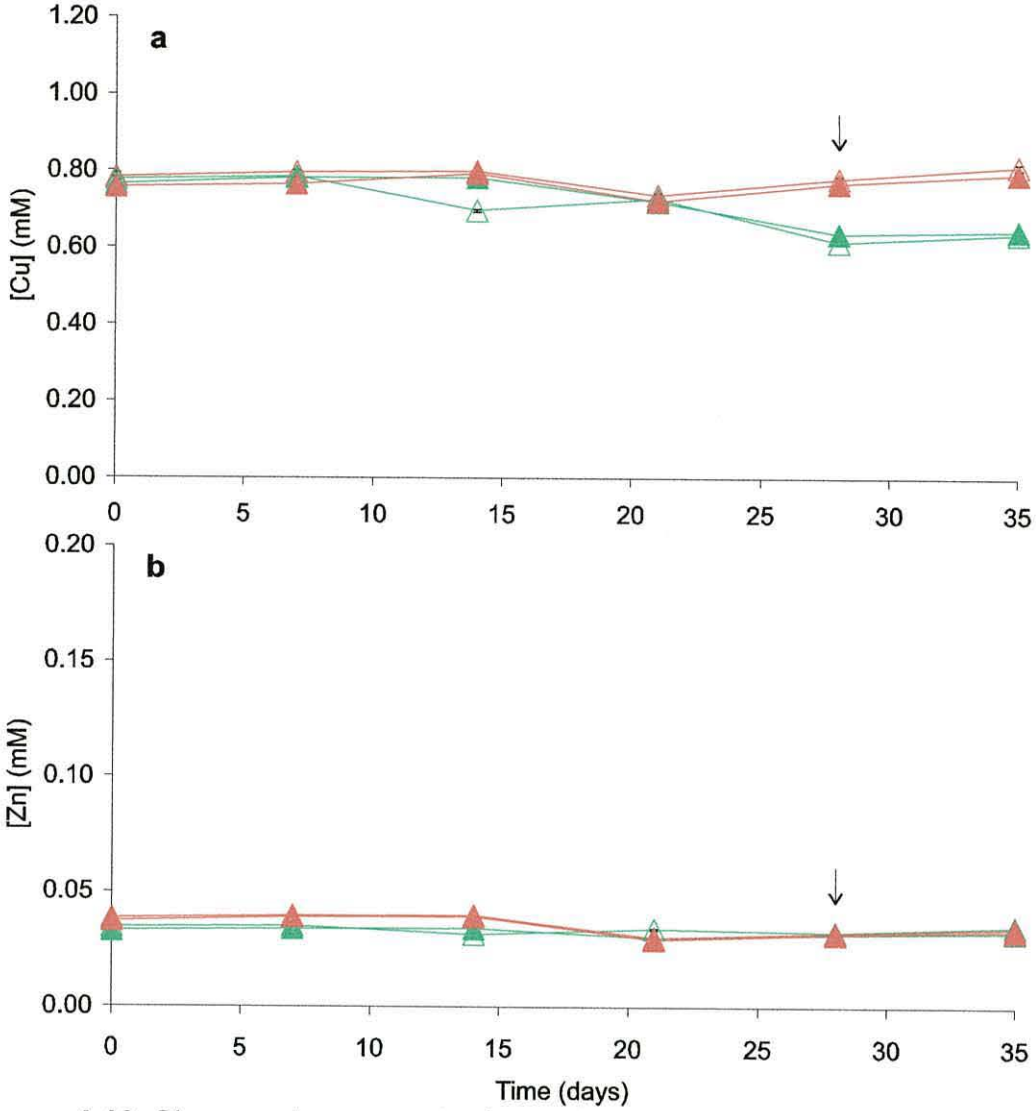

Figure 4.13 Changes in concentrations of soluble (a) copper and (b) zinc, during bioleaching of São Domingos tailings by a pure culture of isolate SDE29. (Key: △▲, inoculated with isolate SDE29; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; *n* = 3 for each data point.)

4.3.2.3.4 Isolate SDKH and Acidiphilium sp. SJH

Isolate SDKH shared 98.5% 16S rRNA gene sequence identity with *Acidithiobacillus ferrooxidans* NO37. It was isolated from the pool at the base of the Achada do Gamo tailings, but was not found in the tailings themselves. During the process of subculturing, the SDKH culture became contaminated with *Acidiphilium* sp. SJH from the under-layer of the Feo plates. As defined, mixed cultures of an obligate chemolithotroph and a non mineral-oxidising heterotroph have been shown to form highly effective bioleaching consortia

(e.g. Wichlacz and Thompson, 1988), the ability of this mixed culture to accelerate the oxidative dissolution of the São Domingos tailings was investigated.

Total soluble iron concentrations increased rapidly initially. The rate was slower than that observed with the nutrient-amended tailings bioleaching culture, following the acceleration phase (see Figure 4.20). Iron concentrations in the SDKH/*Acidiphilium* sp. SJH-inoculated cultures reached a plateau at day 35 and remained at similar levels for the rest of the experiment. Sulfate concentrations increased steadily for the duration of the experiment, although not quite at the same rate as that observed with the nutrient-amended culture inoculated with the enrichment culture from the tailings. Neither iron nor sulfate concentrations were affected by the addition of yeast extract to the cultures. The rapid increase in sulfate concentrations observed after day 63 occurred in both inoculated cultures, despite additional YE (equivalent to 0.02% w/v) having been added to the YE-amended culture only.

Soluble ferrous iron concentrations declined rapidly in inoculated cultures, and from day 7 onwards were maintained at around 0.5 mM. Addition of YE had no discernable effect on the dissolution of the tailings. The culture *p*H decreased substantially in the inoculated cultures during the course of the experiment from just over 2.0 to approximately 1.5, and decreased only very slightly in the uninoculated controls. These data are summarised in Figure 4.14.

Both total soluble copper and zinc concentrations increased in inoculated cultures relative to uninoculated controls. The greatest effect was seen in zinc concentrations in the YE-free culture (Figure 4.15).

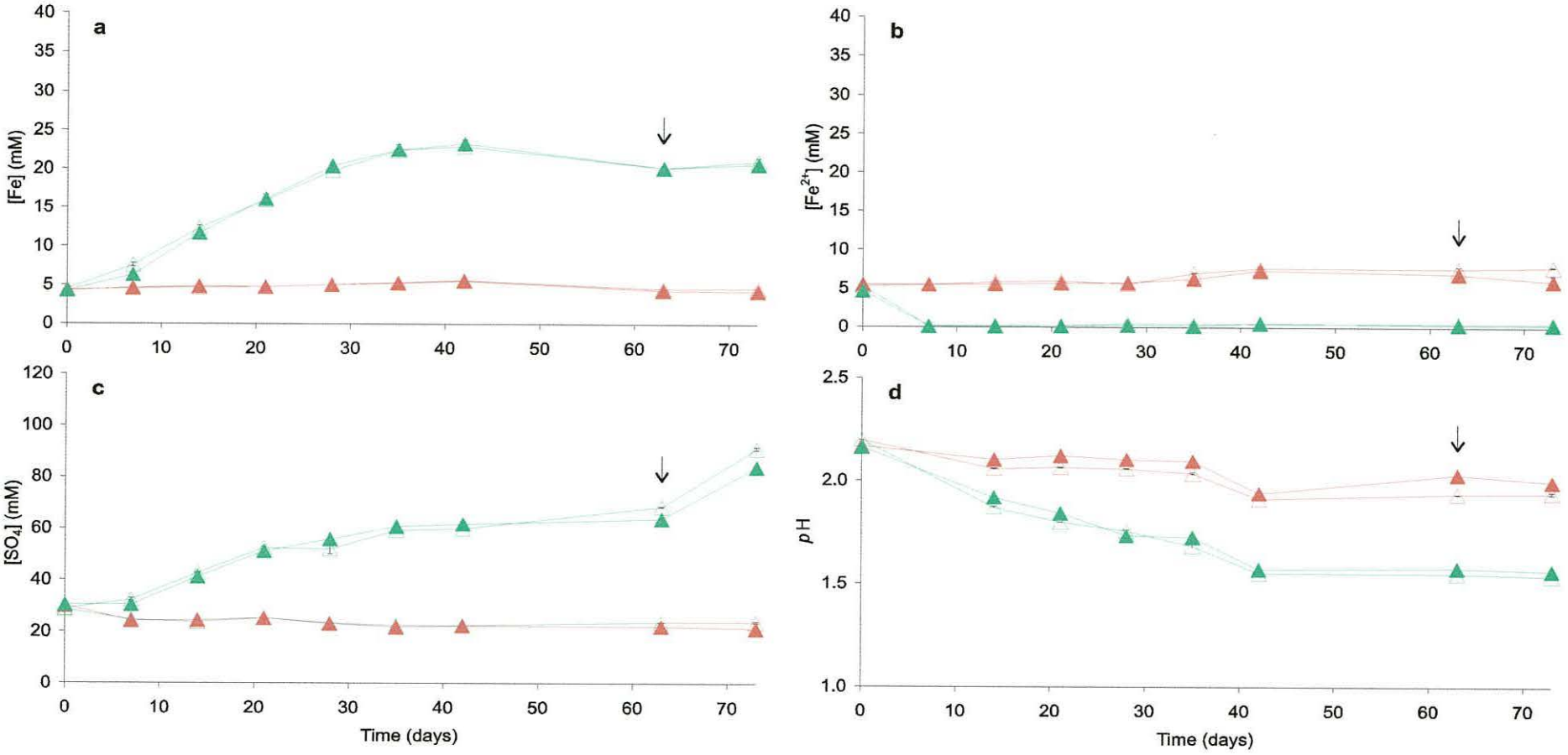

Figure 4.14 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and of (d) *p*H, during bioleaching of São Domingos tailings by a mixed culture of isolate SDKH and *Acidiphilium* sp. SJH. (Key: △▲, inoculated with SDKH and *Acidiphilium* sp. SJH; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; $n = 3$ for each data point.)

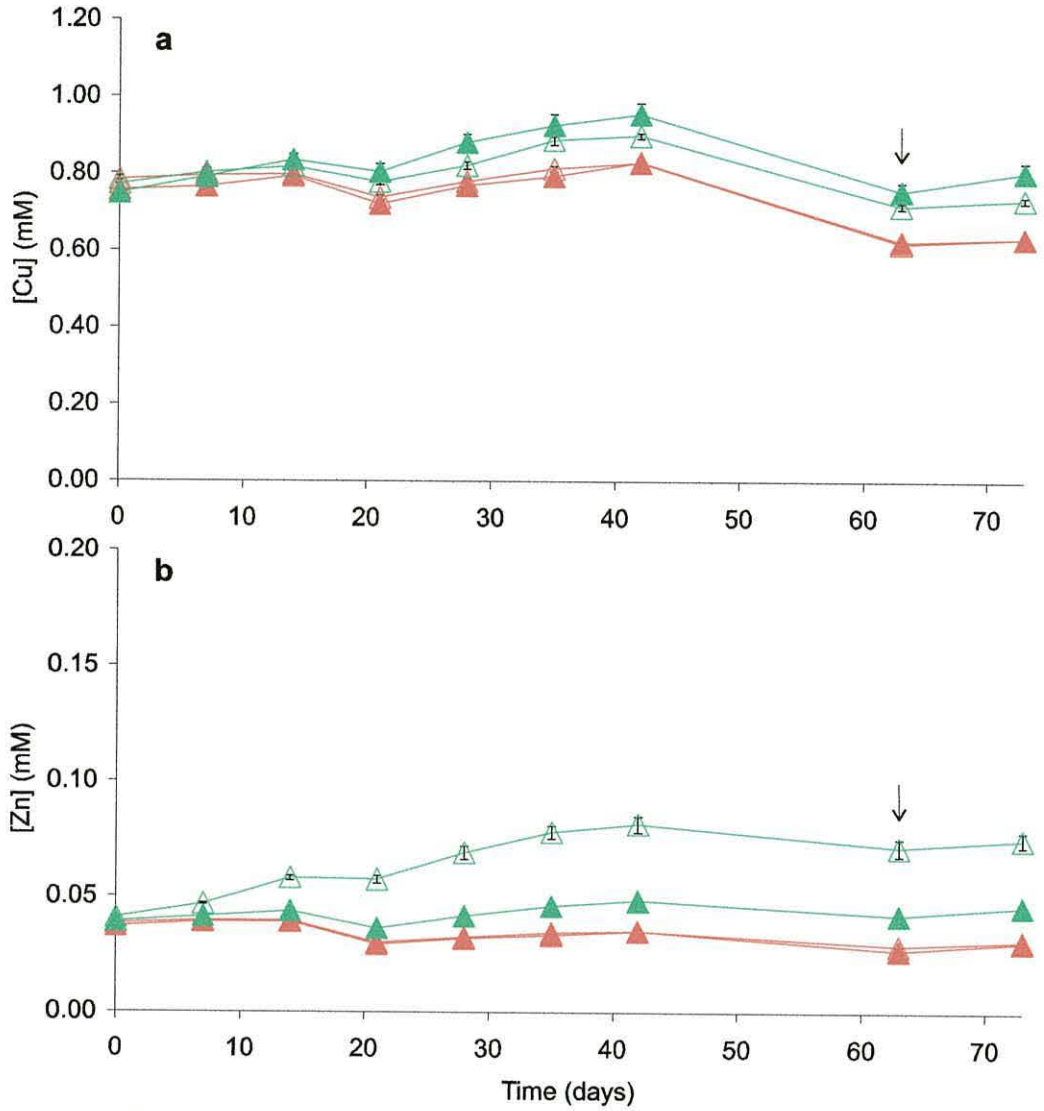

Figure 4.15 Changes in concentrations of soluble (a) copper and (b) zinc, during bioleaching of São Domingos tailings by a mixed culture of isolate SDKH and *Acidiphilium* sp. SJH. (Key: △▲, inoculated with SDKH and *Acidiphilium* sp. SJH; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; n = 3 for each data point.)

4.3.2.3.5 SDEfour

The SDEfour culture comprised isolates SDE2, SDE4, SDE13 and SDE17, and was an attempt to reconstruct the microbial population of the São Domingos tailings, as elucidated by culture data from the enrichment cultures. This mixed culture was assessed for its potential to mobilise metals from the São Domingos tailings. Acceleration of mineral oxidation by this culture, to the same extent as was observed with the pyrite enrichment culture from the

tailings, would imply that it is the population as a whole, rather than specific individuals that is responsible for the extensive metal mobilisation occurring within the tailings *in situ*.

Total soluble iron concentrations in YE-amended cultures increased steadily, following an acceleration phase of approximately 14 days. The rate increased further with the addition of extra YE at day 63. Total iron concentrations in YE-free cultures remained more or less constant for the duration of the experiment, increasing only slightly compared to uninoculated controls. Sulfate concentrations in both YE-amended and YE-free cultures increased steadily, compared to the controls, with no apparent acceleration phase. The rate of increase was consistently higher in YE-amended cultures

In YE-amended cultures, soluble ferrous iron concentrations fell initially, but began to increase slowly after day 14. Following the subsequent addition of YE at day 56, concentrations fell again. Ferrous iron concentrations remained constant for the duration of the experiment in YE-free cultures.

The *p*H of the inoculated cultures was consistently lower than that of the controls, but decreased by a similar amount. There were no considerable differences between YE-amended and YE-free cultures. These data are summarised in Figure 4.16.

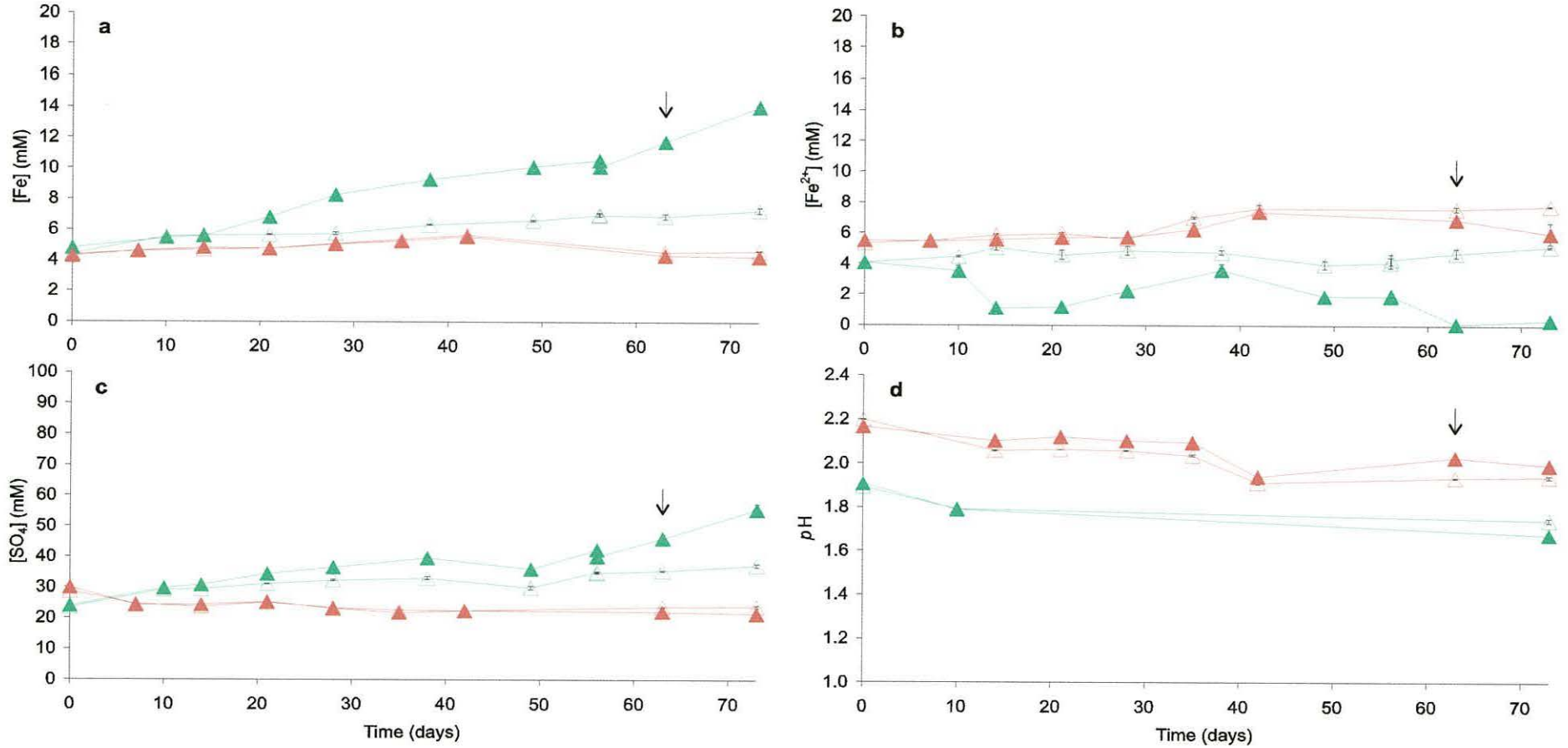

Figure 4.16 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and of (d) *p*H, during bioleaching of São Domingos tailings by the SDEfour consortium. (Key: △▲, inoculated with the SDEfour consortium; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; n = 3 for each data point.)

Bioleaching of the tailings by this consortium was more effective, in terms of increasing total iron and sulfate concentrations and iron oxidation, than either of the two pure cultures tested. Trends in iron oxidation followed those of isolate SDE2 more closely than those of the tailings enrichment culture. However, bioleaching was less effective by this consortium than by a culture of SDKH and *Acidiphilium* sp. SJH (see Figure 4.19).

Analysis of the SDEfour culture inoculum, and bioleaching cultures at days 56 and 73 by T-RFLP showed that despite the *Alicyclobacillaceae* isolates SDE13 and SDE17 having been included in the initial inoculum, they were undetectable in both the inoculum and the bioleaching cultures throughout the experiment. T-RFLP profiles of the bioleaching cultures using the restriction enzyme *Msp* I are shown in Figure 4.17. At the start of the experiment, isolates SDE2 and SDE4 appear almost equally dominant. However, immediately prior to the addition of extra YE on day 56, SDE4 had become the most abundant organism in both cultures. At the end of the experiment on day 73, no DNA could be amplified from the YE-free cultures, whereas the data suggested the relative abundance of the two organisms in the amended cultures had remained unchanged following the addition of more YE.

FeSo plates streaked with bioleaching culture at the end on the experiment were dominated almost entirely with isolate SDE4, with varying, but very low numbers of isolate SDE2 colonies. As with T-RFLP, neither SDE13 nor SDE17 were detected.

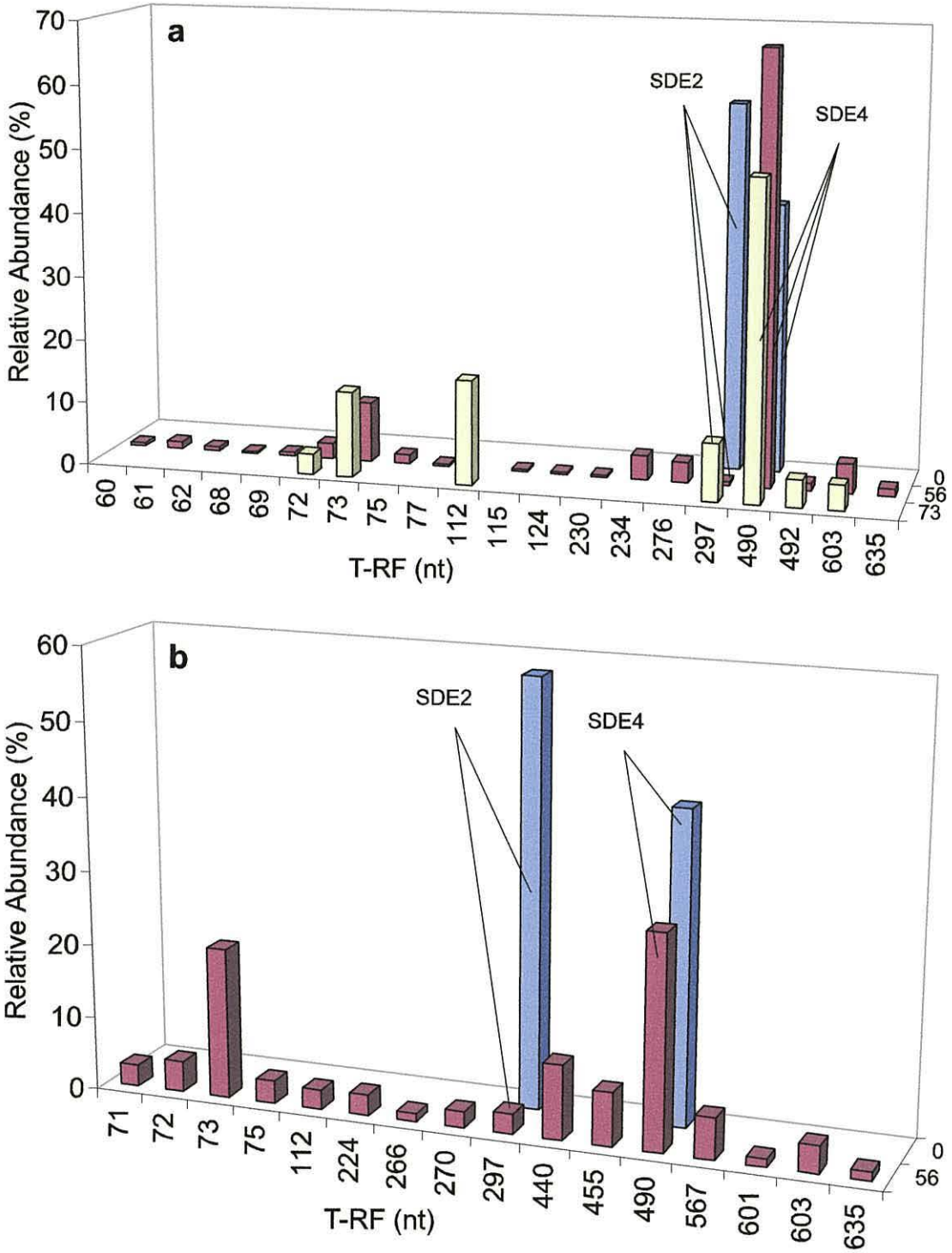

Figure 4.17 T-RFLP profiles for (a) YE-amended and (b) YE-free SDEfour bioleaching cultures using the 27f:1387r primer pair and the restriction enzyme *Msp* I, of the SDEfour inoculum (day 0), and bioleaching culture after 56 and 73 days. No DNA could be successfully amplified from lysates prepared from the YE-free culture at day 73. (Key: , inoculum; , day 56; , day 73.)

4.3.2.3.6 SDEfive

A second consortium, the SDEfive culture, comprised the same four *Bacteria* as the SDEfour culture, plus the archaeal *Fp. acidiphilum*-like isolate, SDE29. Isolate SDE29 was included to elucidate whether, despite being unable to effect accelerated oxidative dissolution of the tailings in pure culture, this iron-oxidising microorganism would affect the rate of mineral dissolution when present in a mixed population. Although isolate SDE29 was isolated from the spoil, rather than the tailings, such *Archaea* are difficult to detect on the solid media used, and might well have been present in the tailings also.

Following a slight acceleration phase of 14 days, total soluble iron concentrations in YE-amended cultures increased steadily for the duration of the experiment. Following an acceleration phase lasting 21 days, concentrations in YE-free cultures also began to increase steadily, though at a slower rate. Sulfate concentrations in both YE-amended and YE-free cultures were almost identical to those in the SDEfour cultures until day 28. After this point, concentrations in the SDEfive cultures increased slightly more rapidly, though still following the same general trends.

Soluble ferrous iron concentrations in YE-free cultures remained fairly constant, as was observed with YE-free SDEfour cultures. In YE-amended cultures concentrations fell to around 1 mM and were maintained at this level. Concentrations dropped further still following the addition of extra YE at day 63.

The *p*H of the inoculated bioleaching cultures was consistently lower than that of the controls, but decreased by a similar amount. There were no substantial differences between YE-amended and YE-free cultures, and no discernable differences between the SDEfour and SDEfive cultures. These data are summarised in Figure 4.18

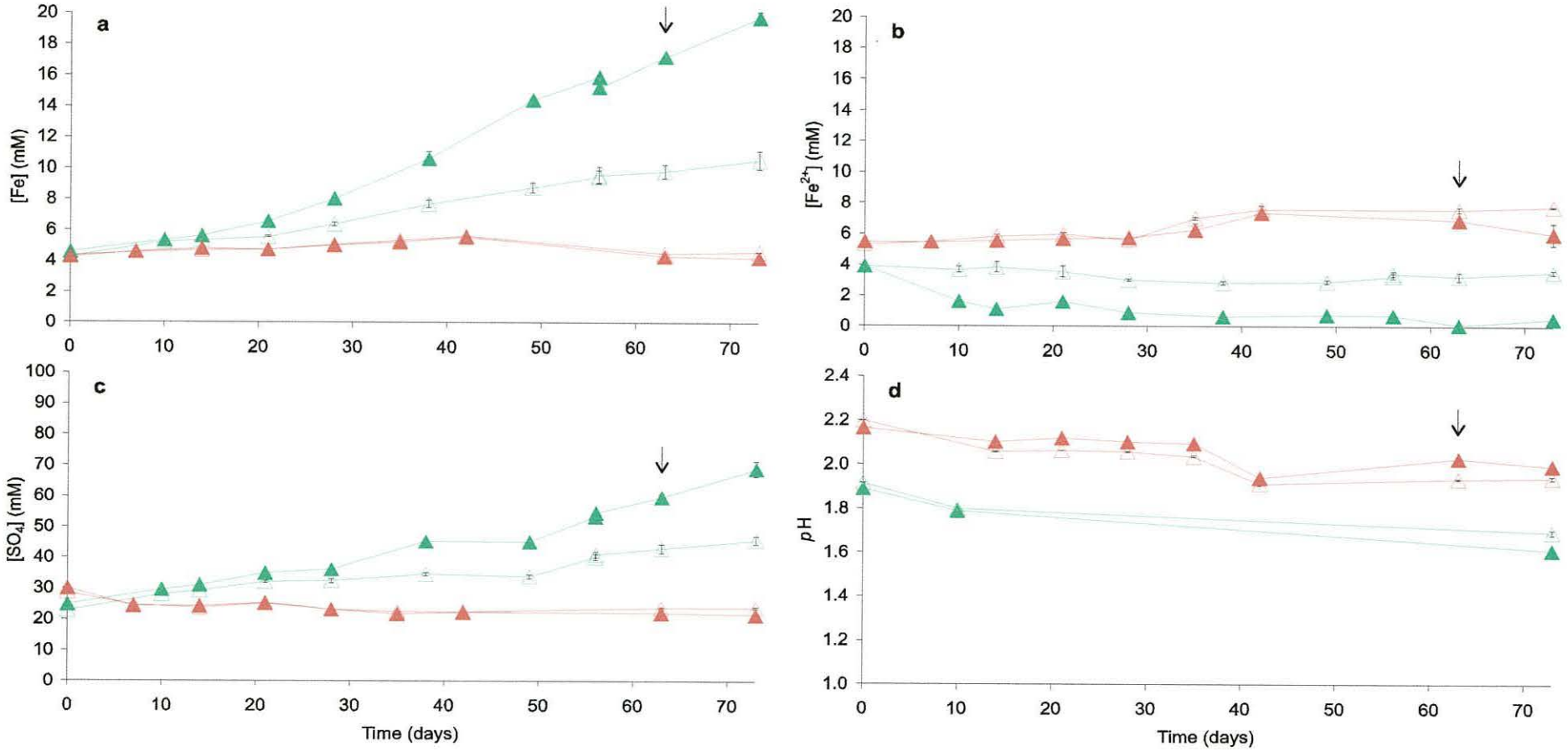

Figure 4.18 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and of (d) *p*H, during bioleaching of São Domingos tailings by the SDEfive consortium. (Key: △▲, inoculated with the SDEfive consortium; △▲, uninoculated controls; Closed symbols, YE-amended; Open symbols, YE-free; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; n = 3 for each data point.)

Bioleaching was more effective by this consortium than by the SDEfour consortium, but was still not as effective as either SDKH/*Acidiphilium* SJH or the initial tailings enrichment culture (see Figure 4.19).

As with the SDEfour culture, the *Alicyclobacillaceae* isolates SDE13 and SDE17 were absent from the bacterial T-RFLP profiles during the course of the bioleaching experiment. Bacterial T-RFLP profiles of the bioleaching cultures using the restriction enzyme *Msp* I are shown in Figure 4.19. At the start of the experiment SDE2 and SDE4 were almost equally abundant, with SDE2 slightly more so. Both YE-amended and YE-free cultures presented similar population dynamics during the leaching operation. Isolate SDE2 had completely or almost completely disappeared from the profiles immediately prior the addition of extra YE at day 56. By the end of the experiment, SDE2 had increased relative to SDE4 in both cultures, despite supplemental yeast extract only having been added to the YE-amended culture. The archaeon SDE29 was detectable for the duration of the experiment in both cultures by archaeal PCR analysis. However, its abundance relative to the *Bacteria* could not be ascertained by this approach. This problem may, in any future experiments, be resolved by the use of fluorescent microscopy methods, such as FISH, or by employing T-RFLP primers universal to both *Archaea* and *Bacteria*.

FeSo plates streaked with bioleaching culture at the end on the experiment were dominated almost entirely with isolate SDE4, with varying, but very low levels of isolate SDE2, as was also found with the SDEfour consortium. Again, neither isolate SDE13 nor isolate SDE17 were detected. Isolate SDE29 could not be detected either, but this is unsurprising given that this archaeon grows much more slowly on this medium than the *Bacteria*.

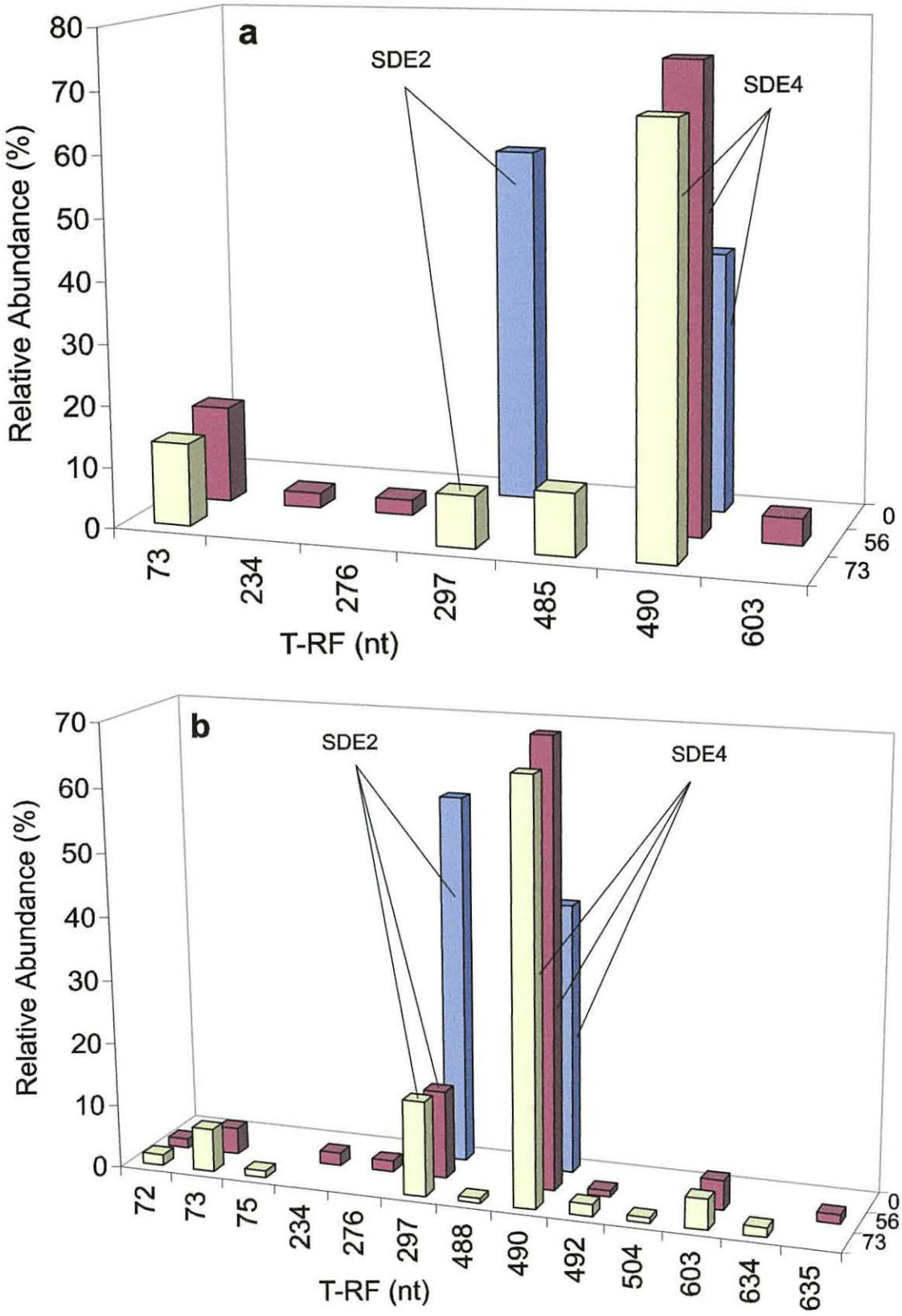

Figure 4.19 T-RFLP profiles for (a) YE-amended and (b) YE-free SDEfive bioleaching cultures using the 27f:1387r primer pair and the restriction enzyme *Msp* I, of the SDEfour inoculum (day 0), and bioleaching culture after 56 and 73 days. (Key: , inoculum; , day 56; , day 73.)

Figure 4.20 shows the comparative bioleaching rates of all the cultures assessed, using changes in soluble total iron concentrations as an indicator of leaching efficacy. Following an acceleration phase, the pyrite enrichment culture form the tailings demonstrated the fastest rate of mineral dissolution, and attained the highest soluble iron concentrations overall. While the mixed culture of isolate SDKH and *Acidiphilium* sp. SJH had leached the same amount of iron from the tailings as the enrichment culture by day 35, leaching by this culture appeared to cease at this point. Of the pure cultures tested, isolate SDE2 was the most effective at accelerating mineral dissolution, while isolates SDE4 and SDE29 were equally ineffective in pure culture. The two consortia tested followed identical trends in changing soluble iron concentrations until around day 28, after which point the rate of mineral dissolution by SDEfour consortium appeared to decline. Leaching of the São Domingos spoil was not accelerated by the pyrite enrichment culture from the spoil. The slight increase in iron concentrations observed was paralleled in the control experiments.

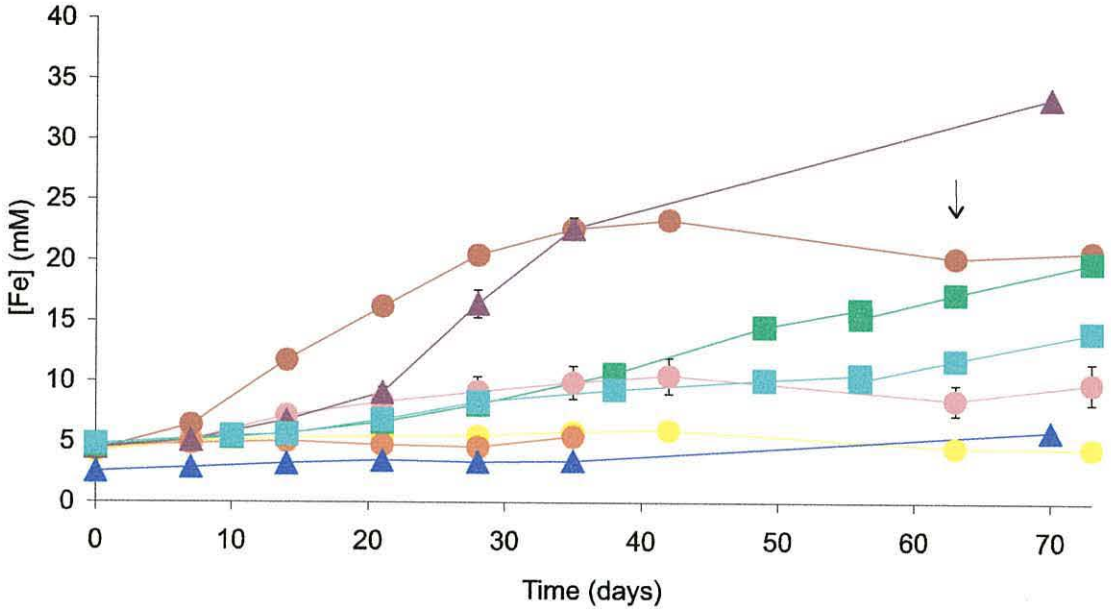

Figure 4.20 Comparative changes in soluble total iron during bioleaching of São Domingos spoil or tailings as an indicator of the bioleaching efficacy of the different cultures tested. (Key: ▲, inoculated with the pyrite enrichment culture from the spoil; ▲, inoculated with the pyrite enrichment culture from the tailings; ●, inoculated with a pure culture of isolate SDE2; ●, inoculated with a pure culture of isolate SDE4; ●, inoculated with a pure culture of isolate SDE29; ●, inoculated with a mixed culture of isolate SDKH and *Acidiphilium* sp. SJH; ■, inoculated with the SDEfour consortium; ■, inoculated with the SDEfive consortium; Triangles, amended with HBS; Circles and Squares, amended with YE; ↓, point at which extra YE was added to YE-amended cultures; Error bars, where visible, show standard errors; n = 3 for each data point).

4.4 DISCUSSION

Analysis of the microbial populations in the São Domingos mine spoil and tailings using a cultivation-based approach suggested that these materials supported only a limited variety of acidophilic microorganisms. The culture-based methods used did not target neutrophilic organisms, but it is unlikely, given the very low average *p*H values of these materials, that such organisms would occur in great numbers, if at all. That isolates could only be obtained directly from the tailings on one occasion and never from the spoil suggests that the acidophilic biota is limited in size as well as complexity. This is also suggested by the inability to amplify DNA from the solid material, even after treatment to attenuate the high acidity. However, PCR may also have been

adversely affected by the high concentrations of soluble metals present in the spoil and tailings.

While an *Acidithiobacillus ferrooxidans*-like isolate was isolated from the pool receiving runoff from the tailings, neither this, nor some other organisms that are commonly isolated from AMD, such as *Leptospirillum* spp., *At. ferrooxidans* or *Acidiphilium* spp., were detected in the spoil or tailings. A similar variety of microorganisms were obtained from the enrichment cultures inoculated with either spoil or tailings, with iron- and sulfur-oxidising "*Firmicutes*" most commonly isolated.

The site as a whole is very extreme in terms of high acidity, readily extractable metals and seasonal changes in temperature and moisture content. The single Gram-negative isolate, SDE4, was shown to be able to survive exposure to temperatures up to 45°C, and to grow well in conditions of high osmotic stress, induced by high concentrations of sodium chloride (500 mM NaCl). Interestingly, Lawson *et al.* (1995) found that *At. ferrooxidans* is unable to grow in NaCl concentrations higher than 5 g L^{-1}, which is just under 100 mM NaCl. They found that the toxicity was due to membrane damage caused by chloride ions, rather than osmotic stress *per se*.

Gram-positive acidophiles are, in general, more osmotolerant than Gram-negative species (e.g. Crane and Holden, 1999) and are able to form highly resistant endospores when conditions become too extreme for growth. It may be that the major limitation to microbial biodiversity in this tailings deposit is extreme osmotic stress. The apparent absence of moderate thermophiles is intriguing at such a site; especially considering most known *Sulfobacillus* spp. and related "*Firmicutes*" are moderate thermophiles. However, mesophilic strains have recently been described, such as the proposed species "*Sb. montserratensis*" (Hallberg and Johnson, 2001).

The high levels of acidity and readily extractable metals in the spoil and tailings suggest that extensive mineral oxidation is occurring. These levels may be exaggerated somewhat due to the often arid nature of the site preventing translocation of dissolved minerals. However, the tailings bioleaching experiments showed that the indigenous acidophiles are capable of extensive mineral mobilisation, though their activities *in situ* may be limited by the availability of one, or more, inorganic nutrients.

Both culture and biomolecular data suggested that the composition of the microbial population remained basically unchanged before and after bioleaching of the tailings in laboratory experiments. However, comparison of the results obtained by isolation on solid media and by T-RFLP analysis showed a major discrepancy. Culture data indicated that isolates SDE2 and SDE4 were the dominant microorganisms in the bioleaching inoculum and in the bioleaching cultures at the end of the experiment, and that they were present in broadly similar numbers. In contrast, although *At. thiooxidans* SDE4 was also detected by T-RFLP analysis, SDE2 was not. Rather, T-RFLP data indicated that SDE4 and the *Alicyclobacillaceae* (SDE13 and SDE17) that had been isolated from enrichment cultures were the most abundant microorganisms. The reason for this anomaly is unclear. On the one hand, it could be because isolates SDE13 and SDE17 did not grow on the selective solid media that were used, though this is highly unlikely as these organisms were first isolated from enrichment cultures using the same solid media. Alternatively, the absence of T-RFs corresponding to isolate SDE2 in the undefined bioleaching culture analysis could have been due to a cell lysis- or PCR-related problem, though again, this is highly unlikely as no problems were encountered in amplifying the 16S rRNA gene from pure cultures of this isolate or mixed consortia. Differing copy numbers of the 16S rRNA gene or the effects of sporulation may be factors. It could be that the *Alicyclobacillaceae* organisms have greater numbers of the 16S rRNA gene in their genome than isolate SDE2. This could cause PCR bias, artificially elevating their relative abundance (von Wintzingerode *et al.*, 1997). Additionally, the majority of these organisms may have formed spores by the end of the bioleaching experiment. Conditions on the FeSo plates may not have favoured germination, but these spores may still be detectable in lysates made from the liquid cultures by PCR. The cause may be any one, none or a combination of the above possibilities. This conundrum could, in theory, be resolved by using fluorescent *in situ* hybridisation (FISH) analysis using 16S rRNA probes designed to specifically-target isolates SDE2, SDE4 or SDE13/17.

Pure cultures of microorganisms isolated from the spoil and tailings were far less efficient at oxidising the tailings, even in the presence of added

inorganic and organic (yeast extract) nutrients, than was the enrichment culture. The mixed culture of SDKH and *Acidiphilium* sp. SJH was much more effective than the pure cultures, but could not maintain mineral oxidation at the same rate as the enrichment population. This is not surprising as microbial consortia, rather than pure cultures, are known to be involved in sulfide mineral oxidation in industrial as well as environmental situations (e.g. Brierley and Brierley, 2001). Synergistic interactions between acidophiles, involving transfer of organic and inorganic carbon and iron/sulfur cycling are thought to have important roles in microbial mineral dissolution in most situations (Okibe and Johnson, 2004). For example, in the present scenario, autotrophic *At. thiooxidans*-like SDE4 could supply organic carbon as cell exudates and lysates to the "heterotrophically-inclined" "*Firmicutes*". In return, ferric iron generated by the latter would attack sulfide minerals generating elemental sulfur and reduced inorganic sulfur compounds such as thiosulfate that are used as electron donors by *At. thiooxidans* for CO_2 fixation (see Figure 4.21). This model was proposed by Bacelar-Nicolau and Johnson (1999), who demonstrated that mixed cultures of iron-oxidising heterotrophs ("*Ferrimicrobium acidiphilum*") and *At. thiooxidans* were able to accelerate mineral dissolution in carbon-free medium. A similar scenario of enhanced arsenopyrite dissolution by a moderately thermophilic consortium of *Sulfobacillus thermosulfidooxidans* and *Acidithiobacillus caldus* has been described by Dopson and Lindström (1999). Additionally, a mixed population may be more adaptive to the changing conditions within the batch cultures used. As conditions become unsuitable for a particular organism, one or more alternative organisms may be able to take its place, essentially occupying their own temporal niche when conditions suit during the batch leaching process.

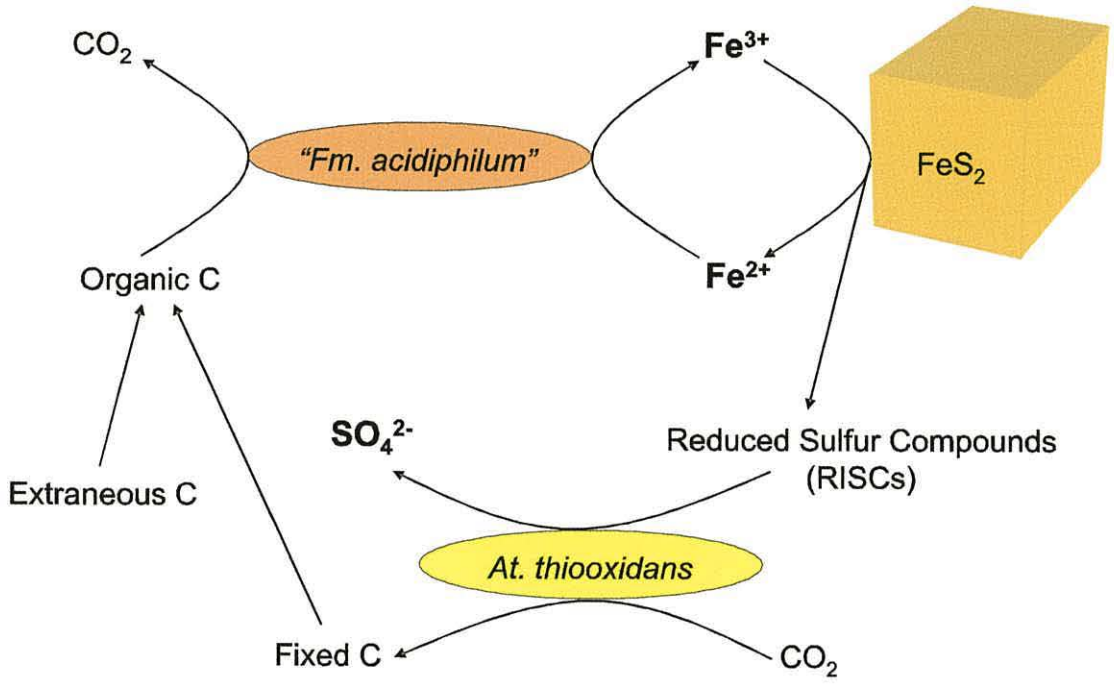

Figure 4.21 Proposed model for mixed culture leaching of pyrite by "*Ferrimicrobium acidiphilum*" and *Acidithiobacillus thiooxidans* (modified after Bacelar-Nicolau and Johnson, 1999).

The 'reconstituted' tailings enrichment population, the SDEfour consortium, was only slightly more effective than the pure isolates at leaching the tailings material. While the addition of the archaeon SDE29 to the consortium ("SDEfive") improved this further, rates of mineral dissolution were still far lower than by the undefined enrichment culture. This indicates that one or more crucial members of the enrichment culture population were missing. One possibility is that these were the *Alicyclobacillaceae* (isolates SDE13 and SDE17), which were included in the SDEfour and SDEfive inocula but never detected in the bioleaching cultures themselves. Although it is impossible to provide evidence of absence, the enrichment culture population that was shown to contain these microorganisms (as well as SDE2 and SDE4) was far more effective than the 'reconstructed' mixed culture.

However, both isolates SDE13 and SDE17 were found to dominate solid media inoculated with the pyrite enrichment cultures from the spoil as often as they were for the tailings, yet the enrichment culture from the spoil was largely ineffective in bioleaching the spoil material. On the other hand, isolate SDE2 was not detected in any of the spoil enrichments. It may

therefore be that successful bioleaching is dependent on the presence of isolate SDE2, or, more likely, on the presence of *all* of these isolates, SDE2, SDE4 and SDE13 and/or SDE17. Unfortunately, it was not possible to grow SDE13 or SDE17 in liquid cultures, so their individual bioleaching capability could not be examined. As mentioned above, in environments that are so extreme as to preclude the growth of 'all-round' highly efficient mineral-oxidising individuals, complex interactions between individually ineffective organisms may create an effective leaching population.

Some other aspect of the spoil may make it less amenable to bioleaching than the tailings. While both total extractable iron and copper concentrations were less in the spoil, there were still high concentrations there. Metal toxicity may be an issue, possibly of metals (and metalloids) that were not analysed. Certainly, DNA could not be amplified from the spoil enrichments at the end of the leaching experiment, potentially suggesting limited microbial biomass. However, concentrations of readily extractable copper were much higher in the tailings, implying that they may be more toxic than the spoil. The only measured analyte that was greater in the spoil than the tailings was zinc, which was present at about twice the concentration as in the tailings.

It may also be possible that an undetected member of the population is key to the bioleaching capacity of the tailings enrichment culture. Despite its isolation from the pool at the base of the tailings, this is unlikely to be the *At. ferrooxidans* NO37 isolate SDKH. This organism is easily cultured on the solid media used, and so would have been readily detected on the FeSo plates at the end of the leaching experiments, had it been present in any great numbers.

The São Domingos mine spoil and tailings were produced when extraction and concentration techniques were less efficient than they are now. Such materials, which were categorised at the time as wastes, are increasingly being considered as potential resources that could, in some cases, be re-processed. Significant concentrations of the base metals copper and zinc are present in the São Domingos spoil and tailings. While the spoil appeared to be somewhat resistant to bioleaching, it is clear that by stimulating the indigenous microflora (biostimulation) both metals could be

readily leached from the tailings. An alternative advantage of bioaugmentation in a situation where metal recovery is not being considered is that the otherwise protracted release of polluting chemicals could be condensed to a relatively brief window, within which leachates could be collected and remediated, e.g. by active treatment with lime.

The spoil and tailings populations were interesting in that they did not contain acidophiles commonly associated with AMD environments, suggesting that organisms such as *Leptospirillum* spp. and *Acidithiobacillus ferrooxidans*/NO37 are not important in such an environment. This is in contrast to a study of the thixotropic pool at the base of the Achada do Gamo tailings, which found the population to be variably dominated by either *L. ferrooxidans* or "*Ferrimicrobium*" spp., with lesser numbers of *At. ferrooxidans*. However, what is interesting is that this population appears to be somewhat transient, with no organisms detectable during periods of low rainfall, when the pool is at its driest (Johnson, unpublished). Interestingly, the "*Firmicutes*" and the archaeon are at least facultatively heterotrophic and, in pure cultures, appeared to require an organic carbon source to facilitate mineral dissolution.

The elevated concentrations of readily extractable metals in both the spoil and tailings, the occurrence of flash floods and continuing uncertainty over climatic trends in the long term mean the São Domingos site represents a significant environmental hazard. While the semi-arid conditions may limit the microbial population in terms of complexity, the wastes, especially the tailings, still host the potential for AMD genesis. The microbiota was limited to a small population of mineral-oxidising acidophiles, well adapted to surviving in the harsh conditions in terms of extreme solute potential and acidity, and seasonally varying environmental conditions.

CHAPTER 5: MICROBIOLOGICAL ANALYSIS OF A DECOMMISSIONED HEAP BIOLEACH STOCKPILE, LOCATED AT THE KENNECOTT BINGHAM CANYON MINE, UTAH

5.1 INTRODUCTION

The Bingham Canyon Copper mine is located close to Salt Lake City, Utah, U.S.A.. Minerals were first discovered in the area in the 1850's, although exploitation didn't begin until 1863. The ore body consists mainly of chalcopyrite, and the mine began producing copper in the early 1900's. It is currently owned by Rio Tinto, via Kennecott Copper and is the largest open pit mine in the world, producing more than 10% of U.S. copper. The open cast void, at 4 km wide and over 1 km deep, is the only man-made structure other than the Great Wall of China to be visible from space. To date it has yielded more saleable metal than any other mine in the world.

Heap leaching operations are usually reserved for the processing of low-grade material that would otherwise be dumped as waste. However, at the Bingham Canyon site some 300 Mt of low-grade ore, which could be efficiently harvested by floatation, was identified as a potential resource for heap leaching. The reasoning was that conventional processing of this material would displace other ores of higher grade. To test the feasibility of this approach a test heap comprising 900 Kt run-of-mine (ROM) ore was constructed and leached commercially from 1995 to 1997. Following a ten month hiatus a second lift was added to this heap and leaching resumed for another 12 months. Overall the pilot study has so far been considered a success, with *ca.* 30% of the total copper in the ore being recovered, using solvent extraction/electrowinning (SX/EW) of the pregnant leach solution (PLS) generated in the heap (Esdaile *et al.*, 1999).

Basic assessments of the heap microbiology were carried out by Bruhn *et al.* (1999). Immediately following the initial acid curing, the microbial populations were found to be of limited diversity and small in numbers, at around 1×10^4 cfu g^{-1}. After a month or so, diversity had increased and numbers were around 1×10^6 cfu g^{-1}. A variety of organisms were putatively identified, including *Acidithiobacillus ferrooxidans*, *At. thiooxidans*, *At. caldus*,

Leptospirillum ferrooxidans, *Acidimicrobium ferrooxidans* and some non-mineral-oxidising heterotrophs.

The heap has not been actively leached since 1999 and provided a unique opportunity to examine the microbial population of a constructed, but currently unmanaged, commercial mineral heap. The objectives of this study are to assess how this population may have changed since irrigation was stopped, and the extent to which it is still capable of mineral dissolution.

5.2 MATERIALS AND METHODS

The heap was sampled in November 2004 at three locations (KB-1, KB-2 and KB-3), at intervals of about 10 m. Holes were cut with a mechanical digger, and samples taken at the surface, and at about 1 and 2 m depths (designated KB-1.0, KB-1.1 and KB-1.2 etc.). The surface samples appeared to be well oxidised, and were straw-coloured. This contrasted with the greyish colours of the sub-surface samples indicating that the heap was not completely depleted in oxidisable minerals at depth. At the time of sampling there was a thick covering of snow on the surface of the heap. Notably, as can be seen in Figure 5.1, snow was absent from around venting pipes that were sunk into the heap. This indicated that sufficient exothermic mineral oxidation was occurring within the heap to maintain above-ambient temperatures. The collected material, even at a depth of just 1 m, was warm to the touch.

Ore samples were collected in sterile plastic bags and processed immediately upon return to the U.K.. The *p*H, readily extractable and total extractable metal concentrations were determined as described in section 2.5.1. A quantity of material was collected from different depths, mixed, dried, crushed (with a geological hammer) and screened to select particles between 63 and 630 µm diameter. This material was used subsequently in bioleaching experiments.

Microorganisms were detached from the mineral matrix, as described in section 2.5.1, using HBS at *p*H 2.5. The resulting liquor was serially diluted, plated onto Fe$\underline{o}$, FeS$\underline{o}$, FeT$\underline{o}$ and YE3$\underline{o}$ solid media and incubated aerobically at 30°C or 45°C for 2-3 weeks. Isolates were described and putatively identified by 16S rRNA gene RFLP and sequence analysis and classified using the RDP Classifier, as described in section 2.2.

Figure 5.1 Photograph of the Kennecott bioleaching heap surface, showing different colours of material (a, surface; b, 1 m depth; c, 2 m depth) and high internal temperatures preventing the snow settling around a venting pipe (d).

Pyrite (P) and iron/yeast extract (FeYE) liquid media were used to enrich for acidophilic microorganisms in the ore samples. The media (50 mL in 100 mL shake flasks) were inoculated with small quantities (*ca.* <0.25 g total) of a mixture of all nine samples from the heap, (which had been homogenised by hand) and incubated aerobically, shaken at 120 rpm, at 30°C. Enrichment cultures were viewed periodically using a phase contrast microscope. When microbial growth was evident they were used as the inoculum for subsequent bioleaching experiments.

Collected material was pre-treated to increase *p*H and to reduce concentrations of soluble metals prior to DNA extraction, as described in section 2.2.1. Crude lysates of both the enrichment cultures and the bioleaching cultures were prepared as described in section 2.2.1.1. PCR and T-RFLP analysis was carried out on community DNA from the enrichments, bioleaching experiments and on individual isolates using the 27f(G):1387r primer pair. Fragment sizes in community samples were compared to those held in the T-RFLP Database, as described in section 2.2.3.

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Bioleaching of material from the Kennecott bioleaching heap was carried out in 100 mL shake flasks containing 1% (w/v) of ground sieved spoil or tailings and 50 mL of either RO water or a basal salts solution (BS+TE), adjusted to *p*H 2.5. Flasks were inoculated with 0.5 mL aliquots from the pyrite enrichment culture. Bioleaching cultures were incubated in a shaking incubator (120 rpm) at 30°C or 45°C and sampled, as described in section 2.5.2, at regular intervals. Concentrations of ferrous iron, total soluble iron, copper and zinc, and sulfate of the culture at each sampling point were measured as described in section 2.4. Concentrations of total soluble metals were measured by ion chromatography.

5.3 RESULTS

Basic geochemical data for each sample are shown in Table 5.1, together with the average values. Zinc concentrations were below the level of detection for the method used. Average values were obtained from the mixed material used in the bioleaching experiments. All the samples were acidic, and the highest acidity and concentrations of readily extractable metals generally occurred in the surface samples, and decreased with depth.

Table 5.1 Basic geochemical data for the Kennecott heap material.

Sample	*p*H*	Readily extractable (µg g^{-1})		Total extractable (µg g^{-1})	
		Fe	Cu	Fe	Cu
KB1.0	2.18	1904	97	n/d	n/d
KB1.1	2.67	627	43	n/d	n/d
KB1.2	3.40	986	284	n/d	n/d
KB2.0	2.49	1471	73	n/d	n/d
KB2.1	2.66	1904	143	n/d	n/d
KB2.2	2.85	659	221	n/d	n/d
KB3.0	2.64	1954	89	n/d	n/d
KB3.1	3.47	1948	92	n/d	n/d
KB3.2	3.08	1195	125	n/d	n/d
Average	2.82 (0.42)	1405 (557)	130 (77)	56033** (2155)	2800** (283)

Bracketed figures are standard deviation; *1:2.5 spoil or tailings to RO water; **Obtained from material used in bioleaching experiments; n/d, no data.

5.3.1 Isolation of indigenous microorganisms

A wide variety of *Bacteria* was isolated from the different samples, along with a single archaeon. Table 5.2 summarises the isolates and their closest relatives. Plate count data suggested an average population size of 2.87×10^6 cfu g^{-1} dry material, but there was much heterogeneity between the samples, with plate counts ranging from below the limits of detection to 1.39×10^7 cfu g^{-1}. Example photographs of some of the Kennecott isolates are shown in Figure 5.2.

Isolate KB15 was very closely related to the archaeon *Ferroplasma acidiphilium*, an iron-oxidising, heterotrophic extreme acidophile. Isolate KB18 could not be identified, but based on its colony morphology during sub-culturing appeared to be an iron- and sulfur-oxidising bacterium. Isolate KB20 was a member of the *Alphaproteobacteria*, probably within the *Acidisphaera* genus, based on 16S rRNA gene sequence identity. It was most closely related to a clone obtained from a study examining hydrocarbon seeps within acidic soils at the Yellowstone National Park, U.S.A..

Isolates KB22, KB23 and KB46 were all, to differing extents, related to the iron- and sulfur-oxidising firmicute, *Sulfobacillus thermosulfidooxidans*. Isolate KB22 was most closely related to a clone obtained from a commercial cobalt bioleaching operation. Isolate KB27 was a member of the *Actinobacteria* and was closely related to the iron-oxidising bacterium "*Ferrimicrobium acidiphilum*". Isolate KB61 could not be identified, but based on colony morphology during sub-culturing, was presumed to be an iron-oxidising microorganism.

The remaining isolates were all related to the more common Gram-negative *Bacteria* of the *Acidithiobacillus* genus, specifically the obligate sulfur-oxidiser, *At. thiooxidans* and the iron- and sulfur-oxidisers *At. ferrooxidans*^{T} (type strain) and *At. ferrooxidans* NO37.

Table 5.2 Descriptions of isolates from the Kennecott heap samples and their closest relatives, based on 16S rRNA gene identity.

Isolate	Description (temp*)	Nearest organism(s) (accession ID)	Identity
KB15	Small, sub-surface iron-oxidising colony from FeSo plate (45°C)	*Ferroplasma acidiphilum* strain DR1 (AY222042)	99.5%
KB18	Large white colony from FeSo plate (45°C)	Unknown	
KB20	Large, off-white, gelatinous colony from YE_3o plate (45°C)	Uncultured bacterium clone RH2-S2 (AY678238)	99.7%
		Acidisphaera rubrifaciens HS-AP3 (D86512)	96.7%
KB22	White/Orange/Brown concentric circular colony from Feo plate (30/45°C)	Uncultured *Sulfobacillus* sp. K55 (AF460984)	100.0%
		Sulfobacillus thermosulfidooxidans strain G2 (AY140233)	97.5%
KB23	Iron-oxidising colony from FeSo plate (45°C)	*S. thermosulfidooxidans* strain G2 (AY140233)	91.3%
KB27	Iron-oxidising colony from FeSo plate (30/45°C)	"*Ferrimicrobium acidiphilum*" (AF251436)	98.0%
KB46	Crystalline sulfur-oxidising, raised, entire colony from FeSo plate (30°C)	*S. thermosulfidooxidans* strain G2 (AY140233)	91.0%
KB47	Large sulfur-oxidising 'fried-egg' colony with Sulfur 'halo' into agar from FeSo plate (30°C)	*Acidithiobacillus thiooxidans*^{T} strain ATCC19377 (Y11596)	100.0%
KB60	Large, almost black, iron-oxidising colony from Feo plate (30°C)	*At. ferrooxidans*^{T} strain ATCC23270 (AF465604)	99.7%
KB61	Small iron-oxidising colony from Feo plate (30°C)	Unknown	
KB64	Sulfur-oxidising, entire, concentric circular colony from FeSo plate (30°C)	Uncultured bacterium clone RA13C21 (AF407401)	99.4%
		Acidithiobacillus ferrooxidans NO37 (AF376020)	99.2%
KB66	Very large, pretty, iron-oxidising colony from FeTo plate (30°C)	*At. ferrooxidans*^{T} strain ATCC23270 (AF465604)	100.0%
KB68	Irregular, iron-oxidising colony from Feo plate (30°C)	Iron-oxidizing acidophile m-1 (AF387301)	99.1%
KB71	Fluffy, white, 'volcano-shaped', orange-tipped colony from FeSo plate (30°C)	*At. ferrooxidans*^{T} strain ATCC23270 (AF465604)	99.7%
KB73	Sulfur-oxidising, concentric circular colony from FeSo plate (30°C)	Uncultured bacterium clone RA13C21 (AF407401)	99.4%
		Acidithiobacillus ferrooxidans NO37 (AF376020)	99.3%

*The temperature(s) at which the organism was originally isolated; ^{T}Type strain.

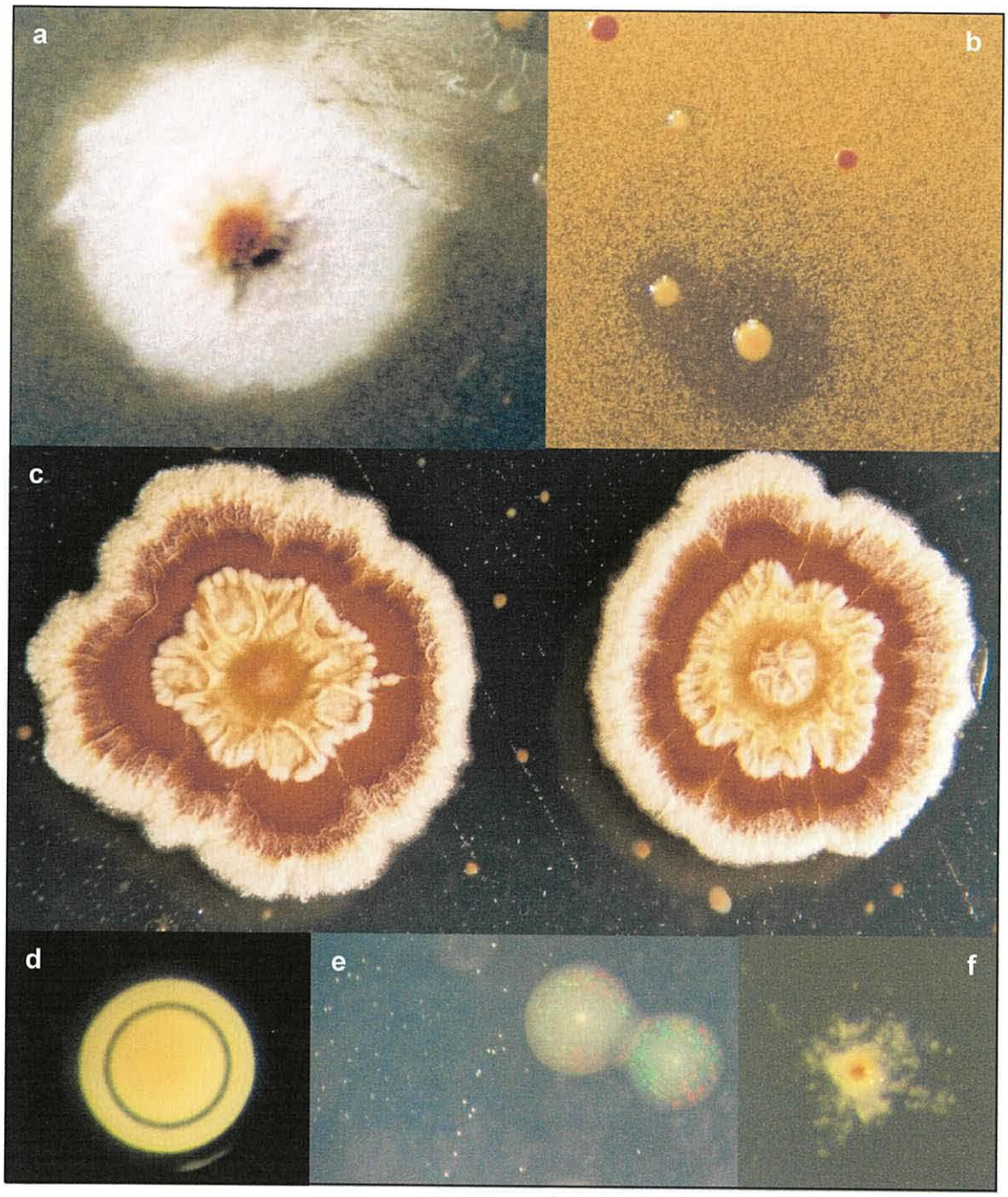


Figure 5.2 Photographs of some Kennecott isolates growing on solid media. (Key: **a**, isolate KB71 growing on FeSo solid medium; **b**, isolate KB22 growing on Feo solid medium (two different colony morphologies); **c**, isolate KB66 growing on FeTo solid medium; **d**, isolate KB64 growing on FeSo solid medium; **e**, isolate KB47 growing on FeTo solid medium; **f**, isolate KB61 growing on Feo solid medium.)

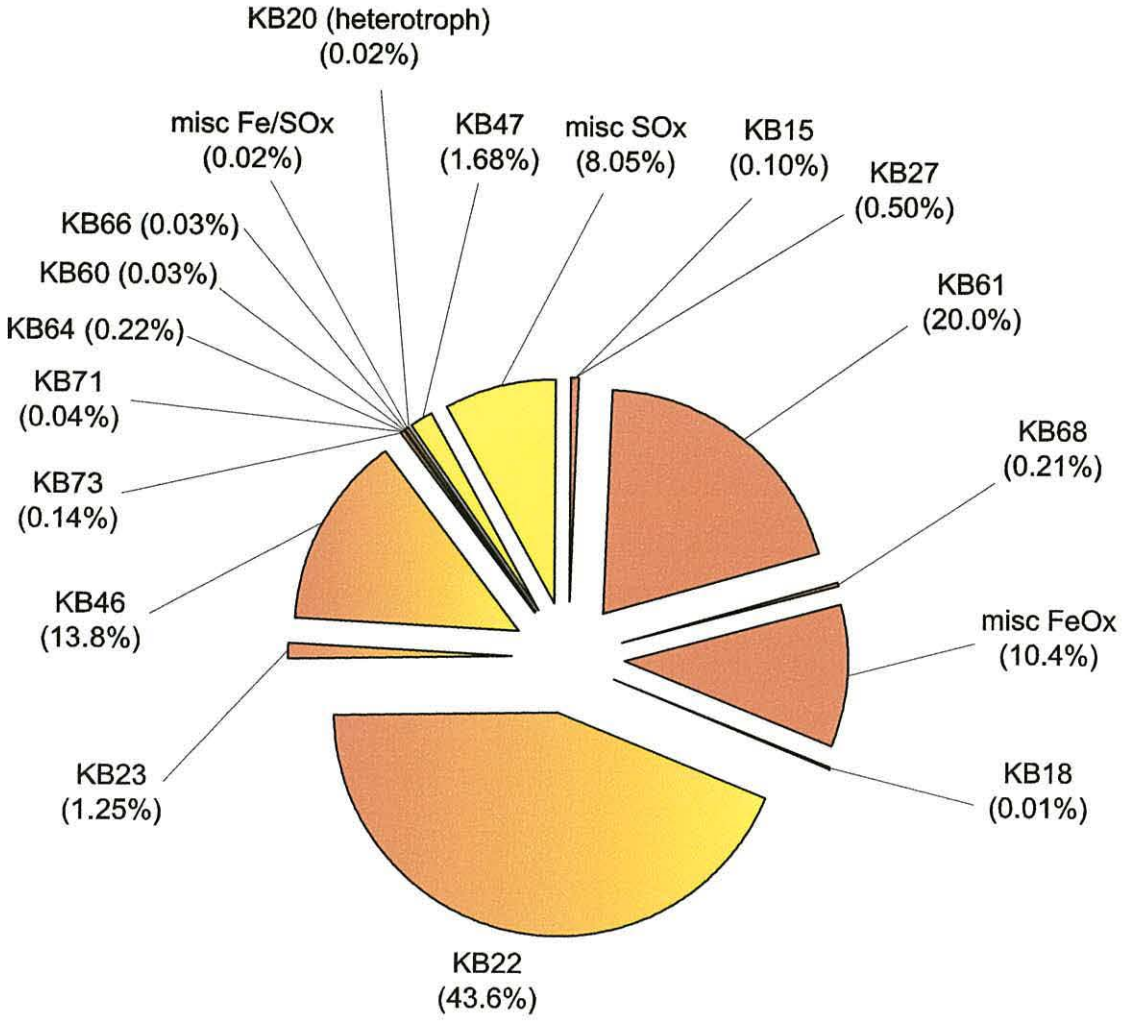


Figure 5.3 Average abundances of Kennecott isolates across all nine samples, grouped by metabolism. (Key: Orange, iron-oxidising; Orange/Yellow, iron- and sulfur-oxidising; Yellow, sulfur-oxidising; Pink (not visible), non-mineral-oxidising heterotroph)

Figure 5.3 shows the average abundance of these organisms across all nine samples. The iron- and sulfur-oxidising "*Firmicutes*" were the dominant isolates, numerically, and accounted for nearly 60% of the cultured population. The acidithiobacilli accounted for only around 2%, with *At. thiooxidans* the most prevalent, which accounted for just over 1.5%. As a group, the iron- and sulfur-oxidising organisms were the dominant fraction of the population, followed by the iron-oxidisers and the sulfur-oxidisers, respectively. Non-mineral-oxidising acidophilic heterotrophs accounted for less than 0.1% of the isolates.

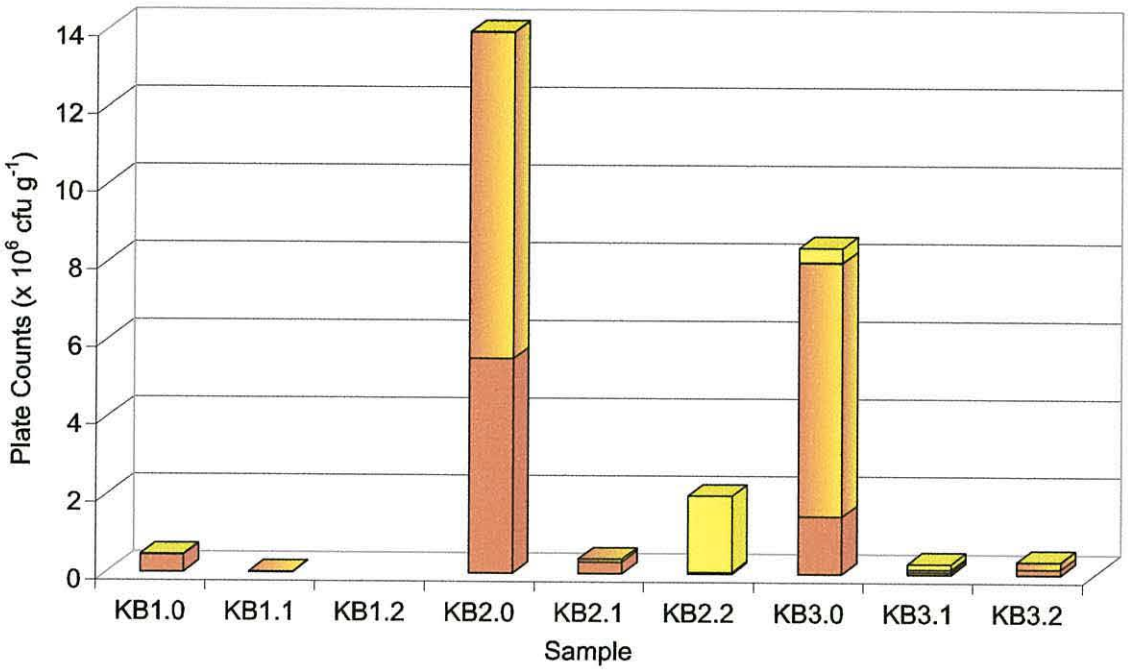


Figure 5.4 Total plate counts of each microbial group (based on iron/sulfur metabolism) in each sample. (Key: Orange, iron-oxidising; Orange/Yellow, iron- and sulfur-oxidising; Yellow, sulfur-oxidising; Pink (not visible), non-mineral-oxidising heterotroph.)

Table 5.3 Distribution of different acidophilic isolates in the Kennecott heap samples, grouped by metabolic capacities.

	KB1.0	KB1.1	KB1.2	KB2.0	KB2.1	KB2.2	KB3.0	KB3.1	KB3.2
Iron-oxidising *Bacteria*									
KB27								+	+
KB68		+							+
KB61				+	+	+		+	
Others*	+	+		+	+		+	+	+
Iron- and sulfur-oxidising *Bacteria*									
KB18						+			
KB22				+	+		+	+	+
KB23							+		
KB46	+			+			+		
KB60, KB66, KB71		+						+	+
KB64, KB73	+							+	+
Others*	+								
Sulfur-oxidising *Bacteria*									
KB47	+			+			+	+	+
Others*						+		+	
Heterotrophic *Bacteria*									
KB20							+		
Iron-oxidising *Archaea*									
KB15					+	+			

*These organisms could not be sub-cultured so metabolism was inferred based on initial colony morphology.

Figure 5.4 shows the total plate counts of the microorganisms of different metabolic groups in each sample. Table 5.3 shows the distribution of the different isolates between the heap samples.

The KB1 samples had the smallest total plate counts and the lowest microbial diversity. Sample KB1.0 was entirely dominated by unidentified iron-oxidising microorganisms with very low numbers of the iron- and sulfur-oxidising firmicute KB46 and the sulfur-oxidising *At. thiooxidans*-like isolate KB47. Sample KB1.1 was dominated by isolate KB68, a bacterium closely related to the iron-oxidising acidophile m-1, but the total cultivated population size was small at 1.8×10^4 cfu g^{-1}. The only other isolates from this sample were the *At. ferrooxidans*^{T}-like isolate KB60 and other unidentified iron-oxidising microorganisms. No microorganisms were isolated from sample KB1.2, suggesting the cultivatable population size in this sample was less than 2.3×10^3 cfu g^{-1}, which was the limit of detection for the culture method used.

The KB2 samples had the highest plate counts of all nine samples analysed. Sample KB2.0 was dominated by iron- and sulfur-oxidising "*Firmicutes*", which accounted for more than 60% of the cultivated population. The unidentified iron-oxidising organism KB61 accounted for over 35%, while miscellaneous iron-oxidisers, which could not be subcultured or identified, accounted for the rest. This sample had the largest cultivatable population of all samples, at approximately 1.4×10^7 cfu g^{-1}. Sample KB2.1 was dominated by miscellaneous iron-oxidising organisms that could not be identified, which accounted for just over 68% of the total isolates. The iron- and sulfur-oxidising firmicute, isolate KB22 accounted for nearly 20% of the total isolates. The sub-cultured, but unidentified, iron-oxidising isolate KB61 accounted for nearly 7% of the total isolates, while the *Fp. acidiphilum*-like isolate KB15 accounted for approximately 5.5% of the total isolates. Sample KB2.2 was dominated by unidentified microorganisms. Miscellaneous sulfur-oxidising organisms that could not be sub-cultured accounted for more than 97% of the cultivatable population, while the iron-oxidising isolate KB61 and iron- and sulfur-oxidising isolate KB18 accounted for just over 2% and just over 0.1% of the total, respectively. The only identifiable organism isolated was the *Fp. acidiphilium*-

like isolate KB15, which accounted for just over 0.2% of the total colony forming units.

The KB3 samples presented the greatest microbial diversity of all the samples at each depth. These samples were of a darker colouration than samples from equivalent depths in the other sample series, suggesting that these samples were less completely oxidised. Sample KB3.0 was dominated by the iron- and sulfur-oxidising "*Firmicutes*" isolates KB22, KB23 and KB46, which accounted for nearly 80% of the cultured population. The *At. thiooxidans* isolate KB47 accounted for nearly 5% of the cultured population, while the *Acidisphaera* isolate KB20 accounted for less than 0.1%. Unidentified iron-oxidising isolates accounted for the rest of the cultured population. Sample 3.1 had a much more even distribution of organisms. This was the only sample in which the *Acidithiobacillus* isolates clearly dominated the cultured population. The *At. thiooxidans*-like isolate KB47 accounted for just over 11% of plate isolates, the *At. ferrooxidans*^{T}-like isolate KB60 accounted for just over 1.5%, while the *At. ferrooxidans* NO37-like isolate accounted for 20%. The iron-oxidising firmicute KB22 accounted for just 1.6% of the cultured population. The unidentified, iron-oxidising isolate KB61 and other unidentified miscellaneous iron-oxidising organisms accounted for just over 10% of total colony forming units, while unidentified miscellaneous sulfur-oxidisers accounted for the rest of the cultured population, just over 41%. Sample KB3.2 presented a diverse population, but was dominated by isolate KB22 and the iron-oxidising *Actinobacteria* isolate KB27, accounting for 36% and 27% of the cultured population, respectively. The iron-oxidising acidophile m-1-like isolate KB68 accounted for just over 12% of the cultured population, while other unidentified iron-oxidising isolates accounted for just over 5%. The *Acidithiobacillus* isolates accounted for just under 19% of the cultured population, with *At. ferrooxidans*^{T}-like isolates KB66 and KB71 accounting for just under 5%, *At. ferrooxidans* NO37-like isolate KB73 accounting for nearly 11% and *At. thiooxidans*-like isolate KB47 accounting for just over 3%.

5.3.2 Bioleaching experiments

The results of the Kennecott heap material bioleaching experiments are described below. In general, neither differences in incubation temperature nor

the presence of additional nutrients had a major effect on the efficacy of mineral leaching.

Total soluble iron concentrations increased steadily with incubation time in all cases, compared to the uninoculated controls. Concentrations in nutrient-amended cultures appeared initially to increase slightly more rapidly than in nutrient-free cultures, but later appeared to stabilise somewhat. Concentrations in the nutrient-free cultures increased at a steadier rate, and by day 66 these cultures had the highest total iron concentrations overall. Ferrous iron was barely detectable in the inoculated flasks after day nine, while concentrations remained constant in uninoculated controls. Incubation at 30°C or 45°C in the presence or absence of added inorganic nutrients made no discernable difference to changes in ferrous iron concentrations over time. Sulfate concentrations in the inoculated cultures increased steadily for the duration of the experiment compared with the uninoculated controls. The rates of change in sulfate concentrations differed little between the various conditions, but those cultures at 45°C ultimately presented slightly higher relative concentrations. The *p*H decreased fairly steadily in the inoculated cultures, whereas an initial increase was observed in the uninoculated controls. The increase levelled off at around *p*H 3.0 after day nine, and remained fairly constant for the rest of the experiment. This initial increase was presumably due to abiotic proton-consuming reactions, which were exhausted within the first few days. Despite a substantial difference in the *p*H between the 30°C and 45°C cultures at day 23, previous and subsequent measurements were fairly similar for the duration. These data are summarised in Figure 5.5.

Concentrations of zinc (data not shown) were extremely low and changed little for the duration of the bioleaching experiment. The fact that from the start, the control flasks tended to contain concentrations of this metal that were lower than the detection limit for the procedure used, suggests that the minute amount of zinc present in the inoculated cultures was simply due to carry-over from the inoculum. Copper concentrations, shown in Figure 5.6, were quite low, and there was little difference in concentrations between the various conditions. Concentrations in the inoculated cultures increased initially, but reached a plateau after day nine. This suggested that the

leachable copper fraction was solubilised rapidly while the rest was more recalcitrant.

On average, the bioleaching cultures were able to leach approximately 55% of the total iron and 30% of the total copper over the duration of the experiment. Table 5.4 summarises the readily extractable and leachable metal concentrations as a percentage of the total extractable concentrations. Clearly, the readily extractable fraction of each metal constitutes only a small amount of the total solubilised during bioleaching.

Table 5.4 Percentages of total extractable iron and copper from the Kennecott ore that were readily extractable or leached during bioleaching.

Metal	Total extractable	Readily extractable*	Total bioleached*
Fe	56 mg g^{-1}	2.5%	55%
Cu	2.8 mg g^{-1}	4.6%	30%

*As a % of total extractable metal concentrations.

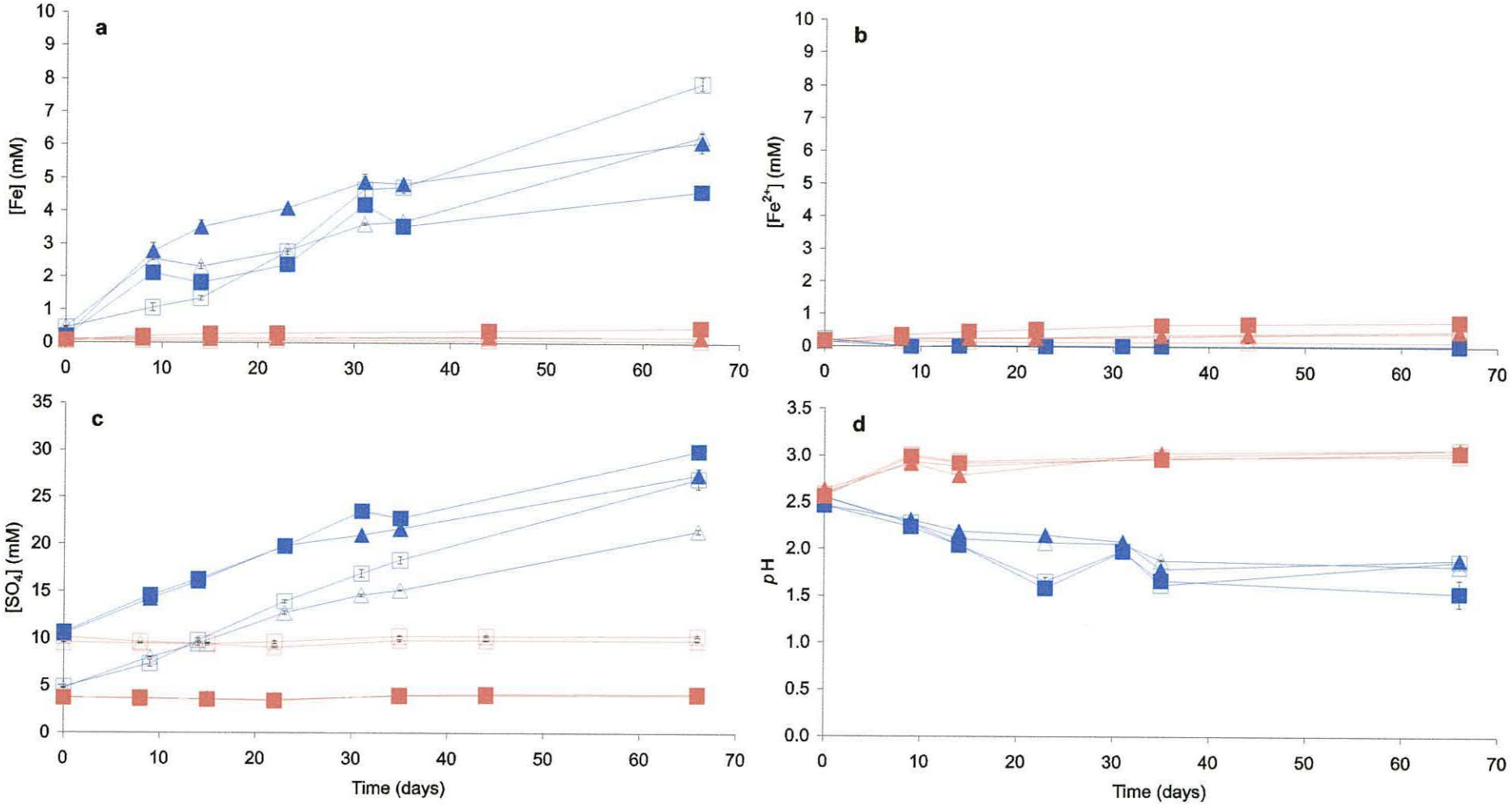


Figure 5.5 Changes in concentrations of soluble (a) total iron; (b) ferrous iron; (c) sulfate and (d) *p*H, during bioleaching of Kennecott heap material by the pyrite enrichment culture. (Key: △▲, inoculated, incubated at 30°C; □■, inoculated, incubated at 45°C; △▲, uninoculated, incubated at 30°C; □■, uninoculated, incubated at 45°C; Closed symbols, nutrient amended; Open symbols, nutrient-free; Error bars, where visible, show standard errors; n = 3 for each data point.)

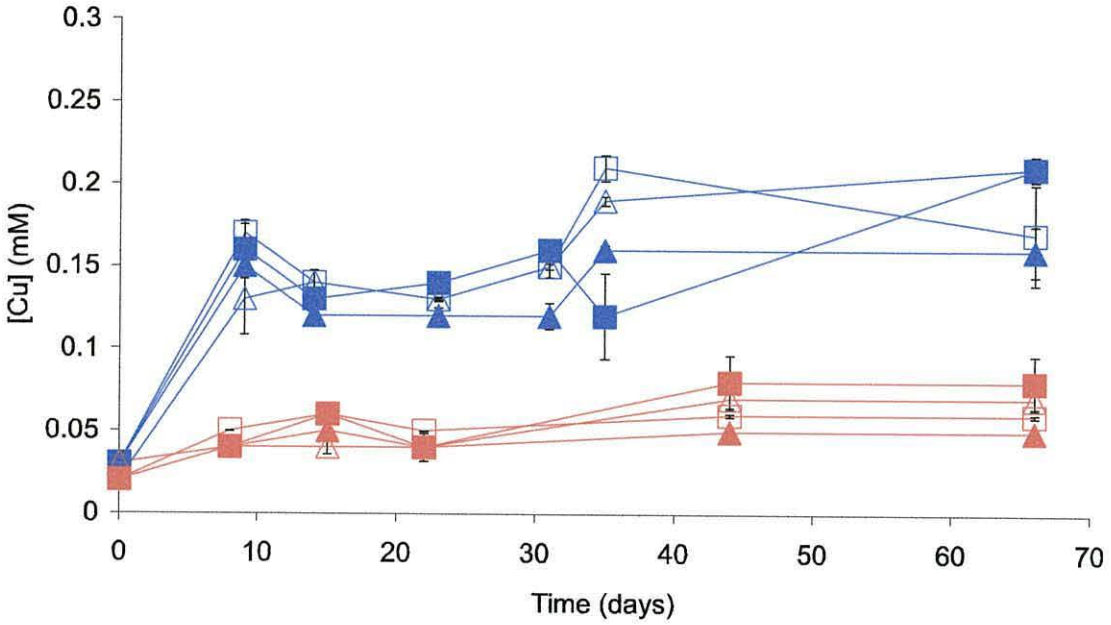


Figure 5.6 Changes in concentrations of soluble copper during bioleaching of Kennecott heap material by the pyrite enrichment culture. (Key: △▲, inoculated, incubated at 30°C; □■, inoculated, incubated at 45°C; △▲, uninoculated, incubated at 30°C; □■, uninoculated, incubated at 45°C; Closed symbols, nutrient amended; Open symbols, nutrient-free; Error bars, where visible, show standard errors; n = 3 for each data point.)

T-RFLP profiles of the enrichment culture used as the inoculum, and of the bioleaching cultures at the end of the experiment, using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I, show how the population changed following bioleaching for 66 days. Due to the poor quality of the PCR product yielded from these cultures, a large proportion of the T-RFs appear to be erroneous, i.e. artefacts created due to the sub-optimal PCR conditions (e.g. Osborne *et al.*, 2005). Consequently, almost every T-RF shorter than 100 nt was discounted from the profile.

Figure 5.7 shows the profile of the enrichment culture. The profile suggests that the culture was relatively simple. T-RFs corresponding to *Leptospirillum* spp. appear to dominate, despite no members of this genus having been isolated directly from the Kennecott heap material. T-RFs corresponding to the *Acidithiobacillus* isolates appear consistently, while T-RFs corresponding to "*Firmicutes*", specifically iron- and sulfur-oxidising "*Firmicutes*", appear in two of the three restriction enzyme profiles.

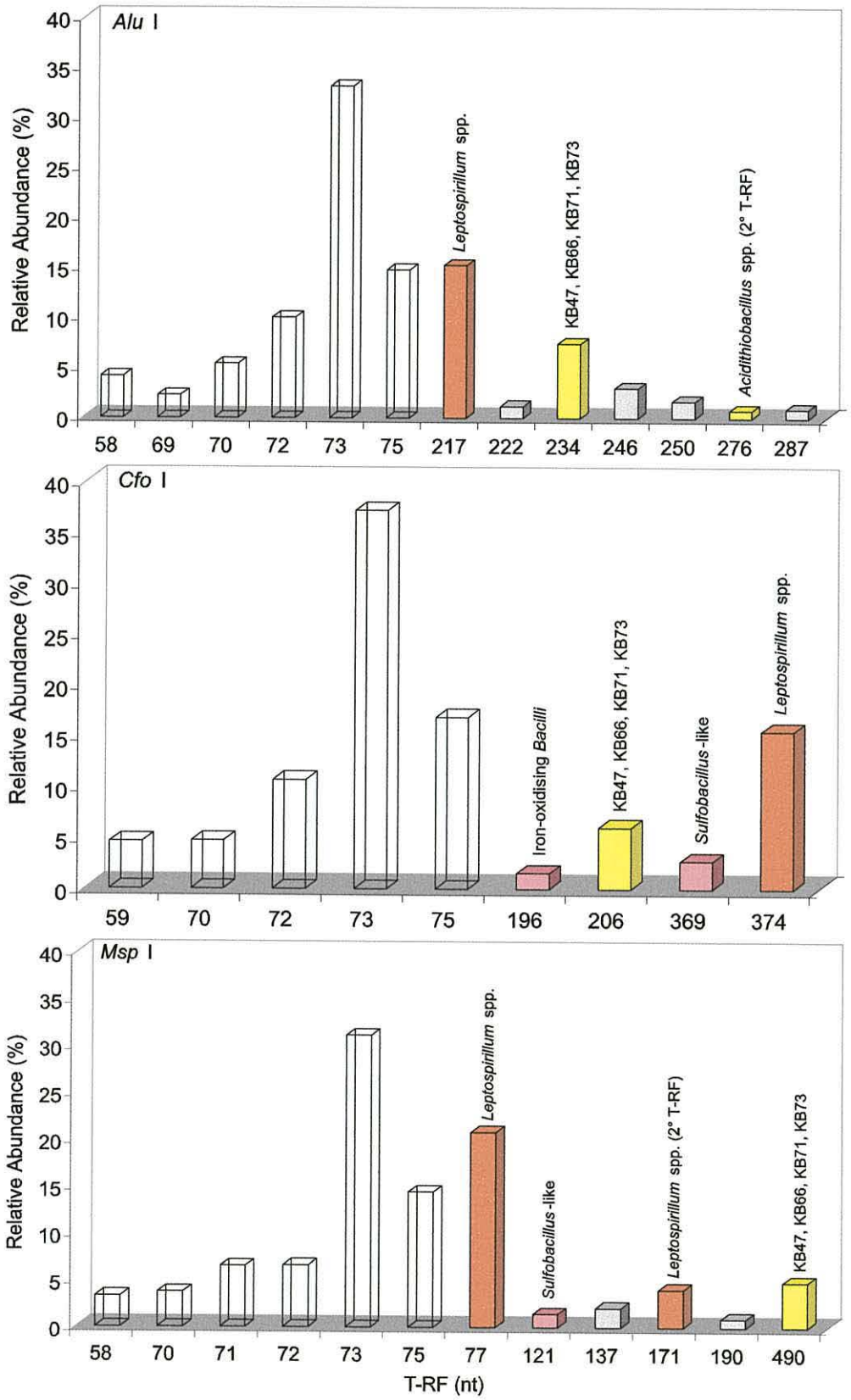


Figure 5.7 T-RFLP profile of the pyrite enrichment culture, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Acidithiobacillus* spp.; , *Alphaproteobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp.; , erroneous T-RF (PCR artefact).)

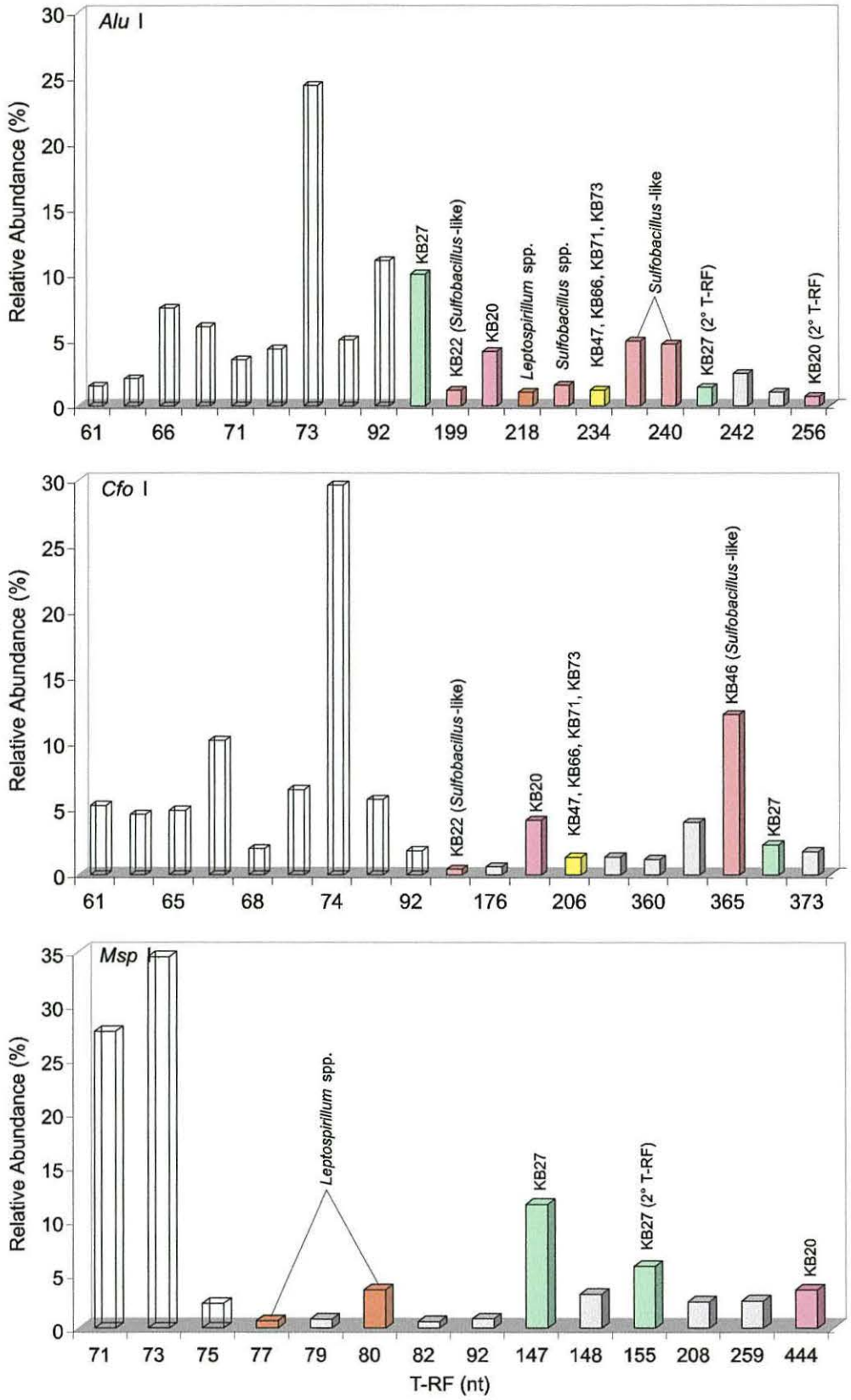


Figure 5.8 T-RFLP profile of the nutrient-free bioleaching culture following incubation at 30°C for 66 days, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Acidithiobacillus* spp.; , *Alphaproteobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp.; , erroneous T-RF (PCR artefact).)

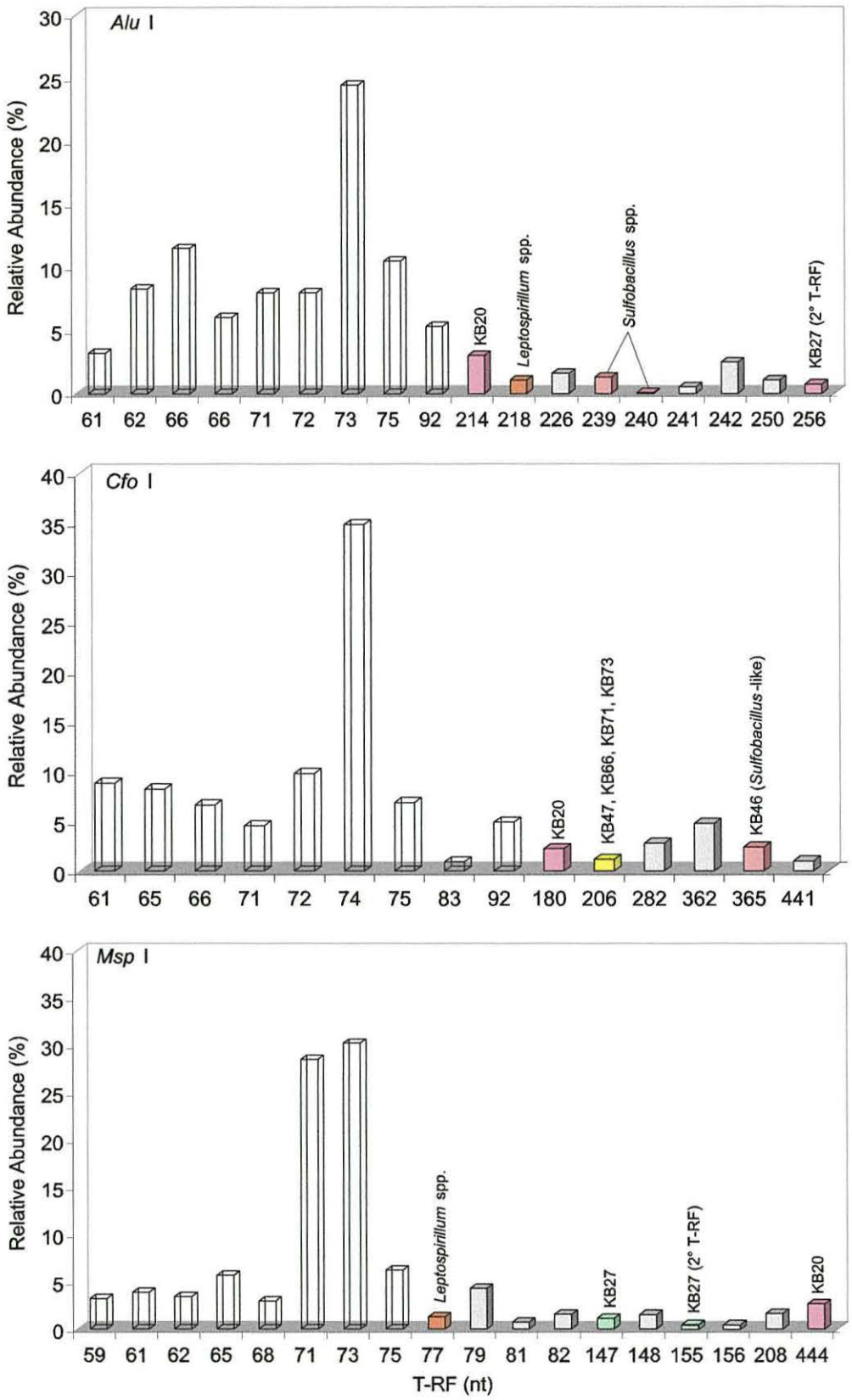


Figure 5.9 T-RFLP profile of the nutrient-amended bioleaching culture following incubation at 30°C for 66 days, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Acidithiobacillus* spp.; , *Alphaproteobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp.; , erroneous T-RF (PCR artefact).)

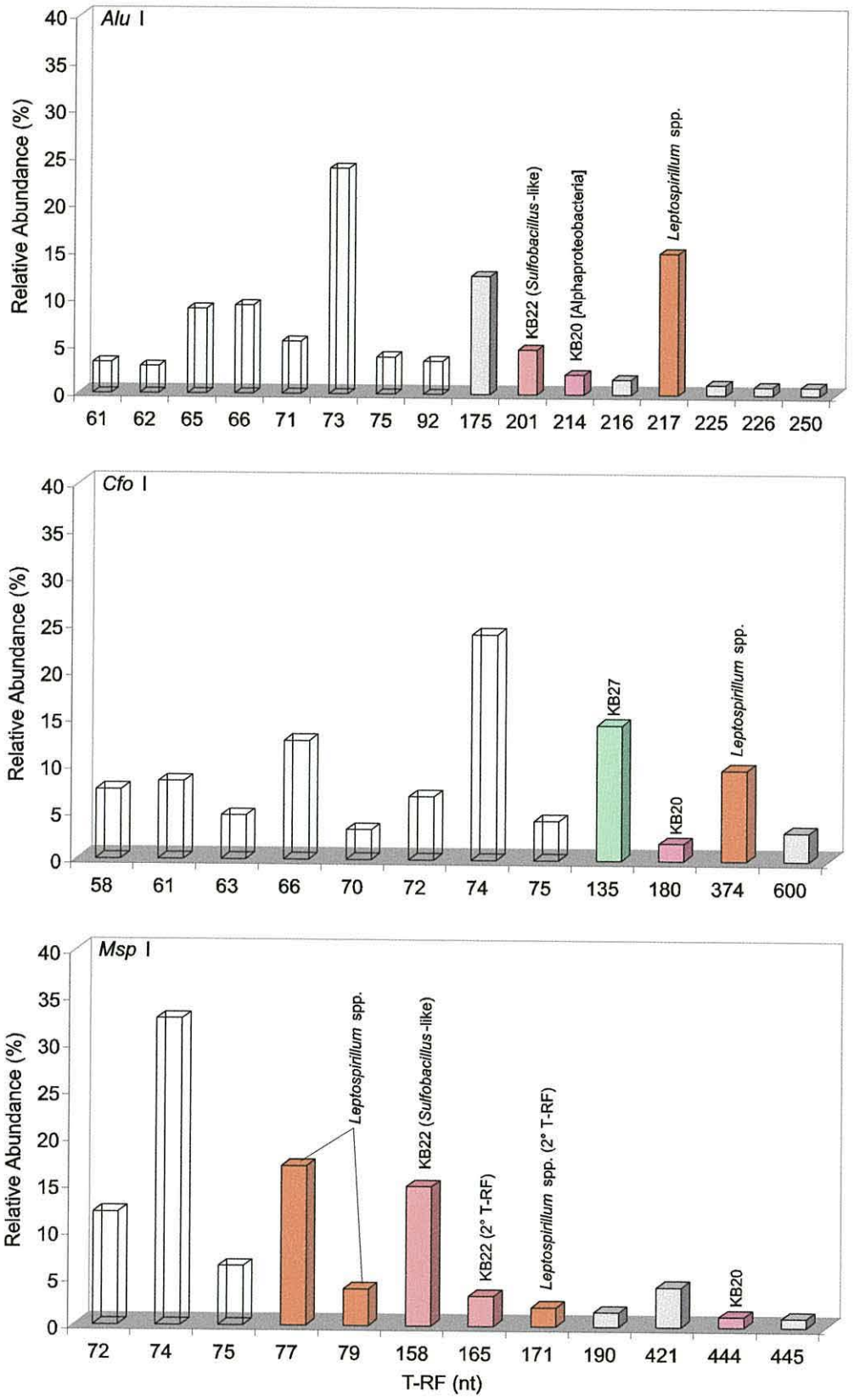


Figure 5.10 T-RFLP profile of the nutrient-free bioleaching culture following incubation at 45°C for 66 days, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Acidithiobacillus* spp.; , *Alphaproteobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp.; , erroneous T-RF (PCR artefact).)

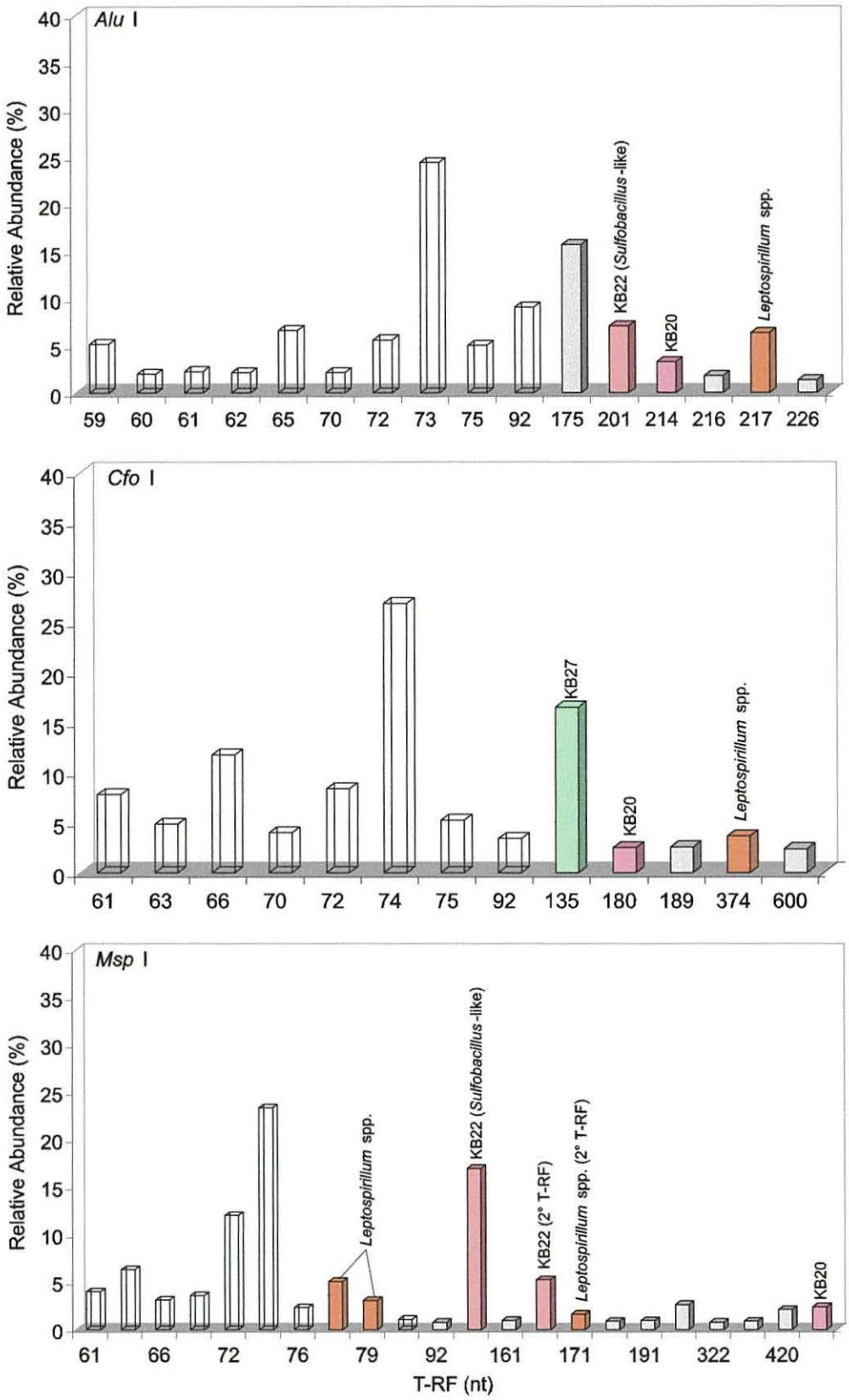


Figure 5.11 T-RFLP profile of the nutrient-amended bioleaching culture following incubation at 45°C for 66 days, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Acidithiobacillus* spp.; , *Alphaproteobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp.; , erroneous T-RF (PCR artefact).)

Figure 5.8 shows the profile of the 30°C nutrient-free bioleaching culture. Population complexity appeared to increase during bioleaching. Primary and secondary T-RFs corresponding to those obtained from the *Actinobacteria* isolate KB27, and those corresponding to iron- and sulfur-oxidising "*Firmicutes*", including isolate KB22 and others not isolated from the material itself, appeared to dominate most of the individual restriction enzyme profiles. T-RFs corresponding to those of the heterotrophic *Acidisphaera*-like isolate KB20 were relatively abundant in two of the three restriction enzyme profiles. T-RFs corresponding to the *Acidithiobacillus* isolates were detected in two of the three restriction enzyme profiles, as were T-RFs corresponding to *Leptospirillum* spp., to varying extents.

The T-RFLP profile for the nutrient-amended 30°C bioleaching culture, shown in Figure 5.9, suggested a slightly less complex population, with T-RFs corresponding to *Acidisphaera*-like isolate KB20 appearing most consistently, with varying levels of detection of the iron- and sulfur-oxidising firmicute, isolate KB46, the *Acidithiobacillus* isolates, *Actinobacteria* isolate KB27 and *Leptospirillum* spp..

The profiles from the 45°C bioleaching cultures were generally similar for both nutrient-free and nutrient-amended cultures, shown in Figures 5.10 and 5.11, respectively. No *Acidithiobacillus* isolates were detected, which is not surprising given that none of those isolated grew at 45°C. T-RFs corresponding to *Leptospirillum* spp. and the heterotrophic *Acidisphaera*-like isolate KB20 were detected consistently. T-RFs corresponding to isolate KB22 were detected with quite high relative abundances in some restriction enzyme profiles but not others. The "*Firmicutes*" were solely represented by KB22 in these profiles, contrary to the 30°C profiles. T-RFs corresponding to the actinobacterium isolate KB27 were only detected in the *Cfo* I restriction digest profiles, which given the relatively high abundance of this T-RF in this profile, suggests this T-RF may actually be derived from a different organism.

In all profiles, there was a variety of presumably genuine T-RFs that could not be identified, suggesting that the complexity was greater than that assumed by the cultivation methods.

5.4 DISCUSSION

Culture-based analysis of the Kennecott heap found that, while the most familiar acidophiles such as *At. ferrooxidans* (both the type strain and NO37 sub-groups) and *At. thiooxidans* were detected, they formed only a minor component of the cultured population. The population was mostly dominated by iron- and/or sulfur-oxidising "*Firmicutes*", most of which were closely related to the genus *Sulfobacillus*. Heterotrophic, non-mineral-oxidising *Bacteria* were detected, but only in relatively small numbers. These findings are slightly at odds with a previous study (Bruhn *et al.*, 1999), which found that Gram-negative mineral-oxidisers such as *At. ferrooxidans*, *At. thiooxidans* and *L. ferrooxidans* dominated the cultured population. However, at the time of the previous study, the heap was actively irrigated, whereas at the time of this study, it had not been actively leached for several years. As a result, the drier and more variable conditions within the heap may favour the growth of Gram-positive organisms over the Gram-negatives, accounting for the possible shift towards a population dominated by "*Firmicutes*". In addition, the previous study relied entirely on colony morphology for the identification of isolated organisms; no molecular analyses were undertaken. This may have led to erroneous identification of organisms, and incorrect conclusions.

Interestingly, the molecular profiles of the enrichment cultures suggested that these populations were dominated by *Acidithiobacillus* spp. and *Leptospirillum* spp.. However, while the enrichments were run effectively as batch cultures, and as such were subject to a certain amount of temporal variation in terms of nutrient availability and osmotic potential, they were inherently more abundant in water and more homogenic than the heap. This essentially suggests that the enrichment conditions favoured the growth of Gram-negative acidophiles. Therefore, the enrichment cultures cannot directly represent the indigenous population, as the enrichment conditions will, to some extent, dictate the resulting population.

Both this study and the previous work by Bruhn *et al.* (1999) found substantially higher numbers of organisms in the surface samples compared to sub-surface samples. A report in 1999 (Esdaile *et al.*, 1999) found that oxygen concentrations in the heap atmosphere were highest at the base of

the heap, and decreased as the gas rose to the surface. However, materials at the surface of the heap would be predicted to contain substantial amounts of oxygen in their pore space, simply due to their proximity to the air above. Therefore, the 1 m and 2 m depth samples potentially contained the lowest oxygen pressures in the heap. As both this and the Bruhn study used only aerobic culture conditions, the differences in plate-counts between the surface and sub-surface samples is most likely explained by these changes in oxygen partial-pressures, although it must be noted that many iron- and/or sulfur-oxidising "*Firmicutes*" are facultative anaerobes, as is *At. ferrooxidans*. The surface samples also appeared to be the most active with regard to mineral-dissolution, based on the geochemical data obtained. It would be interesting to study the distribution of anaerobic microorganisms at different depths and their role in cycling oxidised metals, in terms of heap leaching efficiency.

During its operation, the test heap was found to be effective at leaching copper from the ROM ore, extracting 27% of the total copper over 18 months. While the efficacy of the heap leaching cannot be compared directly to that observed in the bioleaching cultures, the cultures clearly demonstrated that the indigenous population was still capable of mineral oxidation. While much of the residual copper appears to be quite recalcitrant, the bioleaching studies showed a further 30% could be readily mobilised. This is a reasonable amount considering the nature of the material, which appears well weathered visually, and predominately clayey, weathered silicates. The addition of inorganic nutrients or incubation at the two different leaching temperatures appeared to make little difference to leaching efficacy, suggesting that the leaching population was able to adapt to the different conditions, and that nutrients such as nitrogen and phosphorus were not limiting. In general, the population diversity appeared to be greater following bioleaching at 30°C, and more limited following leaching at 45°C. This would suggest that microbial diversity is affected more by operating temperature than nutrient availability.

The detection of T-RFs corresponding to *Leptospirillum* spp. in the 45°C bioleaching culture T-RFLP profiles is interesting as no leptospirilla were isolated directly form the solid-phase samples. While they must have been present in the heap, they must form just a small fraction of the population. However, their relative abundance increased during enrichment. Both the

leptospirilla and the iron- and sulfur-oxidising "*Firmicutes*" appeared to be the dominant organisms following bioleaching at 45°C, suggesting that these organisms are important members of moderately thermophilic leaching operations. It may be that these "*Firmicutes*" are fulfilling the role of the sulfur-oxidiser in this partnership. The presence of the heterotrophic *Acidisphaera*-like isolate KB20 was consistently inferred by T-RFLP analysis in bioleaching cultures at both 30°C and 45°C. This suggests that such heterotrophic organisms are important in both mesophilic and moderately thermophilic bioleaching. They were undetected in the enrichment culture used as the inoculum, suggesting that they may emerge over time during leaching, their numbers increasing relative to the other organisms present. This may be due to the increasing concentrations of organic carbon produced by the mineral-oxidising autotrophs and facultative heterotrophs. Therefore, they may play an important role in reducing the toxicity of the culture to the autotrophs through the consumption of the accumulating organic carbon substrates, such as organic acids, and so prolong the viability of the bioleaching process. In this way, it is likely that they are very important constituents of batch-run bioleaching operations.

The isolation of a bacterium closely related to the iron-oxidising acidophile m-1 is unique, as, as far as is known, this organism has only been isolated once before, from coal strip mine refuse in Missouri, U.S.A. (Harrison, 1982) and has since been lost. It appears to be an iron-oxidising autotroph, and grew well on "inorganic" iFeo plates. Phylogenetic analysis of a partial (~550 nt) sequence of its 16S rRNA gene places it within the *Gammaproteobacteria* class (Section 8.3.3). The *Acidisphaera*-like isolate KB20 is very interesting in that this organism appeared to be moderately thermophilic, in contrast to *As. rubrifaciens* (currently the only designated species in the genus) which is mesophilic. The most closely related characterised moderate thermophile to KB20 is the recently described species, *Acidicaldus organivorans*, which was isolated from a geothermal site in the Yellowstone National Park (Johnson *et al.*, 2006b). It may be that isolate KB20 represents a novel genus, based on partial 16s rRNA gene sequence identity, and its ability to grow at 45°C, although further characterisation is needed to confirm this.

The fact that all attempts to amplify DNA from the heap samples directly failed is surprising, considering that the plate-count data suggested a population size of at least 1.4 x 10^7 cfu g^{-1}. Therefore, it is not likely an issue with cell numbers, but rather some aspect of the mineral material itself. Washing the samples to reduce acidity and, therefore, soluble metal concentrations did not solve this problem. DNA could be successfully amplified from other materials, such as samples from the Shilbottle Brass heap (section 6.3), which, in terms of analytes measured, were quite similar. Therefore, it was thought that some other component of the Kennecott material must have interfered with the DNA extraction procedure at some stage. Whether this is to do with cell lysis or PCR is unclear, but needs to be resolved to allow accurate molecular profiling of the indigenous population.

The Kennecott Bingham Canyon Copper mine bioleach test heap is a highly heterogeneous environment, and hosts a diverse microbial population, dominated by mineral-oxidising "*Firmicutes*". The diversity of the culturable population is likely a product of the variable conditions found within the heap, which have been exacerbated since active irrigation ceased. Yet as a whole, the population retains the potential for bioleaching. It is apparent from the temperature of the heap that the exothermic processes of mineral oxidation are still taking place. Resumption of heap irrigation may well stimulate microbial mineral oxidation, and provide somewhat more homogeneous conditions. It would be interesting to observe whether this would also lead to a subsequent change in the population away from the Gram-positive organisms towards the more common Gram-negatives, as suggested by the T-RFLP profiles of the enrichment and bioleaching cultures.

CHAPTER 6: THE MICROBIAL ECOLOGY OF A HIGHLY PYRITIC COAL SPOIL HEAP AND AN ABANDONED PYRITE MINE

6.1 INTRODUCTION

6.1.1 The Shilbottle 'Brass Heap'

The village of Shilbottle is located in the North East of England, in mid Northumberland just South East of Alnwick close to the North Sea. Mining for coal in the area began in the early 1700's, but the earliest records for the Shilbottle Colliery date from 1882. Production increased in the early 1900's when the Cooperative Wholesale Society took over the mine. At its peak the mine produced 272'000 tons of high-quality household coal *per annum*. Production decreased steadily in the latter half of the 20th century until its closure in 1982 (CoSTaR, Shilbottle, http://www.ncl.ac.uk/minewater/CoSTaR/sites/shilbottle/index.html).

The mine worked a single coal seam, the roof bed of which comprised highly pyritic shale, with an overlying carboniferous limestone stratum. The spoil produced by the mine does not contain any calcareous material as only the pyritic material was excavated. As a result, waters draining the waste heap from the mine workings are acidic, while waters draining the mine are relatively neutral and well buffered (Jarvis *et al.*, 2006). The unusually high quantity of orange iron-rich shales in the spoil heap led to it becoming known locally as the 'Brass Heap', and it is the major source of AMD at the site. Diffuse seepages from the spoil caused severe pollution of the nearby Tyelaw Burn with ochres, aluminium hydroxysulfate foams and manganese deposits.

A reclamation programme was instigated in the late 1990's to remedy the situation. The former colliery yard was cleared, restored and returned to woodland use. Much of the spoil heap was removed and the land restored to agricultural use. The remaining heap was re-graded and mixed with paper mill waste and planted with trees (CL:AIRE site bulletin, march 2006). While the paper mill waste may act to condition the spoil and improve water retention, it is typically low in nitrogen and phosphorus, with a high carbon to nitrogen ratio. The major complex organic elements are lignin (around 15%) and cellulose (around 10%) (Mark Nason, personal communication). However,

waters draining the spoil still caused substantial pollution of the local area. Younger (2003) has suggested that these waters may represent the worst quality mining-related pollution in the U.K.. This drainage is now effectively remediated by a passive treatment system comprising several permeable reactive barriers, settling lagoons and an aerobic wetland (Jarvis *et al.*, 2006). However, this is a treatment rather than prevention, and the spoil leachate remains extremely poor quality. Despite the establishment of a macrobiological population, significant levels of mineral oxidation are still occurring within the heap. This site provided an interesting opportunity to examine the microbial population of a sulfide mineral-rich spoil heap that has been substantially reclaimed. The aim was to elucidate the microbial population, and to examine how the addition of trees and paper mill waste has affected it. The absence of a population capable of sulfide mineral oxidation would indicate that the addition of the paper mill wastes and establishment of a macrobiological population has retarded the generation of AMD within this stratum of the heap.

6.1.2 Cae Coch Sulfur Mine

The abandoned Cae Coch sulfur mine is located above the village of Trefriw in Gwynedd, North Wales. The first reference to the Cae Coch mine comes from 1607 when it was described as having a "great store of brimstone" (pyrite) (Bennet and Vernon, 1997). Production was small scale until the 1800's after which the mine saw sporadic activity, producing at most 80 tons of pyrite per month, for the production of sulfur. Between 1914 and 1918, during the First World War, the mine was acquired by the Ministry for Munitions under the Defence of the Real Act, and extensive exploration and construction followed. During the latter half of 1918 its output was greater than half the total output from U.K. mines.

Mining ceased in 1919, and the mine remained idle until the build up to the Second World War in the late 1930's. Further exploration was undertaken and it was classified as an emergency reserve but was never called upon. It was explored again in the 1950's and 60's but no significant reserves were found and it has remained abandoned ever since. The mine is drained by a

single stream with confluences with other small streams before flowing into the Conwy River in the bottom of a steep-sided valley.

An earlier study found that the water within the mine was dominated by the iron-oxidising *Bacteria*, *At. ferrooxidans* and *L. ferrooxidans*. The numbers of these organisms were found to decrease as the water flowed further down stream from the mine. The mine was found to have no detectable detrimental effect on the Conwy River ecosystem (M^{c}Ginness and Johnson, 1993). Abundant 'acid streamer' growths are found within the mine. A recent study cultivated *At. ferrooxidans*, *L. ferrooxidans*, the iron-oxidising gammaproteobacterium WJ2 and other heterotrophs such as *Acidocella* spp. and *Acidiphilium* spp. Molecular data indicated that the streamer populations were dominated by an uncultured betaproteobacterium. However, the structures were very complex and heterogeneous, with other *Proteobacteria*, *Actinobacteria,* "*Firmicutes*" and *Acidobacteria* detected (Kimura, 2005). Interestingly, the unidentified betaproteobacterium has since been isolated. Denoted betaproteobacterium PSTR, it appears to be an iron-oxidising autotroph that grows as large flocs and filaments *in vitro* (Hallberg *et al.*, 2006).

The roof of the underground mine workings is covered in red liquid droplets where water percolates through the overlying pyritic shale before dropping onto the floor of the void. These droplets represent the early stages of the genesis of AMD at this site. Comparison of the microbial populations of these droplets with those of the water and streamers below could therefore provide an insight into microbial succession in AMD from its genesis onwards. Additionally, the floor of the mine is littered with secondary mineral formations. The presence of microorganisms in these may indicate the extent to which the microbial population may be directly responsible for their formation.

6.2 MATERIALS AND METHODS

6.2.1 Shilbottle Brass Heap

Spoil samples were collected from the surface of the Shilbottle Brass Heap in November 2002 and May 2003. Approximately 300 g of surface material was collected in sterile bags and transported back to the laboratory. Samples were

homogenised by hand, and stored at 4°C or processed within 24 hours. The *p*H and readily extractable metals for all samples were determined as described in section 2.5.1. Figure 6.1 shows the site and sampling location.

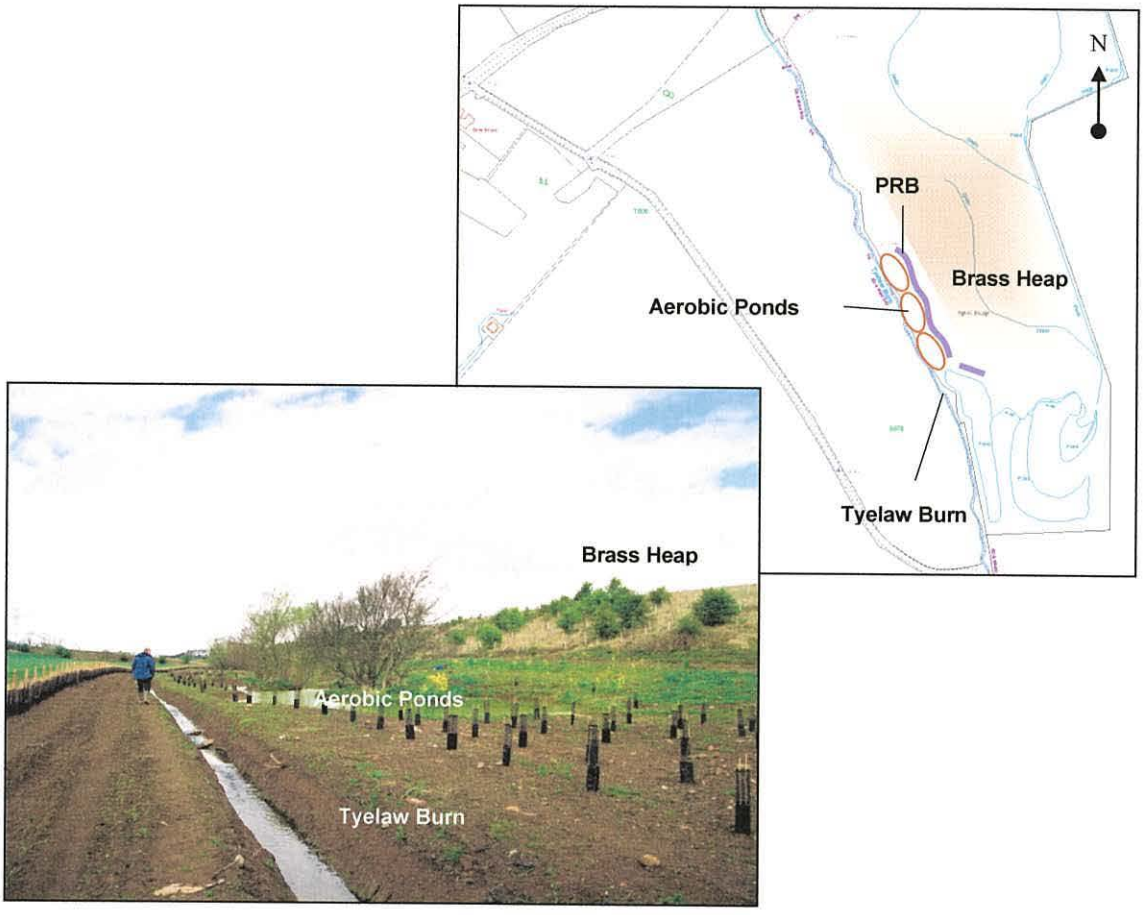


Figure 6.1 Map detailing the Shilbottle Brass Heap site, and a photograph taken at the time of sampling, showing some of the bioremediation scheme and the extent of the reclamation of the heap.

Cells were detached from the spoil matrix by shaking in HBS at *p*H 2.5 for 30 min, as described in section 2.5.1. The cells were serially diluted and plated onto Fe$\underline{o}$, FeS$\underline{o}$ and FeT$\underline{o}$ solid media and incubated aerobically at 20°C for 3-4 weeks. Isolates were categorised by colony morphology, and identity and classification inferred by 16S rRNA gene RFLP and sequence analysis, as described in section 2.2. Cell counts of the liquor used in the plating exercise were determined using the DNA-staining dye DAPI for sample 2, as described in section 2.6.2.

DNA was extracted from 1 g spoil samples using MoBio Soil DNA extraction kits. Microbial 16S rRNA genes were amplified using either

bacterial or archaeal primers, as described in section 2.2.1. Bacterial PCR used either the 27f(G):1492rG or the 27f(G):1387r primer pair.

The indigenous microbial population was examined by T-RFLP analysis using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I, and the construction and analysis of 16S rRNA gene clone libraries, using amplified DNA (sections 2.2.2-2.2.3).

6.2.2 Cae Coch

Samples were collected from within the Cae Coch mine in June 2003. Approximately 20 mL of liquid that formed as droplets on the roof of the underground mine was collected in a sterile container. The *p*H and redox potential of this sample (CC-S1) were determined using an Accumet *p*H meter 50, coupled to a BDH pHase Rapid Renew combination *p*H electrode and a platinum Mettler Toledo InLab 501 Redox electrode with a Ag/AgCl reference. Redox values were converted to E_h, i.e. relative to a hydrogen electrode. Approximately 10 mL of sample was filtered through a 0.2 µm cellulose-nitrate filter (Whatman, U.K.) and dissolved organic carbon (DOC) analysed using a LABTOC DOC analyser (Pollution & Process Monitoring Ltd., U.K.). Metal concentrations were determined by AAS and sulfate concentrations determined turbidometrically, as described in section 2.4. Figure 6.2 shows some details of the site.

Samples CC-S2 and CC-S3 were collected from two distinct secondary mineral formations that were found on the floor of the mine. CC-S2 was a grey, putty-like substance, previously identified as fibroferrite ($Fe(SO_4)(OH)$ $5H_2O$), while CC-S3 was predominately composed of blue crystals, previously identified as melanterite ($FeSO_4 \cdot 7H_2O$) (Johnson *et al.*, 1979). Microorganisms were detached from the mineral matrices as described above. Readily soluble metals and *p*H were determined as described in section 2.5.1. DNA was extracted from 1 g of material using the MoBio Soil DNA extraction kit, and analysed as described above.

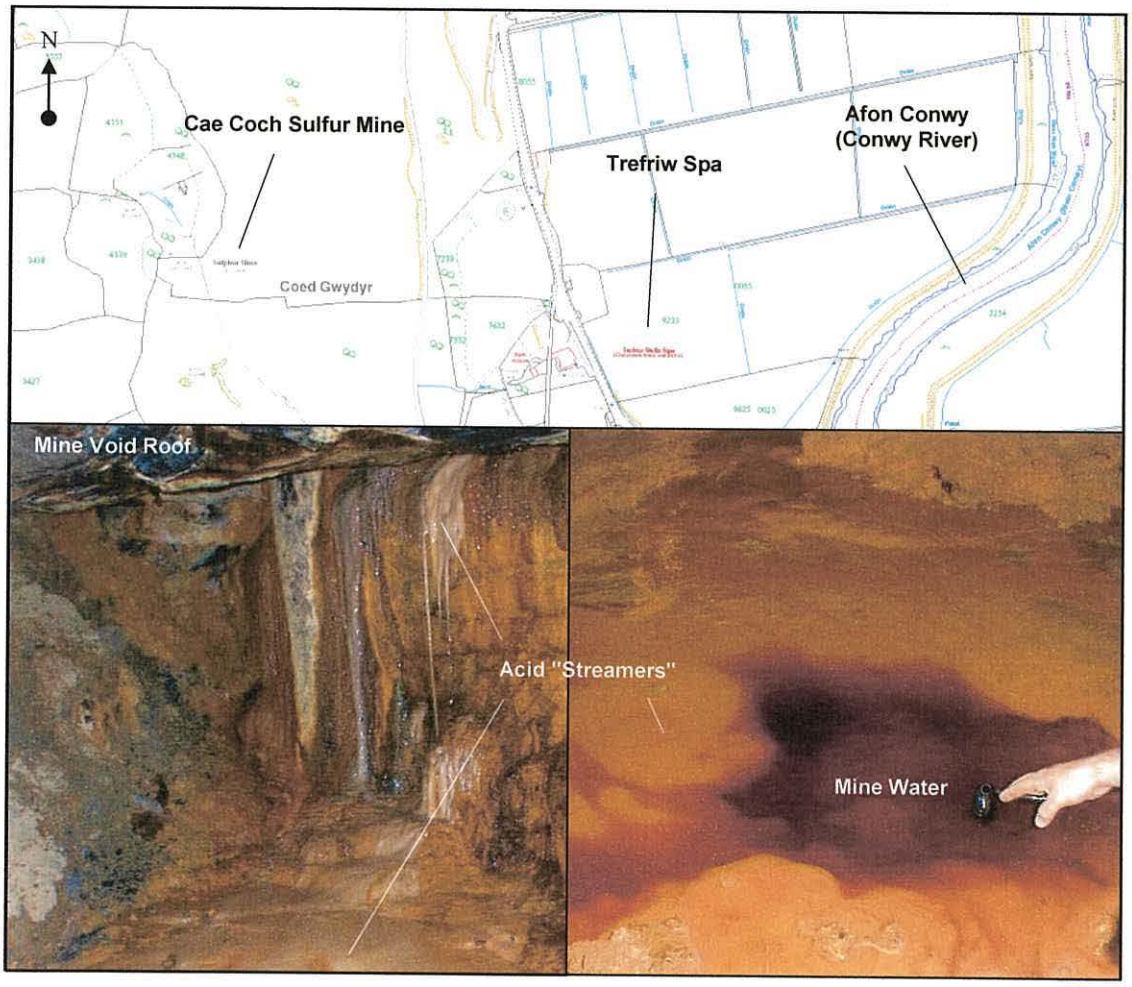


Figure 6.2 Map detailing the Cae Coch sulfur mine site, and photographs taken at the time of sampling, showing the inside of the mine workings

Counts of metabolically active microbial cells in sample CC-S1 were determined using fluorescent *in situ* hybridisation (FISH). Fixed samples were simultaneously stained with a group-specific Cy3-labelled probe, a general bacterial fluorescein (Alexa-fluor 488)-labelled probe, and DAPI. In this way, counts of cells testing positive with the group-specific probes were made against counts of the bacterial probe. This gave relative numbers of various specific active *Bacteria* to the total number of active *Bacteria*. The total numbers of active cells were compared with counts for DAPI-stained cells to obtain viable cell counts. Archaeal cell counts were made relative to total DAPI-stained cells. Probes targeting the *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* classes and the *Leptospirillum* spp. and *At. ferrooxidans* groups were used. Details of the various probes

used are summarised in Table 6.1 All probes were labelled with the indocarbocyanine dye Cy3, except EUB338 which was labelled with the fluorescein derivative Alexa-fluor 488. Probes were labelled at the 5' end.

Table 6.1 Details of oligonucleotide probes used in FISH analysis.

Probe Name	Sequence (5 '- 3')	Target	Formamide	Reference
EUB338[a]	GCTGCCTCCCGTAGGAGT	*Bacteria*	10-50%	Amann *et al.*, 1990.
ARCH915	GTGCTCCCCCGCCAATTCCT	*Archaea*	40%	Stahl and Amann, 1991.
ALF1B	CGTTCG(CT)TCTGAGCCAG Competitor: GCCTTCCCACTTCGTTT	*Alphaproteobacteria* and some *Deltaproteobacteria*, most spirochetes	20%	Manz *et al.*, 1992.
BET42a[b]	GCCTTCCCACTTCGTTT	23S rRNA of most *Betaproteobacteria*	35%	Manz *et al.*, 1992.
GAM41a[b]	GCCTTCCCACATCGTTT	23S rRNA of most *Gammaproteobacteria*	35%	Manz *et al.*, 1992.
LF655	CGCTTCCCTCTCCCAGCCT	*Leptospirillum* groups I, II and III[c]	35%	Bond and Banfield, 2001.
TF539	CAGACCTAACGTACCGCC	*At. ferrooxidans*	20%	Schrenk *et al.*, 1998.

[a]This probe was labelled with the fluorescein derivative Alexa-fluor 488; [b]Due to the single nucleotide difference between the 2 probes, 250 ng of the unlabelled version of the other (a competitor oligonucleotide) was also added for hybridisation; [c]*Leptospirillum* grouping is based on phylogenetic analysis; group III is represented only by environmental clones from the Iron Mountain site.

Fixed samples were spotted or spread onto gelatine-coated glass slides (made by dipping slides into a 70°C solution (comprising (g L^{-1}) gelatine (0.75) and $CrK(SO_4)_2 \cdot 12H_2O$ (0.1) in MilliQ water) and air-dried. Samples were then dehydrated sequentially in 50%, 80% and then 90% ethanol, for 3 minutes each, and allowed to dry. Hybridisation-buffer, containing 25 ng of each labelled probe, was applied to the fixed smear and covered with a cover slip. Unlabelled "helper oligonucleotides" (added to 25 ng) targeted positions either side of the labelled probe target sequence, and acted to increase the amount of fluorescence produced (Fuchs *et al.*, 2000). Unlabelled competitor probes (Manz *et al.*, 1992) were added to 10-fold excess when using labelled probes that had very similar sequences. For example, when using BET42a-Cy3 for the detection of *Betaproteobacteria*, 250 ng of unlabelled GAM41a

was also added to the hybridisation-buffer. Slides were incubated at 46°C for 2 hours in a vessel saturated with hybridisation buffer. A range of hybridisation buffers were prepared; these comprised 20 mM Tris-HCl *p*H 7.4, 0.01% SDS, 900 mM NaCl, and variable concentrations (0-60%, v/v) of formamide, in MilliQ water. The addition of different concentrations of formamide allowed hybridisation to be carried out under a constant temperature regardless of differing annealing temperatures of the various oligonucleotide probes used. After hybridisation, the cover slips were discarded and the slides washed in appropriate pre-warmed wash buffer at 48°C for 15 minutes. Wash-buffer comprised 20 mM Tris-HCl *p*H 7.4, 0.01% SDS, 5 mM EDTA (*p*H 8.0), and 14-900 mM NaCl in RO water. The NaCl concentration in the wash-buffer was changed to match that of the formamide in the hybridisation-buffer so that, for each 5% formamide used, there was an incremental 29.3% decrease in NaCl concentration.

Slides were then rinsed thoroughly with filtered MilliQ water and counter-stained with a 1 μg mL^{-1} DAPI solution for 10 minutes at room temperature. The DAPI solution was washed off with MilliQ water and the slide dried. A mounting medium was applied to the smear (as for DAPI staining but with 70% glycerol) and a cover slip put on top. Cells were visualised using an ECLIPSE E600 (Nikon, Japan) fluorescence microscope. Details of the light properties of fluorophores and the microscope filters used in this study are detailed in Table 6.2. At least 10 randomly selected fields of view were counted per sample to gain an average cell count.

Table 6.2 Light properties of fluorophores and UV filters used in FISH analysis.

Fluorophore	Excitation Maximum (nm)	Emission Maximum (nm)	Filter used	Excitation filter bandwidth (nm)	Barrier filter (nm)
DAPI	358	461	UV-1A-LP	355-375	400
Cy3	550	570	HQ Cy3-BP	510-560	570-650
Alexafluor 488	495	519	HQ FITC-LP	460-500	510

The excitation filter bandwidth refers to the range of wavelength of light allowed to pass by the filter. The barrier filter specifies the range of light generated by the specimen allowed to pass. Long-pass (LP) filters block all wavelengths shorter than that boundary. Band-pass (BP) filters allow light within a certain range to pass.

6.3 RESULTS

6.3.1 Shilbottle Brass Heap

The mean geochemical data from the Shilbottle Brass Heap samples are shown in Table 6.3. The spoil was highly acidic and metal-rich with high concentrations of readily extractable iron and aluminium. The spoil material was a mixture of shale-like stones, intimately mixed with the paper waste that was added during reclamation of the spoil heap. The spoil contained a number of large ants, suggesting a colony must have been established near to the sample location.

Table 6.3 Basic geochemical data from Shilbottle Brass Heap spoil (mean values from all samples).

*p*H*	Readily extractable metals (μg g^{-1})				
	Fe	Cu	Zn	Mn	Al
3.66	1237	3.51	7.81	47.3	141
(0.13)	(11.61)	(0.02)	(1.17)	(4.87)	(15.76)

Bracketed figures are standard deviation; $n = 4$; *1:2.5 spoil to RO water.

No DNA was obtained from the spoil collected in November 2002. Culture data suggested a relatively small microbial population of approximately 1.8 x10^4 cfu g^{-1}. Of these, isolates Sb10 and Sb4 accounted for 54% and 13% of the total colonies obtained, respectively. No identification of the remaining 33% could be made but they all appeared to be heterotrophic acidophiles, based on their colony morphologies. Isolate Sb4 shared 99.6% 16S rRNA gene DNA sequence identity with an *Acidobacteriaceae* (accession ID: AY765993) isolated from a macroscopic 'streamer' growth within the former Mynydd Parys copper mine (Hallberg *et al.*, 2006). Isolate Sb10 shared 99.1% sequence identity with *Acidithiobacillus ferrooxidans* NO37. Unfortunately, no cell count data using DAPI could be obtained for the cell suspension used for plating, so no estimate of plating efficiency was possible.

Bacterial PCR product was amplified from DNA extracted from the May 2003 sample, but no archaeal DNA products were obtained. The *Cfo* I T-RFLP profile for this sample, using the 27f:1492rG primer pair, is shown in Figure 6.3. Interestingly, T-RFs obtained for isolate Sb4 were not present in the community profile. However, T-RFs obtained for other *Acidobacteria* were

found, suggesting that as a class, the *Acidobacteria* were a relatively diverse section of the community. T-RFs corresponding to those obtained for isolate Sb10 represented about 10% of the total T-RFs. A T-RF matching that of the iron-oxidising autotrophic genus *Leptospirillum* was also observed, suggesting that iron- and/or sulfur-oxidising autotrophs make up a substantial proportion of this population. The profile also suggested that the *Gammaproteobacteria* may account for around 4% of the population. While most T-RFs could not be linked with known organisms, the *Actinobacteria* appeared to dominate those that could, and accounted for nearly 20% of the total T-RFs.

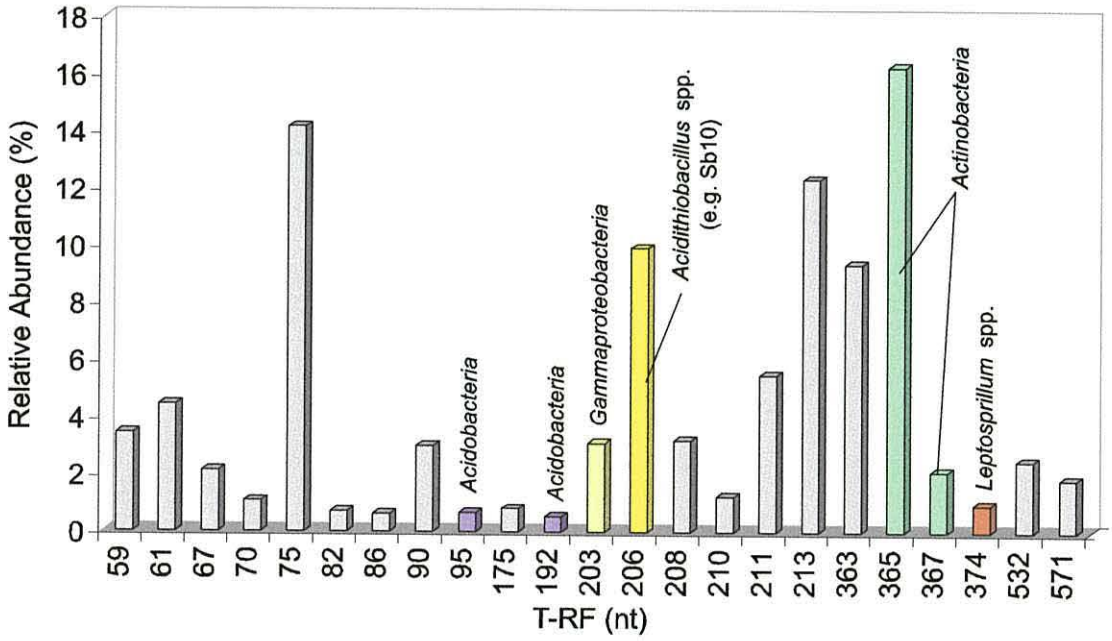


Figure 6.3 T-RFLP profile of the May 2003 Brass Heap sample, using the 27f:1492r primer pair and the restriction enzyme *Cfo* I. (Key: , *Acidobacteria*; , *Actinobacteria*; , *Gammaproteobacteria*; , *Acidithiobacillus* spp.; , *Leptospirillum* spp..)

A small 16S rRNA gene clone library, consisting of 20 clones, appeared to confirm that the *Actinobacteria* class dominated the population of the Shilbottle spoil. The class was represented by half of the total clones, with an unknown *Actinomycetales* clone being the most dominant individual, representing four out of the 20 clones of the population. Three clones where found to be chimeric. Two *Alphaproteobacteria* were identified, one clone being closely related to the genus *Sphingomonas*. One clone was distantly related to a *Clostridia* clone, in the "*Firmicutes*" phylum, though it was not

classified as a member of the *Clostridia* itself. One clone could not be classified and was related to a clone found in acidic forest soil. No *Acidobacteria* were found, but a single clone with almost identical sequence identity to *At. ferrooxidans* NO37 (as had isolate Sb10) was found. These data are summarised in Table 6.4.

Table 6.4 Abundance and nearest relatives of 20 clones obtained from a 16S rRNA gene clone library constructed using DNA amplified from the Shilbottle Brass Heap spoil using the 27fG:1492rG bacterial primer pair, grouped by taxonomic class.

Clone	Nearest relative (accession ID)	Identity	No. of clones
Actinobacteria			
pCBSb2-2	Uncultured soil bacterium clone Tc120-F04 (AY242776)*	97.4%	1
pCBSb2-11	Uncultured *Actinomycetales* clone TM262 (X92710)	96.5%	4
pCBSb2-20	Uncultured bacterium clone fb11 (DQ303258)*	94.7%	1
pCBSb2-22	Agricultural soil bacterium clone SC-I-92 (AJ252667)*	97.6%	2
pCBSb2-29		97.5%	1
pCBSb2-27	Uncultured bacterium clone SK314 (DQ179000)*	97.0%	1
			10
Alphaproteobacteria			
pCBSb2-13	*Sphingomonas* sp. M3C203B-B (AF395031)	99.7%	1
pCBSb2-17	Uncultured bacterium clone 290 (DQ158105)	99.3%	1
			2
Gammaproteobacteria			
pCBSb2-26	Uncultured bacterium clone RCP2-22 (DQ003167)*	92.7%	1
pCBSb2-30	*Legionella gresilensis* (AF122883)	88.1%	1
			2
"*Beta/Gammaproteobacteria*" (acidithiobacilli)			
pCBSb2-31	*Acidithiobacillus ferrooxidans* NO37 (AF376020)*	99.9%	1
			1
Unclassified "*Firmicutes*"			
pCBSb2-10	Uncultured bacterium clone H70 (DQ328625)*	84.9%	1
			1
Unclassified			
pCBSb2-14	Uncultured forest soil bacterium clone DUNssu259 (AY913460)	95.8%	1
			1
Chimerae			
pCBSb2-15	-		1
pCBSb2-18	-		1
pCBSb2-21	-		1
			3

*Associated with mine-impacted or sulfide mineral-impacted environments.

The T-RFLP profile constructed using the 27f:1387r bacterial primers and the restriction enzymes *Alu* I, *Cfo* I, and *Msp* I is shown in Figure 6.4. The *Cfo* I profile was essentially identical to the one generated using the 27f:1492r primer pair (Figure 6.3) indicating that the change of reverse primer had not adversely affected complexity, as determined by PCR-based assays.

The profile again suggested that the class *Actinobacteria* made up at least 20% of the population, while *Acidithiobacillus* spp., such as isolate Sb10, accounted for around 10%. The *Gammaproteobacteria* accounted for around 5%, with the T-RFs in the three restriction enzyme profiles specifically matching those obtained for the moderately acidophilic iron-oxidising heterotrophic *Gammaproteobacteria* obtained from Mynydd Parys and the Wheal Jane former tin mine, such as gammaproteobacterium WJ2, as described in section 3. The *Acidobacteria* class and *Leptospirillum* spp. appeared to exist at or around the lower detection limits for T-RFLP, each representing less than 2% of the total T-RFs in two out of the three restriction digests. The detection of a T-RF corresponding to the alphaproteobacterium, *Acidiphilium acidophilum*, in just one restriction digest does not necessarily imply that this species is present, although other, as yet unidentified *Alphaproteobacteria* may be. The majority of the T-RFs could not be matched to organisms in the database used.

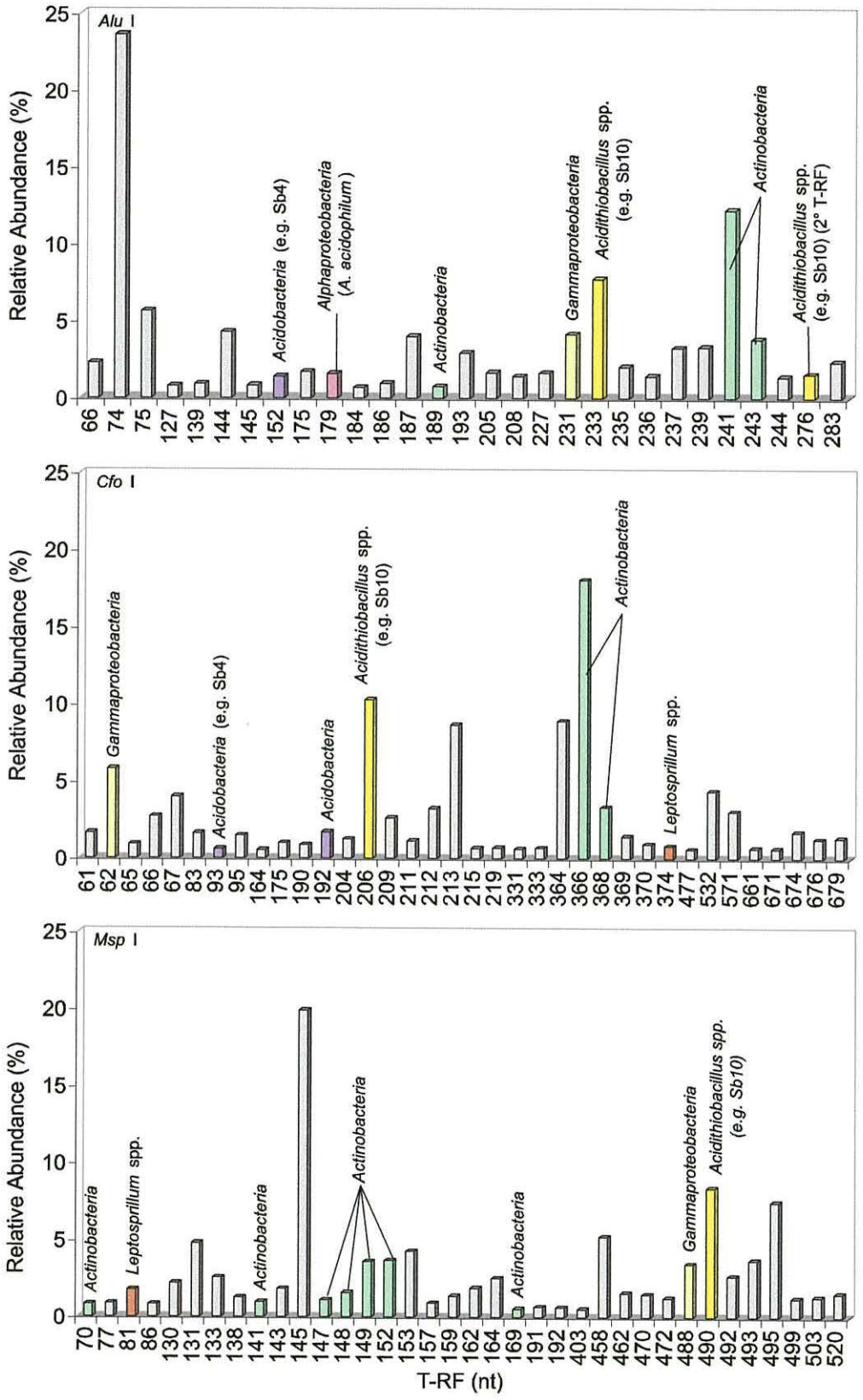


Figure 6.3 T-RFLP profile of the May 2003 Brass Heap sample, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Acidobacteria*; , *Actinobacteria*; , *Alphaproteobacteria*; , *Gammaproteobacteria*; , *Acidithiobacillus* spp.; , *Leptospirillum* spp..)

A second, larger, clone library consisting of 40 clones, using the 27fG:1387r primer pair (Table 6.5) again suggested that the *Actinobacteria* were the dominant class in the Shilbottle spoil sample, with 17 clones, while the *Gammaproteobacteria* also made up a substantial proportion of the library with 13 clones. The *Proteobacteria* phylum made up nearly half the library. Again, *At. ferrooxidans* NO37 was represented by a single clone, as was the genus *Legionella*. A single clone was related to an endosymbiont of the amoeba *Acanthamoeba* spp. The "*Firmicutes*" were represented by 7 clones in total, three of which were members of the *Clostridia* class, while four were could not be assigned to a class. The division *incertae sedis* TM7 was represented by three clones, while a further five clones could not be classified taxonomically. A single clone represented the moderately acidophilic iron-oxidising *Gammaproteobacteria* isolates from Mynydd Parys and Wheal Jane, while three clones were related to *Frateuria* spp. isolated from Wheal Jane and a Norwegian mine site (Hallberg and Johnson, 2003b; Johnson *et al.*, 2001). Again, no *Acidobacteria* were detected. The closest relatives of 25 of the 40 clones in the library come from studies of mine-impacted or sulfide mineral-impacted environments.

Table 6.5 Abundance and nearest relatives of 40 clones obtained from a 16S rRNA gene clone library constructed using DNA amplified from the Shilbottle Brass Heap spoil using the 27fG:1387r bacterial primer pair, grouped by taxonomic class.

Clone	Nearest Relative (accession ID)	Identity	No. of clones
Actinobacteria			
pCBSB206-1	Uncultured eubacterium WCHB1-08 (AF050573)	99.3%	1
pCBSB206-3	Uncultured *Actinomycetales* bacterium clone TM112 (X92698)	95.8%	1
pCBSB206-5	Uncultured *Actinomycetales* bacterium clone TM177 (X92701)	98.5%	4
pCBSB206-8	Uncultured bacterium clone fb11 (DQ303258)*	94.3%	4
pCBSB206-13	Uncultured bacterium clone RCP1-56 (AF523907)*	98.3%	1
pCBSB206-22	Agricultural soil bacterium clone SC-I-92 (AJ252667)	98.5%	1
pCBSB206-27		98.0%	1
pCBSB206-25	Bacterium Ellin5025 (AY234442)	96.6%	1
pCBSB206-42	Uncultured bacterium clone AKAU4215 (DQ125931)*	99.6%	1
pCBSB206-46	Uncultured bacterium clone RCP1-37 (AF523912)*	95.4%	1
pCBSB206-55	Uncultured eubacterium TRA2-10 (AF047642)*	97.2%	1
			17
Alphaproteobacteria			
pCBSB206-16	Uncultured bacterium clone AKAU4071 (DQ125843)*	98.7%	1
pCBSB206-41	Uncultured *Brevundimonas* sp. (AY177781)	99.1%	1
pCBSB206-44	Uncultured bacterium clone AKAU3874 (DQ125760)*	98.8%	1
			3
Gammaproteobacteria			
pCBSB206-6	Acid streamer bacterium PK51 (AY765997)* [a]	94.3%	1
pCBSB206-7	Uncultured bacterium clone EV818SWSAP64 (DQ337087)	93.7%	1
pCBSB206-12	Uncultured clone LiUU-3-167 (AY509435)	91.8%	1
pCBSB206-18	Uncultured bacterium clone MNT-Mm-18 (AY309176)	92.6%	1
pCBSB206-21	Uncultured bacterium clone HTB10 (AF418945)	96.9%	1
pCBSB206-24	*Frateuria* sp. NO16 (AF376025)*	98.9%	1
pCBSB206-36	Uncultured bacterium clone JFJ-ICE-Bact-01 (AJ867747)	97.9%	1
pCBSB206-40	*Lysobacter ginsengisoli* strain Gsoil 357 (AB245363)	99.3%	1
pCBSB206-51		99.3%	1
pCBSB206-45	*Frateuria* sp. WJ64 (AY495957)*	98.5%	2
pCBSB206-53	*Legionella rubrilucens* (WA-270A-C2) (Z32643)	95.8%	1
pCBSB206-60	Uncultured bacterium clone fb10 (DQ303257)* [a]	99.6%	1
			13
"*Beta/Gammaproteobacteria*" (*acidithiobacilli*)			
pCBSB206-9	*Acidithiobacillus ferrooxidans* NO37 (AF376020)*	99.5%	1
			1

Table6.5 Cont..

Clone	Nearest Relative (accession ID)	Identity	No. of Clones
Unclassified *Proteobacteria*			
pCBSB206-11	*Aquicella siphonis* strain SGT-108T (AY359283)	93.6%	1
pCBSB206-19		91.5%	1
			2
Sphingobacteria			
pCBSB206-38	Endosymbiont of *Acanthamoeba* sp. ATCC 30871 (AY549545)	95.8%	2
			2
Clostridia			
pCBSB206-17	Uncultured bacterium clone cD48726 (AJ617915)	99.2%	3
			3
Unclassified "*Firmicutes*"			
pCBSB206-14	Uncultured bacterium clone H70 (DQ328625)*	88.6%	1
pCBSB206-15		86.0%	3
			4
Genus *incertae sedis* TM7			
pCBSB206-29		94.8%	1
pCBSB206-50	Uncultured bacterium clone FW73 (AF524021)*	94.8%	1
pCBSB206-56		95.1%	1
			3
Unclassified			
pCBSB206-23	Agricultural soil bacterium clone SC-I-92 (AJ252667)	98.6%	1
pCBSB206-32	Uncultured bacterium clone 1894a-24 (AY917553)*	88.2%	2
pCBSB206-33	Uncultured *Gemmatimonadetes* bacterium clone AKYG1662 (AY921853)	89.7%	1
pCBSB206-34	Unidentified eubacterium (AF009988)	87.6%	1
			5

*Associated with mine-impacted or sulfide mineral-impacted environments; [a]Related to moderately acidophilic iron-oxidising heterotrophs isolated from Mynydd Parys and Wheal Jane (gammaproteobacterium WJ2/PK51).

6.3.2 Cae Coch

The geochemical data from sample CC-S1 are summarised in Table 6.6. These droplets on the roof of the mine were very acidic, with a high redox potential, and with relatively high concentrations of dissolved organic carbon compared to values of between 1 and 5 mg L^{-1}, typical of AMD. Concentrations of ferric iron and sulfate of 552 mM and 880 mM, respectively, are extremely high. Combining the data for soluble ferrous and ferric iron and sulfate equates to the droplets containing about 10 mM ferrous sulfate

($FeSO_4$) and 280 mM ferric sulfate ($Fe_2(SO_4)_3$). Levels of other soluble trace elements measured were also relatively high. Concentrations of total iron and sulfate were 20 times greater in the droplets than in the mine void water. Additionally, the void water contained much greater concentrations of ferrous iron relative to ferric than the droplets. Concentrations of copper, zinc and manganese were greater in the droplets compared to the void water, while concentrations of aluminium were greater in the mine void water. Despite the abundant 'acid streamers' in the void water, dissolved organic carbon concentrations were much greater in the droplets.

Table 6.6 Basic geochemical data for Cae Coch sample CC-S1 compared to those obtained for the mine void water (S3T).

	*p*H	DOC (mg L^{-1})	E_h (mV)	SO_4^{2-} (mg L^{-1})	Total soluble metal (mg L^{-1})					
					Fe^{2+}	Fe^{3+}	Cu	Zn	Mn	Al
CC-S1	2.00	24	+796	84400	570	30965	37	118	63	19
S3T*	2.26	5.7	+725	4128	224	1344	0.64	1.3	4.95	84

*Kimura, 2005.

The geochemical data for the secondary mineral samples CC-S2 and CC-S3 are summarised in Table 6.7. These minerals were also highly acidic, and contained high concentrations of readily extractable metals, particularly iron. No isolates were obtained from these deposits and no PCR products could be obtained from DNA extracts either, suggesting these deposits were effectively sterile, or hosted only relatively few indigenous microorganisms.

Table 6.7 Basic geochemical data for Cae Coch samples CC-S2 and CC-S3.

Sample	*p*H*	Readily extractable metals (mg g^{-1})				
		Fe	Cu	Zn	Mn	Al
CC-S2	2.04	45.52	0.13	0.11	0.20	1.11
CC-S3	2.65	164.85	0.25	0.07	0.12	0.02

*1:2.5 mineral to RO water.

Culture data from sample CC-S1 suggested a monoculture of isolate CC2, which shared 100% 16S rRNA gene sequence identity with *At. ferrooxidans* NO37. Plate counts indicated a population size of 4.4 $x10^6$ cfu mL^{-1}. Counts using the DNA-staining dye DAPI were 2.7 $x10^7$ cells mL^{-1}, of which FISH suggested 73% were active *Bacteria*, suggesting a plating

efficiency of approximately 24%. No *Archaea* were detected using the archaeal FISH probe. Of the active *Bacteria*, 94% were shown to be *Gammaproteobacteria*, specifically *At. ferrooxidans*, and the rest *Leptospirillum* spp.. No *Alphaproteobacteria* or *Betaproteobacteria* were detected. Therefore, the population was composed of *At. ferrooxidans* and *L. ferrooxidans* only, in a 19:1 ratio.

T-RFLP data using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I (data not shown) suggested a monoculture of *At. ferrooxidans* NO37. The same was true with a bacterial 16S rRNA gene clone library comprising 20 clones, of which all were very closely related (>99% sequence identity) to *At. ferrooxidans* NO37 (data not shown). This appeared to contrast with the FISH data, but was in agreement with the culture data.

6.4 DISCUSSION

6.4.1 Shilbottle Brass Heap

Despite extensive renovation, the Shilbottle Brass Heap still has particularly elevated concentrations of readily extractable metals, and is highly acidic. Concentrations of readily extractable iron were very high, which is not surprising given the highly pyritic nature of the spoil. Concentrations of aluminium and manganese were also quite high. This implies that an active mineral-oxidising population exists within the spoil heap, and that while this remains active, the site is likely to continue producing AMD.

While a single isolate from the *Acidobacteria* class made up 13% of the total isolates from the spoil, T-RFLP and clone library data suggest that the whole class probably accounted for less than 2% of the total population. The only mineral-oxidising isolate obtained was an *At. ferrooxidans* NO37-like organism. However, molecular data suggested that *Leptospirillum* spp. and the iron-oxidising moderately acidophilic *Gammaproteobacteria*, such as PK51 and WJ2 were also present. T-RFLP suggested that *At. ferrooxidans* NO37 accounted for around 9% of the total population, while clone library data suggested around 5%. However, the clone libraries were probably too small and the population too complex to allow accurate quantitative analysis based on clone numbers in the libraries. *Gammaproteobacteria* related to the

iron-oxidising isolate PK51 appeared to represent up to 5% of the population, according to both T-RFLP and clone library analysis. This implies that a sizeable proportion of the population in the stratum analysed, possibly up to 15%, may be capable of iron oxidation, and therefore, if the *p*H is sufficiently low, of accelerating the oxidative dissolution of sulfide minerals.

It is interesting that, while the majority of clones identified were related to uncultured organisms, more than half of the total clones in each library were most closely related to clones from areas impacted either by mining-related activities or other sources of sulfide minerals, such as hot springs and volcanic events. However, without any information on the metabolic activities of these organisms it is not possible to infer their relevance in terms of pollution cause or consequence. It may be that they are responsible for mineral-oxidation, or simply that they are adapted to surviving its consequences (i.e. in a low *p*H, metal-rich environment). Due to the fact that most of the studies in which these related microorganisms have been discovered are yet to be published, little information is available on the conditions of the sites from which they were found, in terms of regeneration, recolonisation etc.. The presence of such organisms does appear to link these sites together and, whether this is a result of the impact of pollution or an indication that the site is recovering, would be an interesting area for further study.

However, the spoil does support a sizeable macrobiological population, in the form of young trees, and insects (ants, and possibly others). Therefore, it is necessary to consider the potential microbial-macrobial interactions occurring within the heap. It is likely that the trees are providing a source of carbon to the microbiota in the form of root exudates, decaying foliage and other detritus. The mycorhizosphere may also offer some protection against the high toxicity of the spoil. This could explain the juxtaposition of iron-oxidising, autotrophic extreme acidophiles, such as *At. ferrooxidans* and *Leptospirillum* spp., with organisms usually thought of as heterotrophic neutrophiles, such as many members of the *Proteobacteria* (e.g. *Leigionella* spp.) and members of the *Clostridia*. The abundant carbon, introduced by the macroflora may also explain, to some extent the occurrence of other

moderately acidophilic heterotrophic organisms such as *Frateuria* sp. NO16 and WJ64 in the clone libraries.

These data suggest that the establishment of trees and other macroorganisms has done little to attenuate the genesis of AMD at this site, and does little more than improve the aesthetics of the area. While they may provide a source of carbon, and allow the establishment of a population of heterotrophic microorganisms which do not rely on lithotrophic sources of energy, this clearly has not precluded the occurrence of a lithotrophic population. On the contrary, it could conceivably be the case that the establishment of a rhizosphere serves to extend the oxic zone further into the heap, allowing mineral oxidation to occur at greater depths. This is known to take place in anaerobic wetlands planted with macrophytes, where ferric iron can be seen to accumulate on root structures (e.g. Batty et al., 2005). This does, therefore, pose an interesting paradox with a view to reclamation. While the outward signs are that the site has been remediated, the truth is far more clandestine. Indeed, the "Discover Northumberland 2003 Countryside Events Flyer" available at the Northumberland Communities' website remarks:

> "The pit heap at Shilbottle has itself been landscaped and the hostile shales covered in wastes from paper-making which have in turn allowed trees to be established. The former railway for the mine has been established as a public bridleway from which the remarkable transformation from polluting industrial wasteland to attractive and benign countryside may now be appreciated."

Yet the drainage from the heap is some of the worst quality mine-related water found in the U.K., and has required the installation of a bioremediative system to prevent gross pollution of the nearby Tyelaw Burn and surrounding environment. This is anything but "benign".

This may have serious implications if these results are common to other spoil and tailings heaps where similar remedial strategies have been applied. However, it is not clear how common this type of treatment is. Successfully remediated waste heaps have involved the application of a low permeability cover, such as clay, prior to the addition of top soil and vegetation (Younger, 2002). Such a technique was used to treat tailings heaps at the former Rum Jungle uranium mine in Australia. Here, the application of a clay layer followed by top soil to provide some protection,

reduced rainfall ingress to less than 5% (Harries and Ritchie, 1990). This reduces the availability of oxygen within the heap, and so dramatically reduces the rate of mineral oxidation. Additionally, reducing the amount of water entering the heap concomitantly reduces the amount of heap drainage. Therefore, it is clear that relying solely on the formation of a vegetation cap at the Shilbottle site without employing a more robust barrier has done little to reduce oxygen influx, at least in the short term.

While the spoil heap has been re-graded and planted with trees, this has not been sufficient on its own to ameliorate the environmental impact of the Shilbottle waste heap. Extensive mineral oxidation still occurs, which is reflected by the geochemistry and microbiology of the site. That the mineral-oxidising microfloral population is apparently dominated by *At. ferrooxidans* NO37 implies that this organism is ubiquitous in acid mine drainage environments, both in AMD itself, and also in the spoil from which it is generated.

6.4.2 Cae Coch

The apparent absence of microorganisms within the secondary mineral deposits was somewhat surprising. Microorganisms are directly responsible for the oxidative dissolution of pyrite, which is a necessary pre-emptive reaction that allows the formation of melanterite and fibroferrite within Cae Coch. However, the results of this study suggest that they are not directly involved in the formation of these secondary minerals, which is presumably an entirely abiotic process. These results suggest that, if microorganisms were to be found associated with such deposits, this would be fortuitous, being entrapped in the minerals as they formed from solution phases.

The droplets from the mine roof are very interesting. The liquid is essentially a 0.25 M solution of ferric sulfate, which is ~0.5 M with regard to ferric iron. It is a highly aggressive solution, with high acidity and a high concentration of ferric iron oxidant, and rich in toxic metals such as aluminium, yet supports quite a large population of extremophilic microorganisms. That this population was dominated by an *At. ferrooxidans* NO37-like organism is somewhat surprising, as it is has been found that *Leptospirillum* spp. rather than *At. ferrooxidans* tend to emerge as the dominant iron-oxidising

microorganisms in conditions of high redox potentials, ferric iron concentrations and extreme acidity (e.g. Rawlings *et al.*, 1999). While *Leptospirillum* spp. were detected, the most quantitative method used in this study, FISH, put their abundance at just 6%, and they were not detected by either cultivation or PCR-based methods. At such low levels, this is not too surprising with the PCR-based assays as the inherent bias of these techniques may have further accentuated the dominance of *At. ferrooxidans* NO37. While *L. ferrooxidans* is readily cultured on the solid media used, a combination of low plating efficiency and low relative numbers could have led to it being missed during analysis of plates covered in iron-oxidising colonies. Additionally, the probes used in FISH analysis stain all characterised *Leptospirillum* spp., and it is conceivable that the *Leptospirillum* sp. present in the droplets was not the more familiar *L. ferrooxidans*, but another species (such as *L. ferrodiazotrophum*) which may be more difficult to culture on the solid media used (though superior solid media have since been devised for all known *Leptospirillum* spp., D. B. Johnson, unpublished data). The relative simplicity of microbial community of the droplets on the mine of the roof contrasts greatly with the complex 'acid streamer' microflora that occur in vast abundance within the main stream that flows through Cae Coch (Kimura *et al.*, 2005), though is similar to the stream water itself (M^{c}Ginness and Johnson, 1993). Table 6.8 summarises the occurrence of different organisms in the Cae Coch mine.

It is proposed that AMD in Cae Coch is formed by the microbially-accelerated oxidative dissolution of the remaining exposed pyrite in the partially worked-out mine. Water percolating through cracks and fissures in the roof of the mine provides the necessary liquid phase for microbial colonisation, while the exposure of the surface minerals to air ensures that the oxygen required for regeneration of ferric iron and thereby the continuation of pyrite dissolution continues. This results in very elevated concentrations of dissolved solutes (principally ferric and ferrous sulfates) in the roof droplets, which is probably the reason why the biodiversity of these droplets is so limited. Many microorganisms found in the acid streamer growths in Cae Coch (such as betaproteobacterium PSTR, gammaproteobacterium WJ2 and *Acidocella* and *Acidiphilium* spp.), are far less tolerant of low *p*H and, in

particular, elevated concentrations of ferric iron, than are *At. ferrooxidans* and *Leptospirillum* spp.. The absence of heterotrophic and mixotrophic microorganisms in these droplets means that there is no immediate sink for the DOC that arises as exudates and lysates from the autotrophic iron-oxidisers present, and it therefore accumulates to much higher concentrations than in the main stream in the mine. Accumulation of DOC, and the persistently relatively low temperature (*ca.* 9°C) within Cae Coch are two possible reasons why, despite the high redox potentials of the roof droplets, *At. ferrooxidans*-like *Bacteria* dominate. *Leptospirillum* spp. tend to be more sensitive to soluble carbon than *At. ferrooxidans* and have higher temperature optima (Hallberg and Johnson, 2001).

Table 6.8 Summary of the organisms detected in the Cae Coch droplets, mine water and 'acid streamers'.

	Droplets	Mine void water[a]	Acid "Streamers"[b]
At. ferrooxidans	+	+	+
L. ferrooxidans	+	+	+
Betaproteobacteria (PSTR)	-	-	+
Gammaproteobacteria (WJ2)	-	-	+
Alphaproteobacteria (*Acidocella*, *Acidiphilium*, *Sphingomonas*)	-	-	+
Acidobacteria	-	-	+
"*Firmicutes*"	-	-	+
Actinobacteria	-	-	+
*p*H	2.00	2.26	1.90
$[Fe^{2+}]$ (mg L^{-1})	570	224	1680
$[Fe^{3+}]$ (mg L^{-1})	30965	1344	2688
$[SO_4^{2-}]$ (mg L^{-1})	84400	4128	5088

[a]M^{c}Ginness and Johnson, 1993; [b]Kimura *et al.*, 2005.

It is interesting to note that it is *At. ferrooxidans* NO37 rather than the type strain of the species that dominates the microbial population in the roof droplets at Cae Coch. This was also found to be the case in the Achada do Gamo pool at São Domingos, which also contained very high concentrations of soluble ferric iron, though its average temperature is significantly higher (and more variable) than that within Cae Coch. Pioneering work by Norris *et al.* (1987) compared the relative substrate affinities and ferric iron inhibition kinetics of *At. ferrooxidans* and *L. ferrooxidans*. They found that *At.*

ferrooxidans was much more sensitive to high ferric iron concentrations than *L. ferrooxidans*, with K_i values of 3.10 and 42.8 mM Fe^{3+}, respectively. However, circumstantial evidence from this study and at São Domingos, suggests that *At. ferrooxidans* NO37, which represents a distinct phylogenetic group within the *At. ferrooxidans* clade (Chapter 8), may have ferric iron inhibition kinetics more similar to those of *L. ferrooxidans*. This would give *At. ferrooxidans* NO37 an advantage over other *At. ferrooxidans* subspecies in the Cae Coch droplets, while its mesophilic temperature optimum allows it to out compete *L. ferrooxidans* in the cool environment. This could provide an interesting niche for NO37-like *Bacteria* for bioprocessing sulfide mineral ores at low temperatures, where high redox potentials and elevated ferric iron concentrations can be important for optimising the rate of mineral dissolution.

Mineralogically, both the Shilbottle Brass Heap and the Cae Coch mine are dominated by pyrite. In both cases, the dominant cultivated iron-oxidising microorganism was found to be most closely related to *At. ferrooxidans* NO37. However, as a whole, the microbial population differed quite markedly between these two sites. This is not surprising given that the Shilbottle Brass Heap has undergone substantial reclamation, and is subject to external influences such as the interactions of the microbiota with the planted trees. On the other hand, the Cae Coch underground mine workings are essentially isolated, and represent the initial stages of AMD formation from within abandoned deep mines.

CHAPTER 7: THE MICROBIAL ECOLOGY OF MINERAL WASTES AT TWO ABANDONED LEAD-ZINC MINES IN WALES, U.K.

7.1 INTRODUCTION

7.1.1 Parc Mine

Parc mine is a former lead-zinc mine located within the Gwydyr Forrest area of Conwy, North Wales, U.K.. The Gwydyr Forrest has hosted several mines, including the Parc, Hafna, Llanrwst, Cyffty, Pandora and Aberllyn mines, all of which are now abandoned. The area has probably been exploited since Roman times, as the Roman road of Sarn Helen passed through the forest.

Parc mine was the most successful of the mines in this area, and was worked intermittently from 1855 up until the 1940s. It was then reopened in 1950 and formally closed in 1954. The mine followed the main lead and zinc lode in the area, the 'Principal Lode', through a complex of underground workings. A large tailings heap, made up of fine calcareous silt-sized particles with high content of lead and zinc, resulted from these activities. Leachate from this waste compounded the uncontrolled drainage flowing from the underground mine workings, and pollution from the site flowed into the Nant Gwydyr, a tributary of the Afon Conwy which flows northwards down the Conwy Valley towards the sea. In periods of heavy rainfall, erosion of the exposed mine wastes and tailings mobilised metal-rich fine-grain particles, resulting in serious contamination of the Conwy River and agricultural land in the valley flood plain below. Between 1954 and 1978 13,000 tonnes of waste, containing 43 tonnes lead, 104 tonnes zinc and 1 tonne cadmium were estimated to have been eroded from the heap (Gao and Bradshaw, 1995).

In 1977 a remediation programme was initiated to stabilise the tailings and to minimise further erosion. This involved landscaping the heap and covering it with a layer of coarse quarry waste and planting the surface with a metal-tolerant grass cultivar. While this has succeeded in its primary objective to stabilise the tailings, it has done little to reduce metal concentrations in the waste leachate. The formation of the vegetation cap may have reduced weathering of the exposed minerals, but the site still contributes an estimated

1 tonne zinc, 0.2 tonnes lead and 0.5 tonnes cadmium to the Conwy River annually (Gao and Bradshaw, 1995).

This site presented an interesting opportunity to examine the microbial population of a site that has been subjected to extensive remediation efforts. It has now been nearly 30 years since the waste was re-graded and mixed with quarry waste. The objective was to determine how the alkalinity imparted on the tailings, due to the addition of the calcareous quarry waste and as a result of the indigenous calcareous minerals, affects the indigenous microbial populations.

7.1.2 Cwm Rheidol

The Cwm Rheidol, in the Ceredigion region of Mid-Wales, U.K., has hosted several lead and zinc mines. Although historical data for this area are scarce, mineral exploitation is thought to have occurred for a considerable period of time (Prichard, 1985). The Cwm Rheidol mine itself is located on the northern side of the Afon Rheidol, and principally mined sphalerite (ZnS) from 1900 to 1912. Ore was crushed on site, and transported, via an aerial ropeway, to the nearby Valley of Rheidol Railway. The ore crusher was also used to process ore from at least four other mines in the area, including the Bwichgwyn, Penrhiw, Ystumteun and Llwynteifi mines. Ore was brought to the site from these mines via a lengthy adit, known as Level Fawr or the Andersons Level adit, which emerges on the side of the valley, high above the Cwm Rheidol spoil heaps. Ore was also brought, via a tramway, from the nearby Tynyfron Mine (Environment Agency Wales, 2002).

Mine drainage from the Cwm Rheidol mine seriously impacts the Afon Rheidol, causing at least 16 km of the river to fall below the objective quality targets set by the Environment Agency. The mine water enters a passive remediation system (a constructed wetland) prior to entry into the Rheidol, but this has proved to be largely ineffective. Temporary works were undertaken in the 1990's in an attempt to reduce the amount of pollution flowing into the site from the Andersons adit. However, these were ineffective and significant erosion of the Cwm Rheidol spoil occurs below this level (Environment Agency Wales, 2002). Further remedial work is planned, but the stability issues associated with the spoil heaps make the implementation of a system

to treat the mine effluent as it emerges from the Andersons adit extremely difficult.

The spoil at this site has itself undergone no form of remediation or re-grading. This site presented, therefore, an interesting opportunity to study the microbial population of spoil, which as well as contributing effluent to the environment, receives AMD from a nearby adit and has not undergone any form of reclamation.

This part of the research project aimed to investigate mine waste at two contrasting abandoned mine sites that, although exploited for similar ores, differed in that one has undergone extensive re-grading (Parc) while the other (Cwm Rheidol) has remained essentially undisturbed for nearly 100 years.

7.2 MATERIALS AND METHODS

7.2.1 Parc Mine

Tailings samples were collected from a clearing in the woodland on the border of the mine, adjacent to a stream running down the re-graded waste heap (Figure 7.1). Three samples (each approximately 300 g) of surface material were collected in sterile polyethylene bags and transported to the laboratory. The first was collected in October 2002, the second in May 2003 and the third in June 2003. The material contained a large amount of stone aggregate, which had been mixed with the tailings during reclamation. Samples were sieved to remove stones larger than *ca.* 1 cm diameter and stored at 4°C or processed within 24 hours. Tailings *p*H and readily extractable metal concentrations were determined as described in section 2.5.1.

Microorganisms were detached from the tailings matrix, as described in section 2.5.1, using HBS adjusted to *p*H 3.0. The resulting liquor was serially diluted, plated onto Fe$\underline{o}$, FeS$\underline{o}$, FeT$\underline{o}$ and YE$_3\underline{o}$ solid media and incubated at 20°C under aerobic conditions for 3-4 weeks. Isolates were categorised by colony morphology, and identity and classification inferred by 16S rRNA gene RFLP and sequence analysis, as described in section 2.2. Cell counts of the liquor used in the plating exercise for both 2003 samples were determined using the DNA-staining dye DAPI, as described in section 2.6.2.

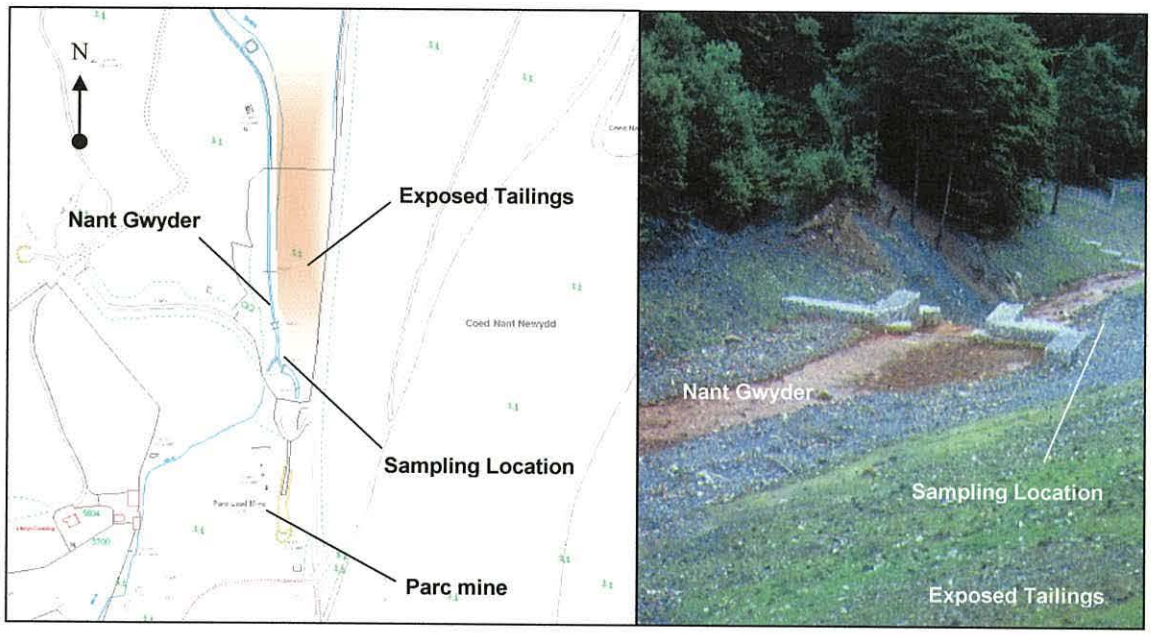


Figure 7.1 Location of the Parc mine, and details of the site. (Source: DigiMap.)

DNA was extracted from 1 g tailings using the MoBio Soil DNA extraction kit. Microbial 16S rRNA genes were amplified by either bacterial or archaeal PCR as described in section 2.2.1. Bacterial PCR used either the 27f(G):1492rG or the 27f(G):1387r primer pairs.

The indigenous microbial population was examined by T-RFLP analysis using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I, and the construction and analysis of 16S rRNA gene clone libraries using amplified DNA (sections 2.2.2-2.2.3).

7.2.2 Cwm Rheidol

Spoil was collected from a section of the Cwm Rheidol spoil heap, slightly upstream of the constructed wetland, near to the banks of the Afon Rheidol (Figure 7.2) in May 2005. Approximately 300 g of surface material was collected in a sterile polyethylene bag and transported to the laboratory. The spoil was homogenised by hand, and either stored at 4°C or processed within 24 hours. Spoil *p*H and readily extractable metal concentrations were determined as described in section 2.5.1.

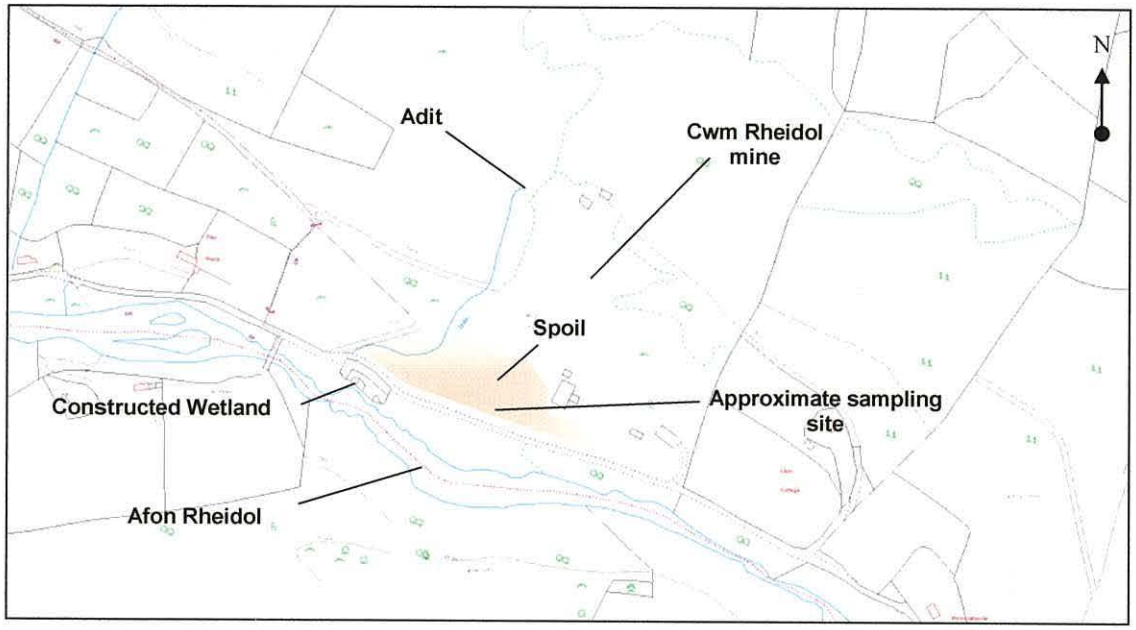


Figure 7.2 Location of the Cwm Rheidol mine, and details of the site. (Source: DigiMap.)

Microorganisms were detached from the spoil material, as described in section 2.5.1, using HBS adjusted to *p*H 3.0. The resulting liquor was serially diluted, plated onto Feo, FeSo, FeTo and YE_3o solid media and incubated at 20°C under aerobic conditions for 3-4 weeks. Isolates were categorised by colony morphology, and identity and classification inferred by 16S rRNA gene RFLP and sequence analysis, as described in section 2.2.

DNA was extracted from 1 g spoil using the MoBio Soil DNA extraction kit. Microbial 16S rRNA genes were amplified by either bacterial or archaeal PCR, as described in section 2.2.1. Bacterial PCR used the 27f(G):1387r primer pair.

The indigenous microbial population was examined by T-RFLP analysis using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I, and the construction and analysis of a 16S rRNA gene clone library, using amplified bacterial DNA (sections 2.2.2-2.2.3).

7.3 RESULTS

7.3.1 Parc Mine

The mean geochemical measurements from each sample and their standard deviations are summarised in Table 7.1. On average the tailings were not very acidic, though the *p*H was highly variable. The *p*H of individual samples ranged from 3.3 to 6.5, demonstrating the highly heterogeneous nature of the site. Concentrations of readily-extractable metals, especially iron and zinc, were high. Site heterogeneity was again indicated by the high standard deviations of each extractable metal concentration.

Table 7.1 Basic geochemical data from the Parc mine tailings (mean values from all samples).

*p*H*	Readily extractable metals (μg g^{-1})				
	Fe	Cu	Zn	Mn	Al
5.38	1017	23.1	1698	227	82.9
(0.2)	(84.9)	(3.06)	(131)	(70.8)	(5.44)

Bracketed figures are standard deviation; $n = 6$; *1:2.5 tailings to RO water.

Plate count data from the October 2002 sample suggested an acidophile population of approximately 4.6 x 10^4 cfu g^{-1}. No cell counts using the DNA-staining dye DAPI could be obtained due to high levels of background fluorescence, and rapid bleaching of the DAPI stain under UV light. Six colony types were observed, but only three of these could be identified by 16S rRNA sequence analysis. Colony morphologies and closest relatives of isolates are shown in Table 7.2, while relative abundances are summarised in Figure 7.3. Isolate Pm2, which was closely related to *At. ferrooxidans*, dominated the cultured population. No molecular data were obtained for 23% of the isolates, so no identification could be made. However, of these, nearly a third appeared to be iron- or sulfur-oxidisers, based on their initial colony morphologies. Isolate Pm3, which was found to be related to the galena-oxidising bacterium *Thiobacillus plumbophilus*, accounted for 10% of the total isolates. This isolate was found to oxidise both iron and sulfur on the moderately acidic FeTo solid medium, but was unable to grow on the low *p*H Feo and FeSo solid media. Indeed, the oxidation of thiosulfate by this isolate in initially moderately acidic solid and liquid media at around *p*H 4.5 caused

the *p*H to drop to a point where the cells lost viability. The only other successfully identified organism from this sample was isolate Pm1, which was closely related to the mixotroph *Acidiphilium acidophilum*. This isolate appeared to oxidise sulfur on the FeSo solid medium, growing as characteristic bright white colonies. No DNA was successfully amplified from this sample, so no molecular community data are available.

Table 7.2 Descriptions of isolates from the October 2002 Parc mine sample and their closest relatives based on 16S rRNA gene identity.

Isolate	Description	Nearest organism(s) (accession ID)	% Identity
Pm1	White, dry colonies with concentric circular patterns from FeSo plate	*Acidiphilium acidophilum* (D86511)	99.7%
Pm2	Iron-oxidising colonies with "fried egg" morphology, from Feo plate	Uncultured bacterium clone G28 (DQ480479);	99.6%
		At. ferrooxidans^{T} strain ATCC23270 (AF465604);	95.2%
		Acidithiobacillus ferrooxidans NO37 (AF376020)	97.7%
Pm3	Iron-oxidising, crystalline colonies from FeTo plate	Uncultured bacterium clone Ra9C4; AF407390	95.0%
		Thiobacillus plumbophilus DSM 6690 AJ316618	94.3%

^{T}type strain.

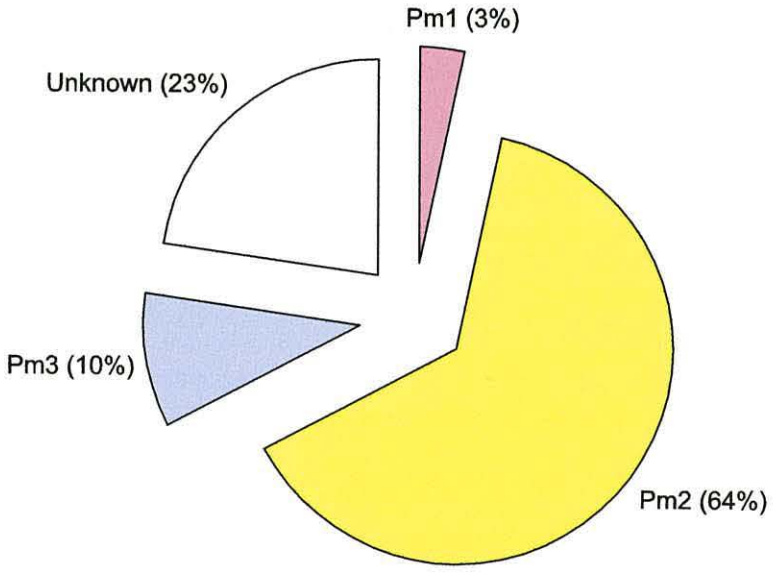


Figure 7.3 The relative abundance of isolates from the October 2002 Parc mine sample, based on plate count data.

No culture data were obtained for the May 2003 sample. Only bacterial DNA could be amplified from DNA extracted from this sample, and no archaeal product was obtained. The T-RFLP profile for this sample using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I is shown in Figure 7.4. The profile suggested a highly complex microbial population, much more so than was suggested by the culture data from the previous sample. The population did not appear to be dominated by one single T-RF, and the vast majority could not be identified using the T-RFLP database.

The *Actinobacteria* appeared to dominate the identifiable peaks, and accounted for somewhere between 18 and 30% of the total population. They only matched 6% of the peaks in the *Alu* I digest, but this is likely to be due to the fact that there are less *Alu* I T-RFs in the database compared to those for *Cfo* I and *Msp* I. The "*Firmicutes*", including the sulfobacilli belonging to the *Bacilli* class, consistently accounted for 6% of the population. The *Proteobacteria* appeared to account for between 4 and 17% of the population, with the *Alphaproteobacteria* seemingly the most dominant class of this phylum. The acidithiobacilli, such as isolate Pm2, accounted for between 1 and 4%, and the *Acidobacteria* seemed to account for a similar proportion. T-RFs corresponding to *Leptospirillum* spp. appeared to exist at or around the limits of detection for T-RFLP, occurring at below 2% in the *Alu* I and *Msp* I digests.

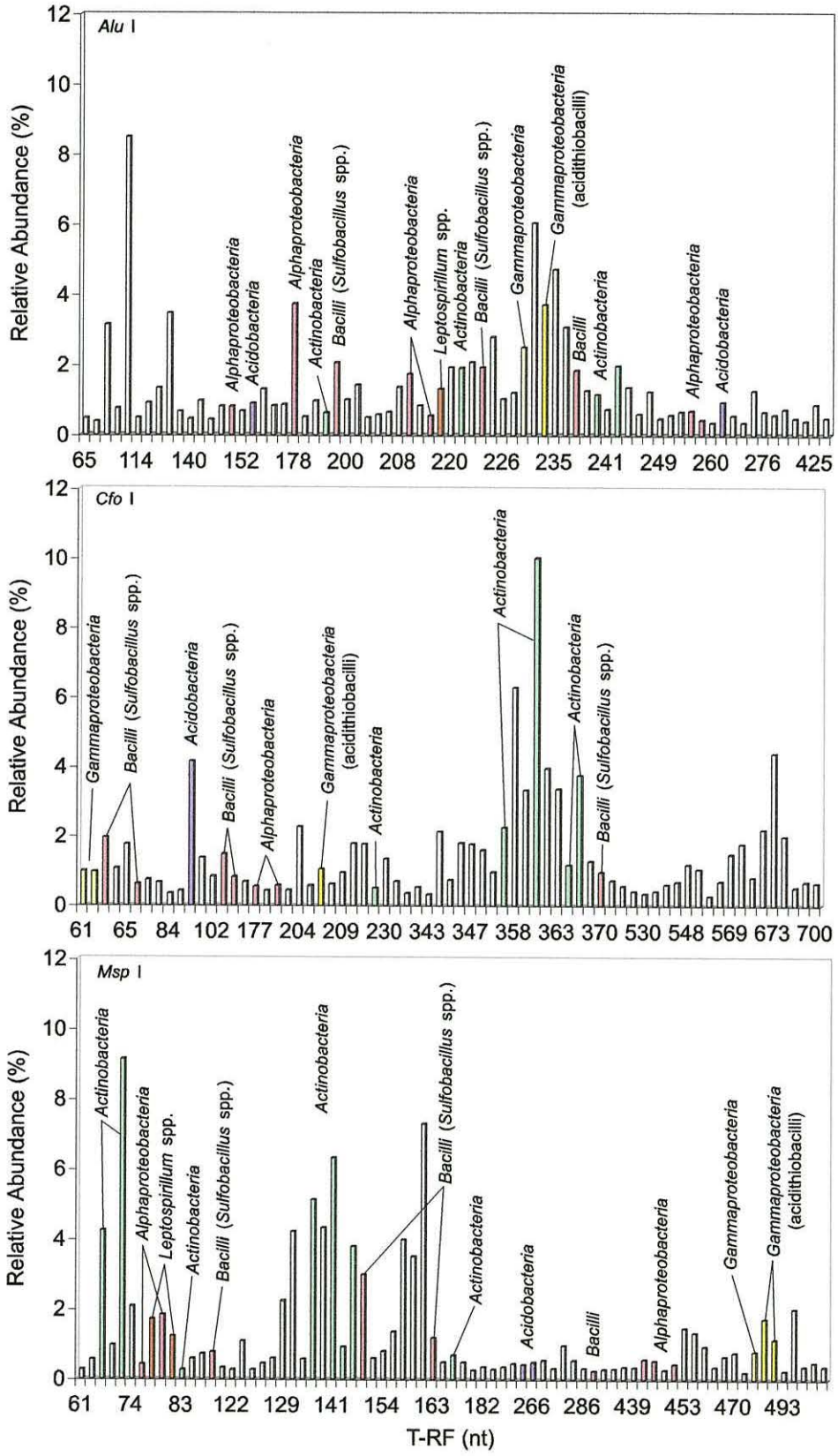


Figure 7.4 T-RFLP profile of the May 2003 Parc mine sample, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Alphaproteobacteria*; , *Gammaproteobacteria*; , acidithiobacilli; , *Acidobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp..)

The results from a small 16S rRNA gene clone library, constructed using DNA from the May 2003 sample, are summarised in Table 7.5. Of 20 colonies picked, 18 had the correct-sized insert. The *Actinobacteria* class accounted for four clones, one of which was most closely related to a clone from a study of a mine- or sulfide mineral-impacted environment. Four clones were classified as *Betaproteobacteria*, of which one was closely related to a clone obtained from a hydrocarbon-impacted environment. Clone pCBPm2b5 had the highest sequence identity with isolate Pm3, but this was very low (approximately 87%). Three clones could not be classified taxonomically; two of whose closest relatives were obtained from a hydrocarbon-impacted environment. Two identical clones belonged to the *Cyanobacteria* class, their closest relative obtained from a sulfide mineral-impacted environment. A single member of the *Alphaproteobacteria* class was found, which shared approximately 92% sequence identity with isolate Pm1. No acidithiobacilli were detected. Four clones were found to be chimeric

A single isolate, Pm10, was obtained from the June 2003 sample. This shared 99.4% sequence identity with the iron-oxidising acidophilic firmicute SLC66 (accession ID: AY040739), a member of the *Bacilli* class. Plate-count data suggested a population of approximately 1.0×10^3 cfu g^{-1} dry tailings; while cell counts using DAPI indicated a population size of 3.8×10^6 cells g^{-1}. This indicates a very low plating efficiency of approximately 0.03%.

Only bacterial DNA could be amplified from this sample. The T-RFLP profile using the 27f:1397r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I are shown in Figure 7.5. As before, the profile suggested a highly complex population with no T-RF, and therefore no organism, singularly dominant. Again, the majority of the T-RFs could not be identified using the T-RFLP database.

The *Actinobacteria* accounted for between 10 and 20% of the total abundance, with the same caveats with regard to enzyme *Alu* I as found previously. The *Proteobacteria* phylum accounted for around 10% of the total abundance, with the *Alphaproteobacteria* accounting for between 3 and 6%, the *Gammaproteobacteria* around 2% and the acidithiobacilli between 1 and 5%. T-RFs corresponding to *Betaproteobacteria* occurred in only 2 of the three restriction enzyme profiles, representing less than 2% of the total

abundance. The "*Firmicutes*", including the sulfobacilli, consistently accounted for at least 6% of the population. In this profile, the *Leptospirillum* genus appeared in each enzyme digest, accounting for between 1 and 3% of the total population.

Table 7.3 Abundance and nearest relatives of 18 clones obtained from a 16S rRNA gene clone library constructed using DNA amplified from the May 2003 Parc mine spoil sample using the 27fG:1492rG bacterial primer pair, grouped by taxonomic class.

Clone	Nearest relative (accession ID)	Identity	No. of clones
Actinobacteria			
pCBPm2b1	*Actinobispora yunnanensis* strain IMSNU 22019T (AJ252822)	97.3%	1
pCBPm2b2		97.0%	1
pCBPm2b9	Uncultured actinobacterium (DQ366007)	96.0%	1
pCBPm2b16	Uncultured bacterium clone 1973e-03 (AY917841)*	99.5%	1
			4
Alphaproteobacteria			
pCBPm2b12	Uncultured bacterium clone MNB-Ms-21 (AY309195)	98.0%	1
			1
Betaproteobacteria			
pCBPm2b20	Uncultured bacterium clone JEG.a11 (DQ228387)	99.5%	1
pCBPm2b3	Uncultured proteobacterium clone 351F (AY571836)[H]	99.5%	1
pCBPm2b4	Bacterium Ellin6095 (AY234747)	96.6%	1
pCBPm2b5	Uncultured betaproteobacterium clone AKYG1851 (AY921995)	96.7%	1
			4
Cyanobacteria			
pCBPm2b17	Uncultured bacterium clone 1959a-08 (AY917645)*	89.6%	2
			2
Unclassified			
pCBPm2b11	Uncultured *Chloroflexi* bacterium clone AKYH1480 (AY922118)	89.8%	1
pCBPm2b7	uncultured eubacterium 2112 (AJ292683)[H]	98.2%	2
			3
Chimerae			
pCBPm2b6	-		2
pCBPm2b10	-		1
pCBPm2b14	-		1
			4

*Associated with mine-impacted or sulfide mineral-impacted environments; [H]Associated with hydrocarbon-contaminated sites.

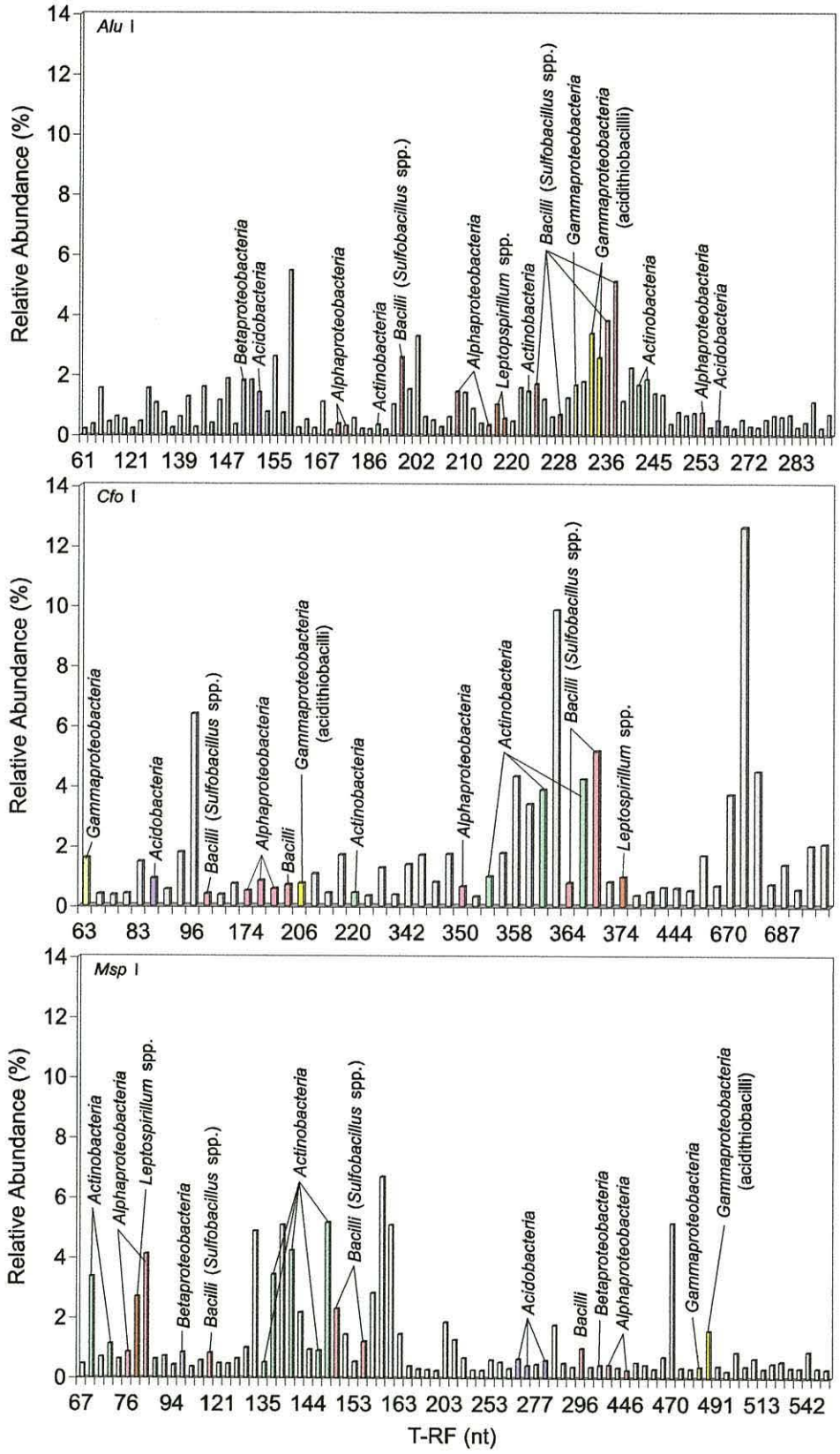


Figure 7.5 T-RFLP Profile of the June 2003 Parc mine sample, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Alphaproteobacteria*; , *Gammaproteobacteria*; , acidithiobacilli; , *Acidobacteria*; , "*Firmicutes*"; , *Leptospirillum* spp..)

A 16S rRNA gene clone library was constructed and 80 clones were picked, of which 77 contained inserts. Unfortunately, of these, 26 were found to be chimeric. This is an unusually large proportion, and is likely due to have been due to too great a concentration of DNA template in the PCR reactions. This would have led to the formation of truncated PCR products, due to partial re-annealing of DNA template or PCR products. These would then effectively act as primers themselves, leading to the formation of chimeric PCR products, as illustrated in Figure 7.6.

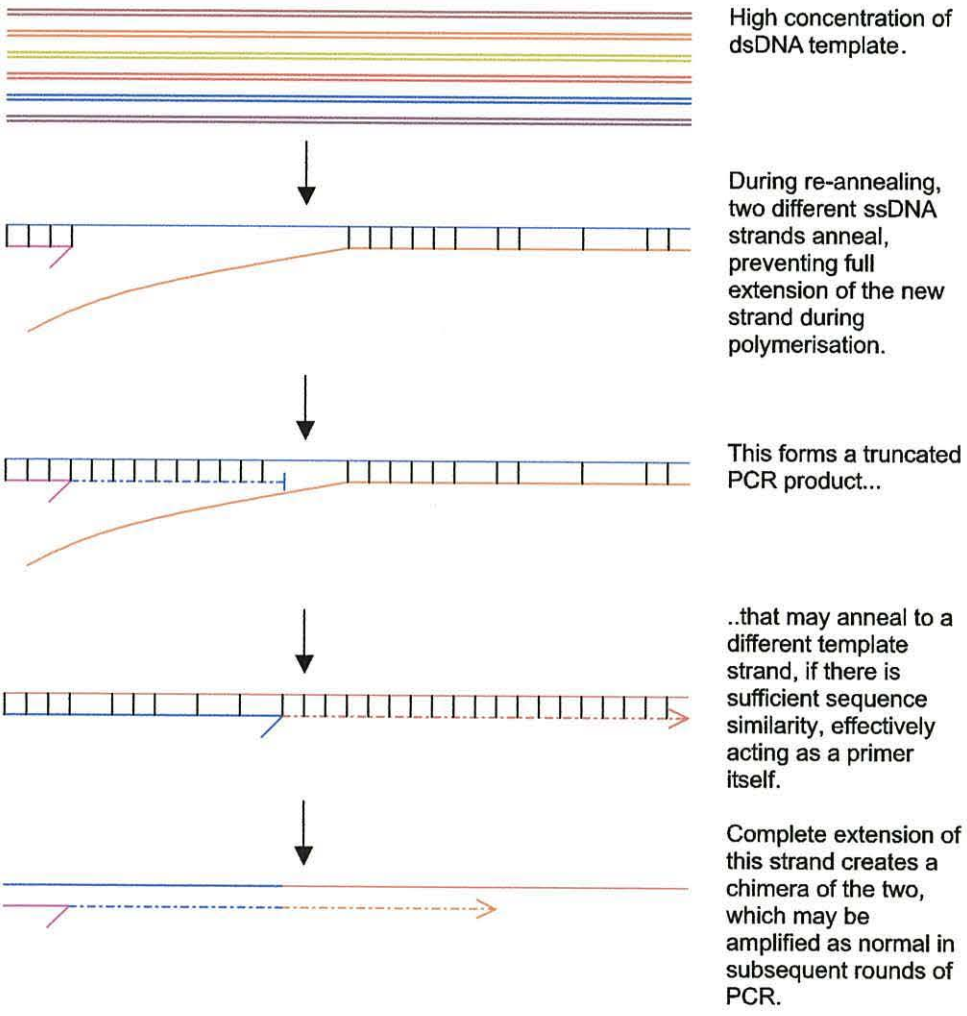


Figure 7.6 Schematic diagram, outlining the possible reason for the formation of Chimerae during PCR reactions saturated with double-stranded (ds) DNA template.

Of the remaining clones, the *Actinobacteria* class was numerically dominant, accounting for 13 clones. One of these was most closely related to a clone obtained from a mining- or sulfide mineral-impacted site. The *Proteobacteria* phylum accounted for seven clones, three of which were *Alphaproteobacteria*, three were *Betaproteobacteria* and one a member of the *Gammaproteobacteria*. Two of the *Alphaproteobacteria* clones and one of the *Betaproteobacteria* clones were related to clones obtained from studies of hydrocarbon-contaminated environments. Seven clones belonged to the "*Planctomycetacia*" class, one of which was related to a clone from a mining- or sulfide mineral-impacted environment. Other classes identified were the *Cyanobacteria*, the *Gemmatimonadetes*, the *Verrucomicrobiae* and the novel class, TM7. Sixteen clones could not be taxonomically classified. Of these, two were related to organisms obtained from hydrocarbon-impacted environments, three were related to organisms obtained from mining- or sulfide mineral-impacted environments and two were related to an organism obtained from a soft metal-contaminated site.

Table 7.4 Abundance and nearest relatives of 77 clones obtained from a 16S rRNA gene clone library constructed using DNA amplified from the June 2003 Parc mine sample using the 27fG:1387r bacterial primer pair, grouped by taxonomic class.

Clone	Nearest relative (accession ID)	Identity	No. of clones
Actinobacteria			
pCBPm1c-15	Uncultured bacterium clone PKD_B5 (DQ190233)	94.8%	1
pCBPm1c-29	Uncultured bacterium ARFS-5 (AJ277689)	96.2%	2
pCBPm1c-35	Uncultured soil bacterium clone 288-2 (AF423245)	92.7%	1
pCBPm1c-36	Uncultured *Conexibacter* sp. clone ACTINO10 (AY494658)	93.8%	1
pCBPm1c-55	Uncultured actinobacterium clone GCPF6 (AY129782)	93.7%	1
pCBPm1c-63	Bacterium Ellin5115 (AY234532)	95.8%	1
pCBPm1c-69	*Friedmanniella* sp. EL-17a (AJ132943)	98.0%	2
pCBPm1c-79	Uncultured bacterium clone C-F-1 (AF443581)	96.0%	1
pCBPm1c-83	Uncultured bacterium clone AKAU3484 (DQ125522)*	96.8%	1
pCBPm1c-84	*Actinoplanes nipponensis* (AB047498)	97.9%	1
pCBPm1c-86	Uncultured bacterium clone AKIW774 (DQ129336)	94.0%	1
			13
Alphaproteobacteria			
pCBPm1c-33	uncultured eubacterium WD248 (AJ292598)[H]	93.8%	1
pCBPm1c-82	Uncultured soil bacterium clone PAH-Bio-16 (DQ123686)[H]	98.7%	1
pCBPm1c-87	Uncultured *Sphingomonas* sp. clone KL-2-4-7 (AF408323)	98.7%	1
			3
Betaproteobacteria			
pCBPm1c-53	Uncultured proteobacterium clone 351F (AY571836)[H]	100.0%	1
pCBPm1c-76		99.7%	1
pCBPm1c-77	Uncultured soil bacterium clone 460 (AY493946)	97.1%	1
			3
Gammaproteobacteria			
pCBPm1c-60	Uncultured bacterium clone 184up (AY212636)	97.0%	1
			1
Cyanobacteria			
pCBPm1c-28	Uncultured Antarctic cyanobacterium clone Fr094 (AY151727)	96.4%	1
			1
Gemmatimonadetes			
pCBPm1c-26	Uncultured *Gemmatimonadetes* bacterium clone AKYG759 (AY921885)	97.0%	1
pCBPm1c-43		94.7%	1
pCBPm1c-49	Uncultured *Gemmatimonadetes* bacterium clone AKYH1347 (AY921725)	95.8%	1
			3

Table 7.4 Cont..

Clone	Nearest relative (accession ID)	Identity	No. of clones
"Planctomycetacia"			
pCBPm1c-25	Uncultured soil bacterium clone ABS-368 (AY289462)	92.0%	2
pCBPm1c-40		91.3%	1
pCBPm1c-64		91.4%	1
pCBPm1c-32	Soil clone MC55 (X64377)	91.1%	1
pCBPm1c-71	Uncultured planctomycete YNPRH54A (AF465657)*	89.4%	1
pCBPm1c-81	Uncultured planctomycete clone DSP21 (AJ290180)	93.4%	1
			7
Genus *incertae sedis* TM7			
pCBPm1c-39	Uncultured bacterium clone UTFS-OF09-d22-24 (AB200299)	91.5%	1
			1
Verrucomicrobiae			
pCBPm1c-44	Uncultured soil bacterium clone L1A.9A10 (AY989165)	96.3%	1
pCBPm1c-52		96.2%	2
			3
Unclassified			
pCBPm1c-6	Uncultured soil bacterium clone 1363-1 (AY326532)	94.5%	1
pCBPm1c-30		94.1%	1
pCBPm1c-8	uncultured eubacterium 2112 (AJ292683)[H]	97.3%	1
pCBPm1c-14	Uncultured "*Planctomycetales*" bacterium clone M10Ba61 (AY360649)	93.1%	2
pCBPm1c-16	Uncultured bacterium clone 1790c-37 (AY917528)*	85.0%	1
pCBPm1c-17	Uncultured bacterium clone S-Jos_51 (DQ017939)	94.7%	1
pCBPm1c-19	Uncultured soil bacterium clone HN1-15 (AY221598)[M]	81.5%	2
pCBPm1c-42	Uncultivated soil bacterium clone C002 (AF013515)	96.2%	1
pCBPm1c-47	Uncultured bacterium clone 1979a-39 (AY917924)*	91.2%	1
pCBPm1c-48	Uncultured *Acidobacteria* bacterium clone 351B (AY571792)[H]	99.0%	1
pCBPm1c-54	Uncultured bacterium clone Biofilm_1093d_c13 (DQ058682)	96.7%	1
pCBPm1c-59	Uncultured soil bacterium clone C022 (AF507680)	90.3%	2
pCBPm1c-90	Uncultured bacterium clone 1959a-08 (AY917645)*	99.8%	1
			16

Table 7.4 Cont..

Clone	Nearest relative (accession ID)	Identity	No. of clones
Chimerae			
pCBPm1c-2	-		1
pCBPm1c-4	-		1
pCBPm1c-7	-		1
pCBPm1c-10	-		1
pCBPm1c-11	-		1
pCBPm1c-12	-		1
pCBPm1c-20	-		1
pCBPm1c-21	-		1
pCBPm1c-22	-		1
pCBPm1c-23	-		1
pCBPm1c-27	-		1
pCBPm1c-37	-		2
pCBPm1c-38	-		1
pCBPm1c-41	-		1
pCBPm1c-50	-		1
pCBPm1c-51	-		1
pCBPm1c-58	-		1
pCBPm1c-62	-		1
pCBPm1c-67	-		1
pCBPm1c-68	-		1
pCBPm1c-72	-		1
pCBPm1c-73	-		1
pCBPm1c-74	-		1
pCBPm1c-88	-		1
pCBPm1c-89	-		1
			26

*Associated with mine-impacted or sulfide mineral-impacted environments; [H]Associated with hydrocarbon-contaminated environments; [M]Associated with soft metal-contaminated environments (not from metal sulfides).

7.3.2 Cwm Rheidol

The basic geochemical data obtained are summarised in Table 7.5. Although the spoil is acidic, it contained much lower concentrations of readily extractable metals than found in the Parc mine tailings.

Table 7.5 Basic geochemical data from Cwm Rheidol spoil.

*p*H*	Readily Extractable Metals (μg g^{-1})		
	Fe	Cu	Zn
3.40	65.85	1.52	7.93

*1:2.5 spoil to RO water

Plate count data from this sample suggested an acidophile population of 1.1 x 10^6 cfu g^{-1} dry spoil. No cell counts using DAPI were obtained, so it was not possible to calculate the plating efficiency for this sample. Descriptions and identification of the isolates are summarised in Table 7.6, and plate-count data in Figure 7.7.

Of the isolates obtained, isolate CR5 was the most abundant, and accounted for approximately 65% of the total isolates. This isolate was closely related to a moderately acidophilic iron-oxidising heterotrophic gammaproteobacterium isolated from the former Mynydd Parys (acid streamer bacterium PK51) and Wheal Jane (gammaproteobacterium WJ2) mines. Isolates CR4 and CR8 were closely related to two *Acidobacteria* and together accounted for nearly 22% of the total isolates. Further sub-culturing of isolate CR8 revealed it to be a mixed culture of two almost identical *Acidobacteria*. Denoted 'CR8a' and 'CR8b', they shared 99.1% pairwise 16S rRNA gene sequence identity. Isolate CR10 was closely related to an *Acidiphilium* sp., a member of the *Alphaproteobacteria* class, although this isolate accounted for less than 0.05% of the total population. The remaining isolate, CR7, could not be identified, but appeared to be a sulfur-oxidising isolate, on the basis of its colony morphology. It appeared as large *Bacillus*-like straight rods under the microscope, suggesting it may be a Gram-positive organism, probably a firmicute, rather than one of the more common Gram-negative sulfur-oxidisers, such as *At. thiooxidans*, although no endospores were apparent.

Table 7.6 Descriptions of isolates from the Cwm Rheidol spoil and their closest relatives, based on 16S rRNA gene identity.

Isolate	Description	Nearest relative(s) (accession ID)	Identity
CR4	Large pink colony from Feo plate	Uncultured bacterium clone MCS36 (AJ830726)	98.8%
CR5	Yellowy colony from Feo plate	Acid streamer bacterium PK51 (AY765997)	99.4%
CR8	(a) Large, flat pink colony from FeSo (b) plate	Uncultured forest soil bacterium clone DUNssu362 (AY913535)	98.7% 99.1%
CR10	Yellowy colony from YE_3o plate	*Acidiphilium* sp. PK40 (AY765994)	98.8%

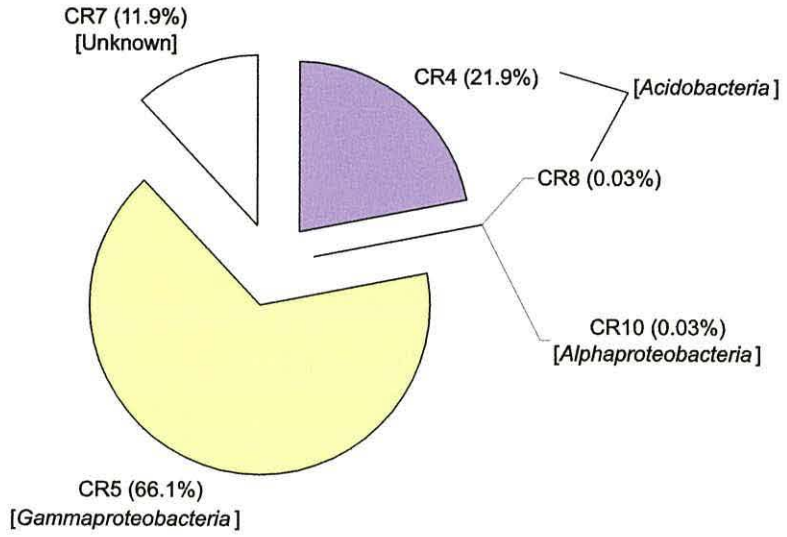


Figure 7.7 The relative abundance of isolates from the Cwm Rheidol spoil, based on plate count data.

The *Acidobacterium* isolate CR8a appeared able to oxidise sulfur, based on its growth as bright white crystalline colonies on FeSo plates, whereas CR8b simply grew as gelatinous white colonies. However, sulfate production and *p*H in TSB_4 medium supplemented with either 2.5 mM potassium tetrathionate or elemental sulfur was similar for all three *Acidobacteria* isolates from this site over the course of 20 days, and differed little from those measured in uninoculated controls.

Only bacterial DNA was amplified from the spoil. The T-RFLP profile using the restriction enzymes *Alu* I, *Cfo* I and *Msp* I is shown in Figure 7.8. The *Gammaproteobacteria*, specifically isolate CR5, accounted for approximately 14% of the total abundance with each enzyme. Consistent abundance with each enzyme and the identification of the secondary T-RFs

obtained for this organism in the profile gave more support to this conclusion. T-RFs corresponding to the *Acidobacteria* isolates CR4 and CR8 (a and b) accounted for around 7% of the total abundance in the *Cfo* I and *Msp* I profiles. A secondary *Cfo* I T-RF for these organisms was also identified, although this could also have been an *Actinobacteria* primary T-RF. No *Alu* I T-RFs were obtained for these isolates, but a T-RF corresponding other known *Acidobacteria* in the database accounted for just over 7% of this profile too. The most abundant T-RFs in the *Alu* I and *Msp* I profiles appeared to be from the *Actinobacteria* class. However, the most abundant T-RF in the *Cfo* I profile could not be identified. T-RFs corresponding to *Leptospirillum* spp., *Alphaproteobacteria* and *Betaproteobacteria* appeared in one or two enzyme digests, and many T-RFs could not be identified.

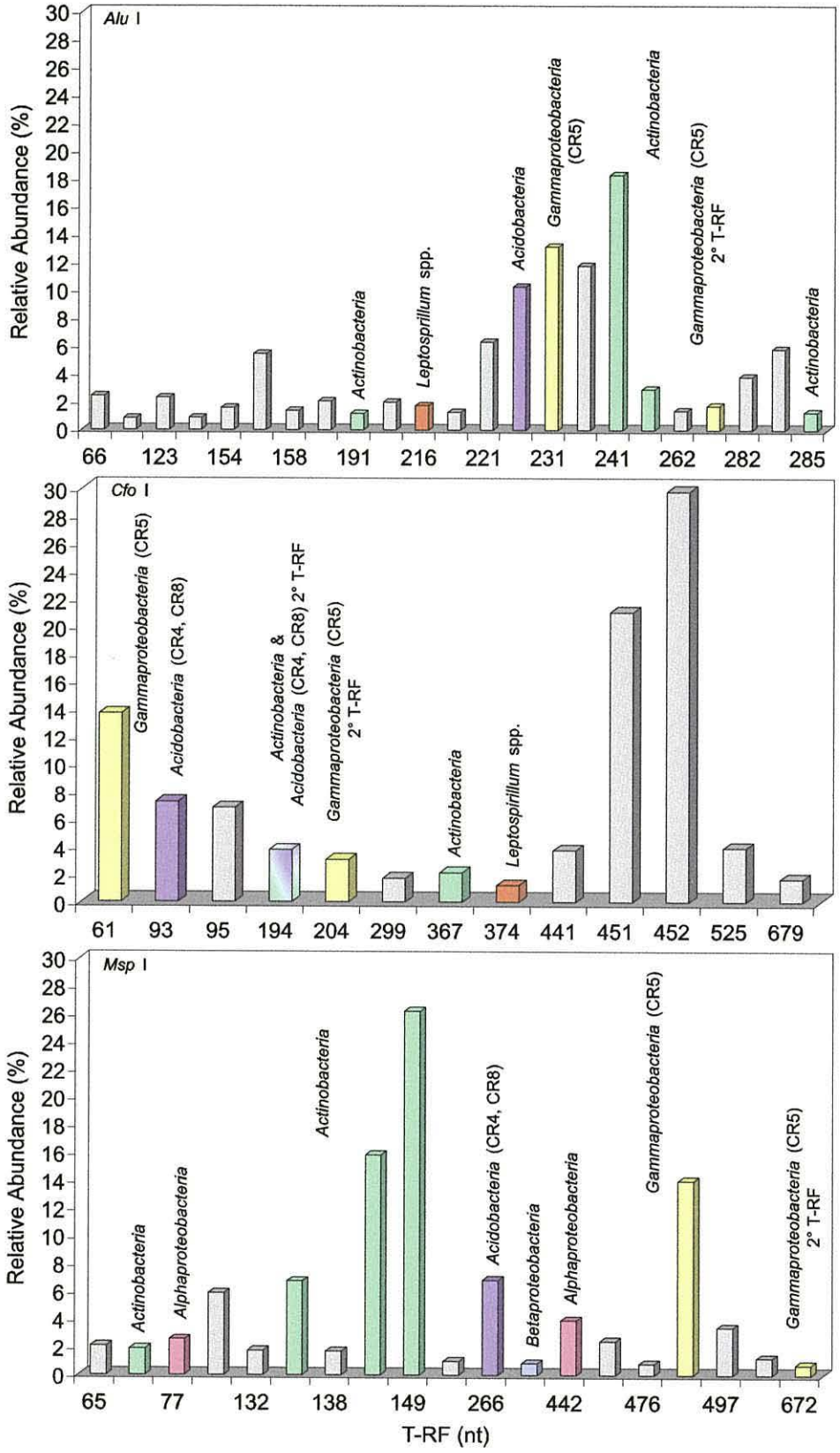


Figure 7.8 T-RFLP Profile of the June 2003 Parc mine sample, using the 27f:1387r primer pair and the restriction enzymes *Alu* I, *Cfo* I and *Msp* I. (Key: , *Actinobacteria*; , *Alphaproteobacteria*; , *Betaproteobacteria*; , *Gammaproteobacteria*; , *Acidobacteria*; , *Leptospirillum* spp..)

The ratio of *Gammaproteobacteria* (CR5) T-RFs to *Acidobacteria* (CR4, CR8a and b) T-RFs, in terms of relative abundance, is broadly in line with that observed with the culture data. Thus, a very rough estimate of total cell numbers can be made by combining the T-RFLP data for these isolates with their relevant cell counts. Assuming the *Gammaproteobacteria* account for roughly 14% of the population, the total population size would be 2.3×10^6 cfu g^{-1} dry spoil. A similar population size of 3.5×10^6 g^{-1} can be inferred using the *Acidobacteria* data. This would imply a plating efficiency of between 21 and 31%. This is a high but not entirely implausible value, considering T-RFs accounting for at least 20% of the total abundance could be matched to isolated organisms.

A 16S rRNA gene clone library was constructed and analysed. These data are summarised in Table 7.7. Of 60 clones picked, 57 had inserts of the correct size, and a single clone was found to be chimeric. The *Gammaproteobacteria* accounted for 23 clones, and were the dominant class in the library. Eight of these clones were related to the iron-oxidising heterotrophic isolate CR5. The single most dominant clone was pCBCR2-8, represented seven times in the library in total. This clone was part of a group of 14 whose closest relative was an uncultured bacterium obtained from uranium mine waste. Four other *Gammaproteobacteria* clones were most closely related to the iron-oxidising acidophile m-1 (originally classified as "*Thiobacillus* m-1"). Other *Proteobacteria* included seven clones related to a single deltaproteobacterium and a single alphaproteobacterium clone.

Members of the *Acidobacteria* class accounted for 11 clones, nine of which were most closely related to a clone found in Hawaiian volcanic sediment. All 11 clones shared 97-98% sequence identity with the *Acidobacteria* isolates. The *Actinobacteria* were represented by just three clones, implying that their abundance was not as high as had been suggested by the T-RFLP analysis. A single "*Planctomycetacia*" clone was identified, while seven clones could not be classified taxonomically.

The closest relatives of 53 of the 56 clones analysed were obtained from mine- or sulfide mineral-impacted environments. Of these, 23 came from a study of microbial succession of volcanic sediments on a Hawaiian island.

Table 7.7 Abundance and nearest relatives of 56 clones obtained from a 16S rRNA gene clone library constructed using DNA amplified from the Cwm Rheidol spoil using the 27fG:1387r bacterial primer pair, grouped by taxonomic class.

Clone	Nearest relative(s) (accession ID)	Identity	No. of clones
Acidobacteria			
pCBCR2-2	Uncultured bacterium clone 1959c1-03 (AY917388)*[V]	99.2%	2
pCBCR2-4		99.5%	1
pCBCR2-13		99.2%	2
pCBCR2-29		98.9%	1
pCBCR2-30		98.7%	1
pCBCR2-46		99.6%	2
pCBCR2-9	Uncultured forest soil bacterium clone DUNssu368 (AY913541)	99.2%	2
			11
Actinobacteria			
pCBCR2-15	Uncultured bacterium clone RCP1-56 (AF523907)*	97.6%	1
pCBCR2-18	Uncultured bacterium clone D134 (AY274140)*	91.1%	1
pCBCR2-42	Uncultured bacterium clone fb11 (DQ303258)*	98.5%	1
			3
Alphaproteobacteria			
pCBCR2-57	Uncultured alphaproteobacterium YNPRH71B (AF465654)*	96.7%	1
			1
Gammaproteobacteria			
pCBCR2-5	Uncultured bacterium clone JG30-KF-CM35 (AJ536882)*	96.1%	1
pCBCR2-7		97.6%	1
pCBCR2-8		98.0%	7
pCBCR2-10		96.7%	1
pCBCR2-16		95.9%	1
pCBCR2-39		96.8%	1
pCBCR2-45		98.0%	1
pCBCR2-59		98.0%	1
pCBCR2-11	Acid streamer bacterium PK51 (AY765997)*[C]	99.6%	4
pCBCR2-12	Iron-oxidizing acidophile m-1 (AF387301)*	94.5%	4
pCBCR2-54	Uncultured bacterium clone fb10 (DQ303257)*[C]	99.1%	3
pCBCR2-56	Uncultured eubacterium TRA5-3 (AF047645)*[C]	99.5%	1
			26
Deltaproteobacteria			
pCBCR2-1	Uncultured bacterium clone 1973d-22 (AY917827)*[V]	93.2%	5
pCBCR2-20		96.0%	2
			7
"*Planctomycetacia*"			
pCBCR2-34	Uncultured bacterium clone 1971a-18 (AY917773)*[V]	95.1%	1
			1

Table 7.7 Cont..

Clone	Nearest relative(s) (accession ID)	Identity	No. of clones
Unclassified			
pCBCR2-17		88.3%	3
pCBCR2-28	Uncultured bacterium clone 1894a-24 (AY917553)*[V]	86.3%	2
pCBCR2-58		89.0%	1
pCBCR2-36	Uncultured forest soil bacterium clone DUNssu389 (AY913561)	97.5%	1
			7
Chimerae			
pCBPm1c-2	-		1
			1

*Associated with mine-impacted or sulfide mineral-impacted environments; [C]Related to isolate CR5; [V]Clone from Hawaiian volcanic sediment.

7.4 DISCUSSION

7.4.1 Parc Mine

The Parc mine tailings were highly variable in terms of *p*H and readily extractable metals. The average *p*H of the tailings samples was relatively high, at around *p*H 5.3, but ranged from *p*H 3.0 to 6.5. Readily extractable metal concentrations were high, and also quite variable. Unsurprisingly, zinc showed the greatest potential for mobilisation, and concentrations of extractable iron were also high. These data point to the possible effects of seasonal variation, but also, as different samples collected at the same time also varied greatly, the probable juxtaposition of different microenvironments within the tailings heap as a whole. In general, significant levels of mineral oxidation must be occurring within the Parc mine tailings, yet there is sufficient neutralising capacity to largely counter the acidity produced during this process. This is presumably due to the calcareous quarry waste added during re-grading and the tailings' inherent neutralising capacity due to indigenous calcareous minerals,

The microbial population isolated from the initial Parc mine sample was dominated by iron- and sulfur-oxidising *Bacteria*, which accounted for up to 80% of the total isolates obtained. However, the isolation of the moderately acidophilic iron- and sulfur-oxidising *Thiobacillus plumbophilus*-like isolate, Pm3, was very interesting, as this isolate was very sensitive to the fall in *p*H

due to sulfur-oxidation in synthetic media. Therefore, its growth in the environment would rely entirely on some form of neutralisation to maintain at least moderately acidophilic conditions. Without cell counts using DAPI, it is not possible to say what fraction of the total population were culturable acidophiles, though the cultured population was quite small at 4.6×10^4 cfu g^{-1} tailings.

An absence of isolates from the second sample obtained suggests that the culturable population, with the media used, must be less than approximately 2.0×10^2 cfu g^{-1}, which is the limit for detection of this method. The culturable population from the third sample was entirely made up of the iron- and sulfur-oxidising firmicute, isolate Pm10. However, again the population was very small, at around 1.0×10^3 cfu g^{-1}, and total cell counts showed this to represent less than 0.05% of the total population. These data suggest that while the culturable populations were phylogenetically very diverse between samples, and largely capable of accelerating mineral oxidation, in general they represented a small fraction of the total population, existing at or around the limits of detection; the total population was potentially much larger, much more complex and probably mainly neutrophilic.

Analysis of T-RFLP data from the latter two samples confirmed this premise, the two profiles were highly complex, and differed greatly between these two samples. No single T-RF, and therefore no single organism, accounted for much more than 10% of the total population. As a class, the *Actinobacteria* appeared to dominate the T-RFs that could be identified using the T-RFLP database, accounting for 10-20% of the total population. In the profile from each sample, the *Bacilli* ("*Firmicutes*"), specifically the sulfobacilli, and the genus *Acidithiobacillus* appeared to account for approximately 6% and between 1 and 5% of the total T-RFs, respectively. However, in the June 2003, sample, clone library data suggested that this was highly unlikely to be the case. No "*Firmicutes*" were represented in the clone library, and the only *Gammaproteobacteria* clone identified was not closely related to the acidithiobacilli. While it must be conceded that the clone library was too small to fully elucidate such a complex population, and that nearly a third of those clones analysed were Chimerae, it would still be expected that an organism

that made up 5% of the population would be represented by two-three clones out of a total of 50.

The *Actinobacteria* represented significant proportions of the 16S rRNA gene clone library from each of these latter samples, while the *Proteobacteria*, specifically the *Alphaproteobacteria* and *Betaproteobacteria*, also represented substantial proportions of the total. No characterised iron- or sulfur-oxidising microorganisms were detected; indeed the vast majority of clones were most closely related to uncultured organisms. However, many clones were closely related to organisms detected in studies of contaminated sites, though, interestingly, these were not necessarily sites impacted by metal sulfide-associated pollution. Many were contaminated with high molecular weight hydrocarbons. It may be that the common denominator between these sites is anthropogenic disturbance, and that the relatively neutral *p*H of Parc tailings, in general, is what has allowed the establishment of such a complex population.

With no information about the potential metabolic capabilities of the population as a whole, it is difficult to draw any conclusions about which organisms are responsible for the extensive mineral dissolution, as evidenced by the high readily extractable metal concentrations, that appears to be occurring at the Parc mine site. Although microorganisms capable of accelerating mineral dissolution have been detected, these populations are small, and probably make up a tiny proportion of the total population. While one of these isolates, the *T. plumbophilus*-like isolate Pm3, is a moderately acidophilic iron- and sulfur-oxidising organism, and it is known that members of the *At. ferrooxidans* group (e.g. isolate Pm2) are able to colonise neutral environments (Mielke *et al.*, 2003) it may be that other, more neutrophilic organisms are responsible for the accelerated mineral dissolution observed at this site. Although no known neutrophilic iron- or sulfur-oxidising microorganisms, such as *Leptothrix* spp., *Gallionella ferruginea* and neutrophilic *Thiobacillus* spp., were detected, it might be that some of the uncultured microbial population are capable of accelerating mineral dissolution.

Therefore, it is likely that at the Parc mine site, in general, the *p*H is such as to preclude the dominance of the microbial population by known

mineral-oxidising acidophiles. While these organisms may exist in suitable microenvironments, with no metabolic information available for the rest of the population it is unclear as to whether the high concentrations of readily extractable metals are a product of a small acidophilic, or moderately acidophilic population or whether a larger, potentially neutrophilic, population of hitherto undescribed mineral-oxidisers is responsible. The utilisation of neutrophilic media during culture-based investigation would help to resolve the issues regarding the occurrence of mineral-oxidising and other neutrophiles, and could form the basis for further studies at this site.

The neutralising capacity of the Parc tailings would certainly allow for the continued existence of mineral-oxidising microorganisms like isolate Pm3, which would otherwise be forced out as the *p*H decreased as a result of oxidative dissolution of minerals and secondary processes (metal hydrolysis etc.). It would be interesting to see how the microflora of this site compares to a recently disturbed environment, where the indigenous microbial population is responding to recently exposed sulfide minerals. In a recently exposed environment, it would be expected that the environment would initially remain at a near neutral *p*H until the residual buffering capacity was depleted, and then become progressively more acidic. It would also be interesting to observe if and how the Parc mine tailings populations change over time. If mineral-oxidation continues, it is likely that the neutralising capacity will eventually be depleted and the site may become acidic in nature. How this would effect the indigenous population would be another area of interest.

7.4.2 Cwm Rheidol

The Cwm Rheidol spoil is highly acidic, but contains relatively low concentrations of readily extractable metals. The cultured microbial population was quite large, but relatively simple. Combination of the culture data with data from the biomolecular analyses allowed for the very approximate estimate of a total population size of around 3 x 10^6 cfu g^{-1} spoil. The gammaproteobacterium isolate CR5 dominated the cultured population, while members of the *Acidobacteria*, isolates CR4 and CR8 (a and b), also made up a large proportion of the total isolates. Interestingly, while isolate CR5 is closely related to an organism that is capable of iron-oxidation, it grew only as a heterotroph in the laboratory, and displayed no ability to oxidise ferrous iron. In contrast, one of the *Acidobacteria* isolates appeared to be able to oxidise sulfur under certain conditions, a trait which has not previously been identified in members of this class.

T-RFLP data showed the *Acidobacteria* isolates and gammaproteobacterium CR5 to be numerically important members of the population in the Cwm Rheidol sample. This was supported by the data from the 16S rRNA gene clone library, which was dominated by the *Gammaproteobacteria* and the *Acidobacteria*. Many of the *Gammaproteobacteria* clones were related to isolate CR5, while the majority were related to an uncultured organism found in a study of uranium mine wastes. The rest were closely related to the iron-oxidising organism m-1, which has only been reported previously in acidic coal mine drainage, and elsewhere in this study at the inactive heap-leaching operation at the Kennecott Bingham Canyon mine (Chapter 5). All of the *Acidobacteria* clones were closely related to isolates CR4 and CR8. Thus, the population as a whole appears to be relatively simple, with no great discrepancy, in terms of detection, between the biomolecular and culture-dependent methods. However, it would have been useful to isolate the dominant *Gammaproteobacteria* organism, as indicated by the clone library data, in order to elucidate its metabolic capacity, and infer its potential role in the Cwm Rheidol and the uranium-mine spoil heaps.

The low concentrations of extractable metals at this site are very interesting in the context of the microbial populations. While most of the cultured organisms were isolated growing heterotrophically on solid media, isolate CR5 is very closely related to an iron-oxidising microorganism, and there is tantalising evidence for sulfur-oxidation by one of the *Acidobacteria* isolates. Additionally, as far as is known, m-1 is unable to grow heterotrophically. Therefore, it is highly probable that iron-oxidation must be occurring within the spoil. Iron and sulfur cycling within the spoil may explain the low concentrations of readily extractable metals observed. Work by Coupland (2005) has shown many members of the *Acidobacteria* class are capable of iron-reduction. More importantly, this was also observed by gammaproteobacterium PK51, which is closely related to isolate CR5, and so to several of the *Gammaproteobacteria* clones from the Cwm Rheidol clone library. Thus, ferrous iron, oxidised to ferric by the iron-oxidising population may be reduced back to ferrous by an iron-reducing population. This would mean less ferric oxidant available in the environment to effect indirect oxidative mineral dissolution and, in addition, would re-supply ferrous iron to the iron-oxidisers. Obviously, this would require a source of energy, which would most likely be organic in nature.

The activities of sulfate-reducing prokaryotes would also suppress the concentrations of dissolved metals, causing their precipitation as insoluble metal sulfides. No such organisms were detected by isolation, but as anaerobic growth conditions were not used during microbial cultivation, this is not surprising. However, several *Deltaproteobacteria* clones were detected in the Cwm Rheidol clone library. Many sulfate-reducing *Bacteria* occur within this class. While membership of this class does not imply the capacity for sulfate reduction, it is not possible to rule out the occurrence of such a population.

With so many of the clones in the clone library having been identified previously in sulfide mineral-impacted environments, it is highly likely that sulfide minerals play a significant role in the metabolism of the population as a whole. It would be inconceivable that the indigenous microorganisms would effectively ignore such a source of energy. Therefore, the explanation for the seemingly inert nature of the Cwm Rheidol spoil, despite its acidity, may be

that mineral oxidisation has gone almost to completion, i.e. nearly all of the oxidisable sulfide minerals in the spoil have been oxidised. This could have been accelerated by the constant disturbance of the spoil heap, due to its instability, and drainage from the Andersons Level above. In such a scenario, the mineral-oxidising microorganisms detected may be a residual population, utilising the remaining sulfide minerals. This would make sense, as many of the organisms detected are mixotrophic, able to derive energy from organic as well as inorganic sources. Alternatively, the spoil may be continuously inoculated with these organisms by AMD draining the Andersons Level adit. The AMD could conceivably also be the source for the low concentrations of extractable metals detected and the low *p*H.

With such low concentrations of readily extractable metals, it is unlikely that the spoil itself contributes greatly to the pollution of the Afon Rheidol from the site. If similar results were observed for more representative number of spoil samples at the site, then limiting the environmental impact of the Cwm Rheidol site could be achieved by treating the mine effluent draining the Andersons Level adit directly. However, this may not be feasible due to the instability of the spoil, which could preclude the construction of a treatment plant at this location, and the extreme acidity of the AMD potentially causing problems in terms of using a pipeline to carry the drainage to a plant located elsewhere.

Comparison of the Cwm Rheidol spoil and the Parc mine tailings is very interesting. At the Parc mine, after an extensive reclamation programme, the tailings host a complex, largely uncharacterised microbial population. The environment is of mildly acidic *p*H, yet presents high concentrations of readily extractable metals, possibly due to the activities of neutrophilic mineral-oxidising microorganisms, and microniches occupied by other, more acidophilic mineral-oxidising organisms. The Cwm Rheidol spoil, on the other hand has not been subjected to any remedial action. It is much more acidic, but presents low concentrations of readily extractable metals. Known iron- or iron- and sulfur-oxidising microorganisms were detected, and made up substantial sections of the microbial community. However, their ability to

accelerate oxidative mineral dissolution *in situ* appears to be restricted, possibly either because of the activities of iron- and maybe sulfate-reducing populations, or because mineral-oxidation within the spoil has progressed almost to completion. In conclusion, it is probable that the introduced and inherent alkalinity within the Parc mine tailings has retarded the rate of bioleaching, potentially extending the time-scale of AMD genesis at this site. On the other hand, at Cwm Rheidol conditions have allowed for efficient bioleaching of the spoil, reducing the duration of AMD genesis, and as such, condensing its long-term environmental impact.

Further studies at each site would help to elucidate the microbial processes associated with sulfide-mineral metabolism. An investigation of potential neutrophilic iron- and/or sulfur-oxidising populations within the Parc mine tailings, and also the occurrence of disparate microclimates within the site is needed. An investigation of the iron-reducing capabilities of organisms from the Cwm Rheidol spoil, along with attempts to detect possible sulfate-reducing organisms, through the use, for example, of PCR primers specific to the APS gene (Hipp et al., 1997), would confirm the presence (or otherwise) of these prokaryotes.

CHAPTER 8: THE PHYLO-GEOGRAPHIC DISTRIBUTION OF THE BACTERIAL POPULATIONS IDENTIFIED IN THE PRESENT STUDY

8.1 INTRODUCTION

A large number of 16S rRNA gene sequences were obtained during this study. The majority of these were classified as belonging to the "*Acidobacteria*", *Actinobacteria* or *Proteobacteria* phyla. Of the remaining sequences, most could not be classified taxonomically.

The "*Acidobacteria*" phylum consists of a single class of the same name, and has only three described cultivated representatives, *Acidobacterium capsulatum*, *Holophaga foetida* and *Geothrix fermentans*. Members of this phylum rarely appear in culture-based surveys. However, using biomolecular techniques, Barns *et al.* (1999) detected "*Acidobacteria*" in every soil and sediment sample they examined and also some hot springs, and suggested that they are as diverse and widespread as the *Proteobacteria*. Sait *et al.* (2002) found that the "*Acidobacteria*" appeared to be numerically dominant and active members of most soils, forming up to 52% of 16S rRNA gene sequences they obtained. At least eight monophyletic groups within this phylum have been described (Hugenholtz *et al.*, 1998), with groups 1, 3, 4 and 6 the most well-represented. However, very little is known about their metabolic characteristics. Both *Holophaga* spp. and *Geothrix* spp. are obligate anaerobes, while *Acidobacterium* spp. are moderately acidophilic heterotrophs. Some "*Acidobacteria*" isolates are able to reduce ferric iron to ferrous (e.g. Coupland, 2005).

The *Actinobacteria* phylum circumscribes the high G+C Gram positive *Bacteria*, and consists of a single class, the *Actinobacteria*. The *Actinobacteridae* are the best characterised subclass, with many cultivated representatives. However, two further subclasses, the *Acidimicrobidae* and the *Rubrobacteridae* have been increasingly detected in biomolecular analyses. Rheims *et al.* (1996) used 16S rRNA gene clone libraries to study an acidic wetland. Many of these clones, the "TM" clones, form three phylogenetic groups. Groups II and III are related to *Acidimicrobium ferrooxidans*, the type species for the *Acidimicrobidae* subclass, while Group I

is related to the genus *Rubrobacter*, in the *Rubrobacteridae* subclass. These three groups, especially groups II and III, have been shown to dominate many different environments, including grassland soils, geothermally heated soils and soft metal-contaminated soils (Felske *et al.*, 1997; Rheims *et al.*, 1999; Gremion *et al.*, 2003), but have very few cultured representatives. Therefore it is not possible to comment on their potential phenotypes, but they appear to be quite ubiquitous in the environment.

The *Proteobacteria* are an incredibly diverse and well characterised phylum. Along with the "*Bacteroidetes*" and the Gram-positive phyla, Hugenholtz *et al.* (1998) found that they accounted for over 90% of all cultivated prokaryotes that had characterised 16S rRNA gene sequences, and approximately 70% of environmental sequences. Brofft *et al.* (2002) studied a forested wetland that had been severely impacted by AMD from reject coal spoil, to the point where it no longer supported any form of vegetation. They found *Proteobacteria* sequences were the most abundant in their 16S rRNA gene clone libraries, and these were mainly in the *Alphaproteobacteria* class. The *Actinobacteria* and the "*Acidobacteria*" phyla were also very abundant in this study.

Phylogenetic analyses were carried out on the sequences obtained during the present study in order to examine the phylogenetic distribution of sequences between the different sites. Of particular importance was to determine which groups of sequences, if any, were common to all sites, or whether phylogenetic groups were unique to each site.

8.2 MATERIALS AND METHODS

Operational taxonomic units (OTUs) were defined on a site-by-site basis as partial 16S rRNA gene sequences from isolates or clones, which had been differentiated on the basis of RFLP analysis and/or colony morphology, where applicable. Phylogenetic analysis was carried out on three groups of OTUs classified as members of the "*Acidobacteria*", *Actinobacteria* or *Proteobacteria* phyla by the RDP Classifier with a confidence interval of greater than 80%. OTUs that were not members of these phyla, or could not be classified above the 80% confidence threshold were analysed as a fourth group, the 'Other *Bacteria*'.

8.2.1 Reference sequence selection

Reference sequences for each of the four groups were obtained from the GenBank sequence repository. Sequences containing unknown nucleotide residues were excluded, as this may indicate that the sequences had not been sufficiently scrutinised prior to submission to the database or that they were of poor quality, which would adversely influence the phylogenetic analysis. Where possible, sequences representing the type genus of each family, as described in Bergey's Manual for Systematic Bacteriology (Garrity *et al.*, 2004), were used.

8.2.1.1 The "*Acidobacteria*"

At present, there is only a single family within the "*Acidobacteria*" phylum. Therefore, reference sequences were chosen from studies of this phylum in the environment, e.g. Brofft *et al.* (2002), Barns *et al.* (1999) and Quaiser *et al.* (2003). These sequences represented groups 1 to 8, as described by Hugenholtz *et al.* (1998). 50 reference sequences were selected, and a total of 69 sequences were used in this analysis.

8.2.1.2 The *Actinobacteria*

DNA sequences for the type genus of each family were obtained, representing every subclass except the *Sphaerobacteridae*. This comprises a single species, for which no reliable sequence data could be found. In addition, sequences from an acidic peat bog (the "TM" clones), which have been shown to be ubiquitous within many different environments (Rheims *et al.*, 1996; Felske *et al.*, 1997; Rheims *et al.*, 1999) and several clones from a forested wetland impacted by AMD from coal spoil (the "RCP" clones) (Brofft *et al.*, 2002) were included. The TM clones have been shown to form three monophyletic groups, TM groups I to III. 126 reference sequences were selected, and a total of 182 sequences were used in this analysis.

8.2.1.3 The *Proteobacteria*

DNA sequences representing the type family were obtained for the majority of orders within this phylum. However, the *Epsilonproteobacteria* have only a

single order, so sequences representing the type genera of each family were included. 41 reference sequences were selected, and a total of 124 sequences were used in this analysis.

8.2.1.4 Other *Bacteria*

Where possible, sequences representing every class within the *Bacteria* were included in the analysis. Occasionally, the sequence data were of too poor quality to allow the inclusion of some classes, or even entire phyla. Some phyla were better represented than others, simply due to having greater numbers of classes, and being generally better characterised. 159 reference sequences were selected, and a total of 224 sequences were used in this analysis.

8.2.2 Phylogenetic analysis

Sequences were aligned using the ClustalX alignment programme (Thompson *et al.*, 1997). Alignments were checked by hand, and sequences shortened to a uniform length based on the shortest sequence length in the alignment, using the BioEdit sequence-editing programme (Tom Hall, Ibis Therapeutics, California, U.S.A.). Maximum likelihood (ML) trees were constructed using the PhyML algorithm (Guindon and Gascuel, 2003). The ModelGenerator model selection programme (Keane *et al.*, 2006) was used to select the optimal nucleotide substitution model for ML analysis. Bootstrap values were determined from 100 replications. Bootstrap values greater than 70% were considered good, values between 50 and 70% were considered moderate while values less than 50% were considered poor.

8.2.3 Clone library richness evaluation

S_{chao1} and S_{ACE} were used to estimate total richness (OTUs) for each 16S rRNA gene clone library, using the interface available at http://www.aslo.org/lomethods/free/2004/0114a.html (Kemp and Aller, 2004). This uses a random sub-sampling method, and plots sub-sample size against estimated richness. If the plots do not show asymptotic distribution, then the richness estimates are not stable. Therefore, the clone library is too small to allow a reliable estimate of the total number of OTUs at the site from which it

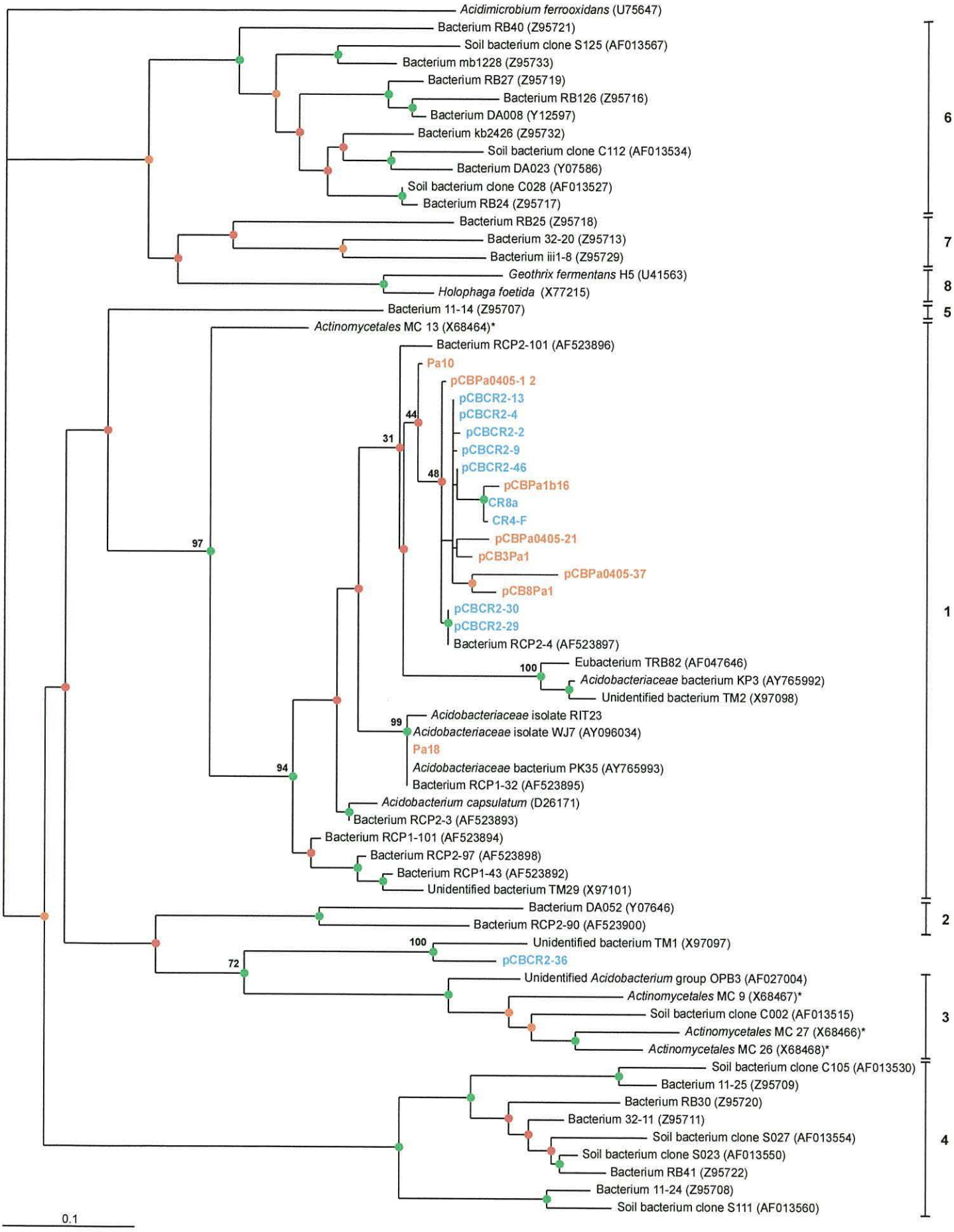
was derived. If the plot reaches an asymptote, then the estimated total OTU number can be compared to the observed number, and it is possible to infer how exhaustively the library has been sampled.

8.3 RESULTS

8.3.1 The "*Acidobacteria*"

Microorganisms belonging to this phylum were found in the Cwm Rheidol, Mynydd Parys and Shilbottle spoil samples (Figure 8.1). However, isolate Sb4, the only *Acidobacterium* detected in the Shilbottle Brass Heap, was not included as the sequence data obtained were not of sufficient quality. The majority of the OTUs included formed a highly interrelated cluster within Group 1. Although bootstrap values did not support the occurrence of this cluster, there was good support for the placement of all the Group 1 taxa within a distinct clade. Isolate Pa18 formed a distinct subgroup with the *Acidobacteriaceae* isolates RTI23, WJ7, and PK35, all of which are able to reduce ferric iron (Coupland, 2005). A group of three microorganisms, including *Acidobacteriaceae* isolate KP3, which was not found by Coupland (2005) to reduce ferric iron, formed a distinct branch from the Cwm Rheidol/Mynydd Parys OTUs, with good bootstrap support. A single clone from Cwm Rheidol grouped with a clone ("TM1") from an acidic peat bog, with good bootstrap support to suggest that they form a separate group from Group 3. Interestingly, when this Cwm Rheidol clone was classified using the RDP Classifier, it was classified with only 55% confidence. However, this may be a reflection of the paucity of "*Acidobacteria*" sequences in the databases used by the RDP.

All of the Cwm Rheidol and Mynydd Parys sequences grouped with sequences obtained from an acidic peat bog (TM) or a wetland severely impacted by acidic coal spoil drainage (RCP) in Group 1 and the unnamed "TM1:pCBCR2-36" group.


Acidimicrobium ferrooxidans (U75647)
Bacterium RB40 (Z95721)
Soil bacterium clone S125 (AF013567)
Bacterium mb1228 (Z95733)
Bacterium RB27 (Z95719)
Bacterium RB126 (Z95716)
Bacterium DA008 (Y12597)
Bacterium kb2426 (Z95732)
Soil bacterium clone C112 (AF013534)
Bacterium DA023 (Y07586)
Soil bacterium clone C028 (AF013527)
Bacterium RB24 (Z95717)
Bacterium RB25 (Z95718)
Bacterium 32-20 (Z95713)
Bacterium iii1-8 (Z95729)
Geothrix fermentans H5 (U41563)
Holophaga foetida (X77215)
Bacterium 11-14 (Z95707)
Actinomycetales MC 13 (X68464)*
Bacterium RCP2-101 (AF523896)
Pa10
pCBPa0405-1 2
pCBCR2-13
pCBCR2-4
pCBCR2-2
pCBCR2-9
pCBCR2-46
pCBPa1b16
CR8a
CR4-F
pCBPa0405-21
pCB3Pa1
pCBPa0405-37
pCB8Pa1
pCBCR2-30
pCBCR2-29
Bacterium RCP2-4 (AF523897)
Eubacterium TRB82 (AF047646)
Acidobacteriaceae bacterium KP3 (AY765992)
Unidentified bacterium TM2 (X97098)
Acidobacteriaceae isolate RIT23
Acidobacteriaceae isolate WJ7 (AY096034)
Pa18
Acidobacteriaceae bacterium PK35 (AY765993)
Bacterium RCP1-32 (AF523895)
Acidobacterium capsulatum (D26171)
Bacterium RCP2-3 (AF523893)
Bacterium RCP1-101 (AF523894)
Bacterium RCP2-97 (AF523898)
Bacterium RCP1-43 (AF523892)
Unidentified bacterium TM29 (X97101)
Bacterium DA052 (Y07646)
Bacterium RCP2-90 (AF523900)
Unidentified bacterium TM1 (X97097)
pCBCR2-36
Unidentified *Acidobacterium* group OPB3 (AF027004)
Actinomycetales MC 9 (X68467)*
Soil bacterium clone C002 (AF013515)
Actinomycetales MC 27 (X68466)*
Actinomycetales MC 26 (X68468)*
Soil bacterium clone C105 (AF013530)
Bacterium 11-25 (Z95709)
Bacterium RB30 (Z95720)
Bacterium 32-11 (Z95711)
Soil bacterium clone S027 (AF013554)
Soil bacterium clone S023 (AF013550)
Bacterium RB41 (Z95722)
Bacterium 11-24 (Z95708)
Soil bacterium clone S111 (AF013560)
44
31
48
97
100
99
94
100
72
6
7
8
5
1
2
3
4
0.1

8.3.2 The *Actinobacteria*

Microorganisms belonging to the *Actinobacteria* phylum were detected in the Cwm Rheidol, Kennecott heap, Mynydd Parys, Parc mine and Shilbottle Brass Heap samples. No microorganisms were found to belong to the *Coriobacteridae* subclass. The phylogenetic dendrogram is shown in Figure 8.2.

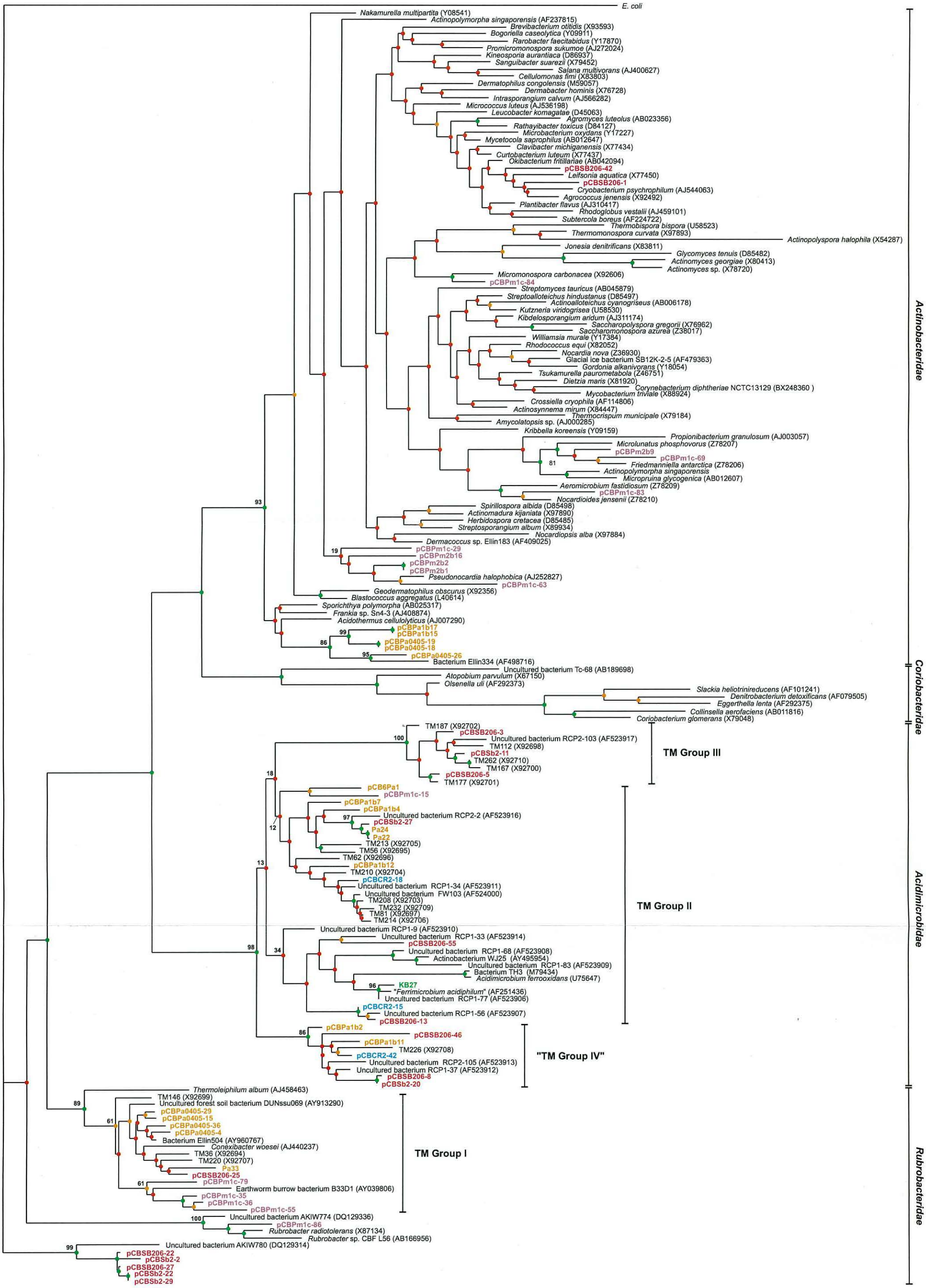
Several OTUs from Mynydd Parys, Parc and Shilbottle were found to belong to the *Actinobacteridae* subclass. The grouping of the *Actinobacteridae* sequences into a distinct clade had good bootstrap support. Five OTUs from Parc mine formed a cluster with the *Pseudonocardia*, a group of organisms often found in soil and manure samples. However, the group was not highly interrelated and bootstrap support for the branch was poor. A group of OTUs from Mynydd Parys formed a cluster with an uncultured organism (Ellin334) obtained from pasture soil. This clone had been found to have no close relatives within the *Actinobacteridae*, and possibly represents a novel suborder (Sait *et al.*, 2002). There was good bootstrap support for the occurrence of this branch, although its classification as a new subclass is not clear from this analysis. Other OTUs from Parc mine and Shilbottle Brass Heap were distributed throughout the rest of the *Actinobacteridae*.

The division of the *Acidimicrobidae* sequences into the TM groups II and III (as described by Rheims *et al.*, 1996) was not supported by the tree topology, although there was good support for the formation of a fourth group, "TM group IV". This group diverged before the group II/III division, but sequences in this group had previously been assigned to TM group II. TM group III was solely represented by OTUs from Shilbottle. Interestingly, when classified using the RDP Classifier, these were only classified as *Actinobacteria* with 70-76% confidence. OTUs from Cwm Rheidol, Kennecott, Mynydd Parys, Parc, and Shilbottle were distributed throughout TM group II. Only the Kennecott isolate KB27 was closely related to either of the two characterised microorganisms in this subclass. This isolate clustered, along with a clone from the RCP study, with "*Ferrimicrobium acidiphilum*", with good bootstrap support. The Mynydd Parys isolates Pa22 and Pa24 grouped with a clone obtained from Shilbottle, with good bootstrap support.

The *Rubrobacteridae* did not form a coherent monophyletic group, and were instead divided into three distinct branches, each with good bootstrap support. The first branch included TM Group I, although the occurrence of this group had only moderate bootstrap support. This contained OTUs from Mynydd Parys, Parc and Shilbottle, including the iron-oxidising isolate Pa33. The OTUs from Parc mine formed a distinct clade away from the rest of this group. A single OTU from Parc clustered in the second *Rubrobacteridae* clade, along with the type genus for this subclass, *Rubrobacter*. Four OTUs from Shilbottle formed the final group within the *Rubrobacteridae*, with a clone obtained from an air sample obtained from above Texas (Andersen *et al.*, unpublished).

The majority of OTUs are distributed within the TM groups I, II, III and "IV", within the *Acidimicrobidae* and *Rubrobacteridae* subclasses. The Parc mine OTUs are unique in that they did not appear to cluster with any of the TM or RCP clones. The Parc and Shilbottle *Actinobacteria* clones showed the greatest phylogenetic diversity. While some OTUs seemed to form some site-specific clusters, where these occurred, they were not highly interrelated.

Figure 8.2► Phylogenetic dendrogram of the *Actinobacteria* phylum, as determined by maximum likelihood analysis of 546 nt of the 16S rRNA gene. *Escherichia coli* served as the outgroup. Only bootstrap values for clusters discussed in the text are included. The scale bar represents changes per nucleotide. (Key: ●, Preceding branch has good bootstrap support; ●, Preceding branch has moderate bootstrap support; ●, Preceding branch has poor bootstrap support; pCB, 16S rRNA gene clone; CR, Cwm Rheidol spoil; KB, Kennecott Bingham Canyon bioleach heap; Pa, Mynydd Parys spoil; Pm, Parc mine tailings; SB, Shilbottle Brass Heap spoil.)


E. coli
Nakamurella multipartita (Y08541)
Actinopolymorpha singaporensis (AF237815)
Brevibacterium otitidis (X93593)
Bogoriella caseolytica (Y09911)
Rarobacter faecitabidus (Y17870)
Promicromonospora sukumoe (AJ272024)
Kineosporia aurantiaca (D86937)
Sanguibacter suarezii (X79452)
Salana multivorans (AJ400627)
Cellulomonas fimi (X83803)
Dermatophilus congolensis (M59057)
Dermabacter hominis (X76728)
Intrasporangium calvum (AJ566282)
Micrococcus luteus (AJ536198)
Leucobacter komagatae (D45063)
Agromyces luteolus (AB023356)
Rathayibacter toxicus (D84127)
Microbacterium oxydans (Y17227)
Mycetocola saprophilus (AB012647)
Clavibacter michiganensis (X77434)
Curtobacterium luteum (X77437)
Okibacterium fritillariae (AB042094)
pCBSB206-42
Leifsonia aquatica (X77450)
pCBSB206-1
Cryobacterium psychrophilum (AJ544063)
Agrococcus jenensis (X92492)
Plantibacter flavus (AJ310417)
Rhodoglobus vestalii (AJ459101)
Subtercola boreus (AF224722)
Thermobispora bispora (U58523)
Thermomonospora curvata (X97893)
Actinopolyspora halophila (X54287)
Jonesia denitrificans (X83811)
Glycomyces tenuis (D85482)
Actinomyces georgiae (X80413)
Actinomyces sp. (X78720)
Micromonospora carbonacea (X92606)
pCBPm1c-84
Streptomyces tauricus (AB045879)
Streptoalloteichus hindustanus (D85497)
Actinoalloteichus cyanogriseus (AB006178)
Kutzneria viridogrisea (U58530)
Kibdelosporangium aridum (AJ311174)
Saccharopolyspora gregorii (X76962)
Saccharomonospora azurea (Z38017)
Williamsia murale (Y17384)
Rhodococcus equi (X82052)
Nocardia nova (Z36930)
Glacial ice bacterium SB12K-2-5 (AF479363)
Gordonia alkanivorans (Y18054)
Tsukamurella paurometabola (Z46751)
Dietzia maris (X81920)
Corynebacterium diphtheriae NCTC13129 (BX248360)
Mycobacterium triviale (X88924)
Crossiella cryophila (AF114806)
Actinosynnema mirum (X84447)
Thermocrispum municipale (X79184)
Amycolatopsis sp. (AJ000285)
Kribbella koreensis (Y09159)
Propionibacterium granulosum (AJ003057)
Microlunatus phosphovorus (Z78207)
pCBPm2b9
pCBPm1c-69
Friedmanniella antarctica (Z78206)
Actinopolymorpha singaporensis
Micropruina glycogenica (AB012607)
Aeromicrobium fastidiosum (Z78209)
pCBPm1c-83
Nocardioides jensenii (Z78210)
Spirillospora albida (D85498)
Actinomadura kijaniata (X97890)
Herbidospora cretacea (D85485)
Streptosporangium album (X89934)
Nocardiopsis alba (X97884)
Dermacoccus sp. Ellin183 (AF409025)
pCBPm1c-29
pCBPm2b16
pCBPm2b2
pCBPm2b1
Pseudonocardia halophobica (AJ252827)
pCBPm1c-63
Geodermatophilus obscurus (X92356)
Blastococcus aggregatus (L40614)
Sporichthya polymorpha (AB025317)
Frankia sp. Sn4-3 (AJ408874)
Acidothermus cellulolyticus (AJ007290)
pCBPa1b17
pCBPa1b15
pCBPa0405-19
pCBPa0405-18
pCBPa0405-26
Bacterium Ellin334 (AF498716)
Uncultured bacterium Tc-68 (AB189698)
Atopobium parvulum (X67150)
Olsenella uli (AF292373)
Slackia heliotrinireducens (AF101241)
Denitrobacterium detoxificans (AF079505)
Eggerthella lenta (AF292375)
Collinsella aerofaciens (AB011816)
Coriobacterium glomerans (X79048)
TM187 (X92702)
pCBSB206-3
Uncultured bacterium RCP2-103 (AF523917)
TM112 (X92698)
pCBSb2-11
TM262 (X92710)
TM167 (X92700)
pCBSB206-5
TM177 (X92701)
TM Group III
pCB6Pa1
pCBPm1c-15
pCBPa1b7
pCBPa1b4
Uncultured bacterium RCP2-2 (AF523916)
pCBSb2-27
Pa24
Pa22
TM213 (X92705)
TM56 (X92695)
TM62 (X92696)
pCBPa1b12
TM210 (X92704)
pCBCR2-18
Uncultured bacterium RCP1-34 (AF523911)
Uncultured bacterium FW103 (AF524000)
TM208 (X92703)
TM232 (X92709)
TM81 (X92697)
TM214 (X92706)
TM Group II
Uncultured bacterium RCP1-9 (AF523910)
Uncultured bacterium RCP1-33 (AF523914)
pCBSB206-55
Uncultured bacterium RCP1-68 (AF523908)
Actinobacterium WJ25 (AY495954)
Uncultured bacterium RCP1-83 (AF523909)
Bacterium TH3 (M79434)
Acidimicrobium ferrooxidans (U75647)
KB27
"Ferrimicrobium acidiphilum" (AF251436)
Uncultured bacterium RCP1-77 (AF523906)
pCBCR2-15
Uncultured bacterium RCP1-56 (AF523907)
pCBSB206-13
pCBPa1b2
pCBSB206-46
pCBPa1b11
TM226 (X92708)
pCBCR2-42
Uncultured bacterium RCP2-105 (AF523913)
Uncultured bacterium RCP1-37 (AF523912)
pCBSB206-8
pCBSb2-20
"TM Group IV"
Thermoleiphilum album (AJ458463)
TM146 (X92699)
Uncultured forest soil bacterium DUNssu069 (AY913290)
pCBPa0405-29
pCBPa0405-15
pCBPa0405-36
pCBPa0405-4
Bacterium Ellin504 (AY960767)
Conexibacter woesei (AJ440237)
TM36 (X92694)
TM220 (X92707)
Pa33
pCBSB206-25
pCBPm1c-79
Earthworm burrow bacterium B33D1 (AY039806)
pCBPm1c-35
pCBPm1c-36
pCBPm1c-55
TM Group I
Uncultured bacterium AKIW774 (DQ129336)
pCBPm1c-86
Rubrobacter radiotolerans (X87134)
Rubrobacter sp. CBF L56 (AB166956)
Uncultured bacterium AKIW780 (DQ129314)
pCBSB206-22
pCBSb2-2
pCBSB206-27
pCBSb2-22
pCBSb2-29
93
19
99
86
95
18
100
12
13
97
81
98
34
96
89
61
61
100
99
86
0.1
Actinobacteridae
Coriobacteridae
Acidimicrobidae
Rubrobacteridae

8.3.3 The *Proteobacteria*

The phylogenetic dendrogram produced using the *Proteobacteria* sequences is shown in Figure 8.3. Microorganisms from this phylum were detected in every study site. The majority of isolates obtained during the study were acidithiobacilli, which branched deeply within the *Gammaproteobacteria*, close to the root between the *Betaproteobacteria* and *Gammaproteobacteria*. These are often referred to as the "*Beta/Gammaproteobacteria*", and considered as a separate class, although this is not recognised in the current issue of Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2004). The acidithiobacilli OTUs that clustered tightly with *Acidithiobacillus thiooxidans* did so in 100% of the bootstrap replicates. The remaining acidithiobacilli OTUs clustered in the *At. ferrooxidans* group, although the formation of this branch did not have good bootstrap support. The *At. ferrooxidan*s group could be divided into three subgroups, while Parc mine Isolate Pm3 was quite divergent, branching close to the root of the group. Subgroup I included the type strain of the species and isolates from the Kennecott heap only. Isolates from the Kennecott heap, Mynydd Parys and Shilbottle Brass Heap formed subgroup II and two clones from Shilbottle and an isolate from Cae Coch formed subgroup III. The OTUs that formed subgroups II and III shared greater 16S rRNA gene identity with *At. ferrooxidans* NO37 than the type strain.

The rest of the *Gammaproteobacteria* class consisted mainly of clones from Cwm Rheidol and Shilbottle. Eight clones obtained from Cwm Rheidol, and a single clone from Mynydd Parys formed a cluster within the *Gammaproteobacteria*, with good bootstrap support. All of these clones were closely related to clones obtained from uranium mine waste (Satchanska *et al.*, unpublished; Selenska-Pobell, 2002). This cluster formed part of a larger branch with the Kennecott heap isolate KB68 and a further clone from Cwm Rheidol, which were both related to the iron-oxidising acidophile m-1. There was good bootstrap support for the occurrence of this larger group. Six clones from Shilbottle formed a cluster which included *Legionella lytica*, although none of these sequences were closely related to each other.

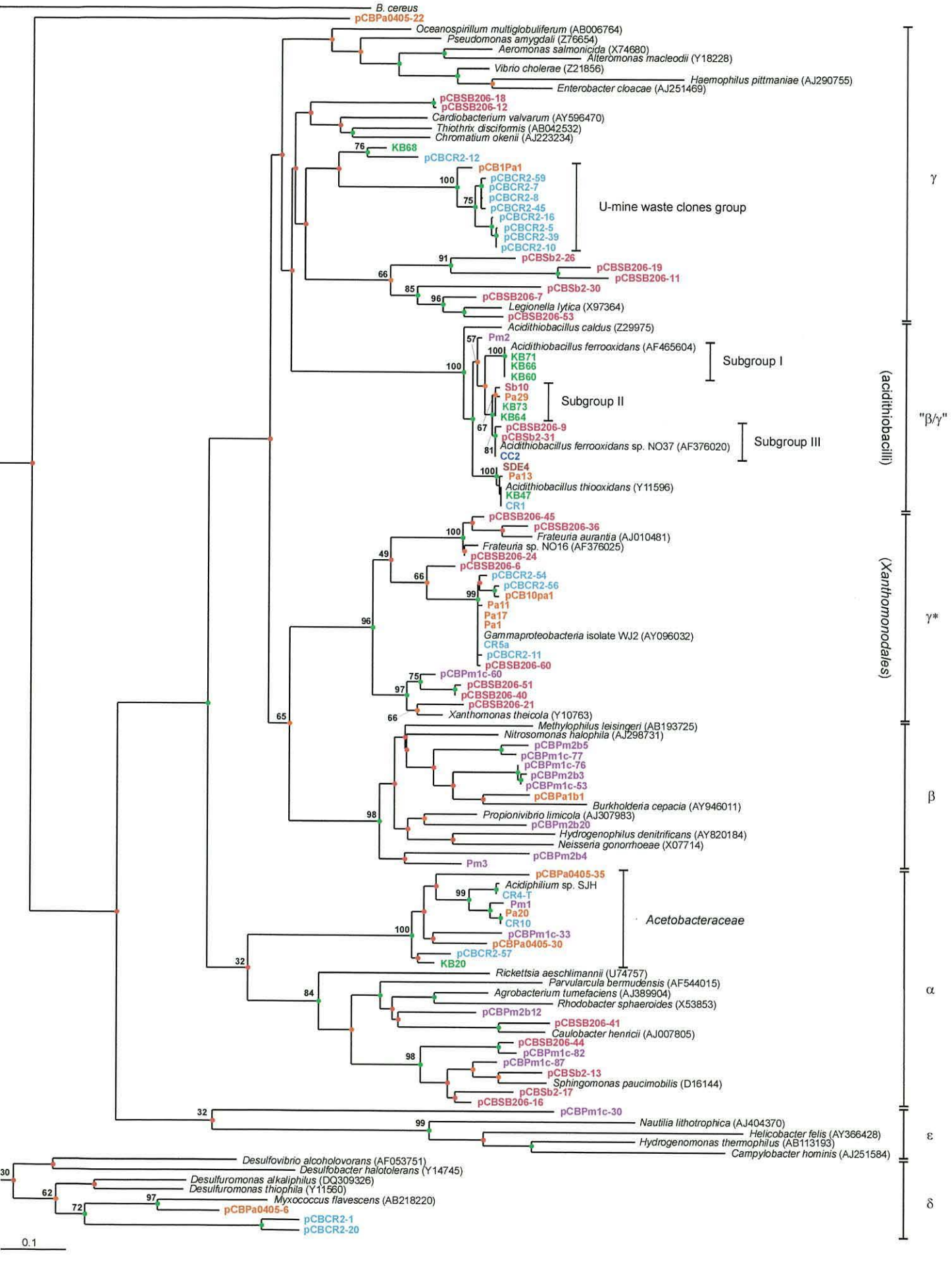
Several OTUs from Cwm Rheidol, Mynydd Parys, Parc mine and Shilbottle grouped within the order *Xanthomonodales*. Like the acidithiobacilli, this order is classified as part of the *Gammaproteobacteria* class, but diverges close to the root between the *Betaproteobacteria* and the *Gammaproteobacteria*. It is suggested in Bergey's Manual for Systematic Bacteriology (Garrity *et al.*, 2004) that the *Xanthomonodales* probably represent a sister group of the *Betaproteobacteria*, and this is supported by this analysis.

Several OTUs from Cwm Rheidol, Mynydd Parys and Shilbottle formed a highly interrelated cluster with the iron-oxidising *Gammaproteobacteria* isolate WJ2, with good bootstrap support. This cluster, along with another clone from Shilbottle, was fairly distantly related to a group of three clones from Shilbottle that clustered with the genus *Frateuria*. Three other clones from Shilbottle, plus a single clone from the Parc mine formed a cluster with the genus *Xanthomonas*, with good bootstrap support.

The *Betaproteobacteria* were mainly represented by OTUs from Parc mine, and these were distributed throughout this class. A number of OTUs from Cwm Rheidol, the Kennecott heap, Mynydd Parys and the Parc mine were found to belong to the *Acetobacteraceae* family, within the *Alphaproteobacteria* class, with good bootstrap support. OTUs from Parc and Shilbottle were distributed throughout the rest of the *Alphaproteobacteria* class, with most lying within the *Sphingomonadaceae* family. A single clone from Parc mine may be part of the *Epsilonproteobacteria*, but the bootstrap values did not support this. The clone could not be classified using the RDP Classifier, and was very distantly related to sequences from this class. The *Deltaproteobacteria* class contained three OTUs from Cwm Rheidol and Mynydd Parys. None of these clustered with known sulfate-reducing genera, such as *Desulfovibrio*, or the *Deltaproteobacteria* sequences obtained by Bond *et al.* (2000) from an extreme acid mine drainage site at Iron Mountain, California, U.S.A. (data not shown).

Whilst most proteobacterial OTUs obtained during this study were widely distributed throughout the phylum, those from the Parc mine tailings were found mainly within the *Alphaproteobacteria* and *Betaproteobacteria* classes.

Figure 8.3► Phylogenetic dendrogram of the *Proteobacteria* phylum, as determined by maximum likelihood analysis of 547 nt of the 16S rRNA gene. *Bacillus cereus* served as the outgroup. Only bootstrap values for clusters discussed in the text are included. The scale bar represents changes per nucleotide. (Key: ●, Preceding branch has good bootstrap support; ●, Preceding branch has moderate bootstrap support; ●, Preceding branch has poor bootstrap support; pCB, 16S rRNA gene clone; CC, Cae Coch droplets; CR, Cwm Rheidol spoil; KB, Kennecott Bingham Canyon bioleach heap; Pa, Mynydd Parys spoil; Pm, Parc mine tailings; SD, São Domingos spoil or tailings; SDE, São Domingos enrichment cultures; SB, Shilbottle Brass Heap spoil; **Xanthomonodales*.)


B. cereus
pCBPa0405-22
Oceanospirillum multiglobuliferum (AB006764)
Pseudomonas amygdali (Z76654)
Aeromonas salmonicida (X74680)
Alteromonas macleodii (Y18228)
Vibrio cholerae (Z21856)
Haemophilus pittmaniae (AJ290755)
Enterobacter cloacae (AJ251469)
pCBSB206-18
pCBSB206-12
Cardiobacterium valvarum (AY596470)
Thiothrix disciformis (AB042532)
Chromatium okenii (AJ223234)
KB68
pCBCR2-12
pCB1Pa1
pCBCR2-59
pCBCR2-7
pCBCR2-8
pCBCR2-45
pCBCR2-16
pCBCR2-5
pCBCR2-39
pCBCR2-10
U-mine waste clones group
pCBSb2-26
pCBSB206-19
pCBSB206-11
pCBSb2-30
pCBSB206-7
Legionella lytica (X97364)
pCBSB206-53
Acidithiobacillus caldus (Z29975)
Pm2
Acidithiobacillus ferrooxidans (AF465604)
KB71
KB66
KB60
Subgroup I
Sb10
Pa29
KB73
KB64
Subgroup II
pCBSB206-9
pCBSb2-31
Acidithiobacillus ferrooxidans sp. NO37 (AF376020)
CC2
Subgroup III
SDE4
Pa13
Acidithiobacillus thiooxidans (Y11596)
KB47
CR1
pCBSB206-45
pCBSB206-36
Frateuria aurantia (AJ010481)
Frateuria sp. NO16 (AF376025)
pCBSB206-24
pCBSB206-6
pCBCR2-54
pCBCR2-56
pCB10pa1
Pa11
Pa17
Pa1
Gammaproteobacteria isolate WJ2 (AY096032)
CR5a
pCBCR2-11
pCBSB206-60
pCBPm1c-60
pCBSB206-51
pCBSB206-40
pCBSB206-21
Xanthomonas theicola (Y10763)
Methylophilus leisingeri (AB193725)
Nitrosomonas halophila (AJ298731)
pCBPm2b5
pCBPm1c-77
pCBPm1c-76
pCBPm2b3
pCBPm1c-53
pCBPa1b1
Burkholderia cepacia (AY946011)
Propionivibrio limicola (AJ307983)
pCBPm2b20
Hydrogenophilus denitrificans (AY820184)
Neisseria gonorrhoeae (X07714)
pCBPm2b4
Pm3
pCBPa0405-35
Acidiphilium sp. SJH
CR4-T
Pm1
Pa20
CR10
Acetobacteraceae
pCBPm1c-33
pCBPa0405-30
pCBCR2-57
KB20
Rickettsia aeschlimannii (U74757)
Parvularcula bermudensis (AF544015)
Agrobacterium tumefaciens (AJ389904)
Rhodobacter sphaeroides (X53853)
pCBPm2b12
pCBSB206-41
Caulobacter henricii (AJ007805)
pCBSB206-44
pCBPm1c-82
pCBPm1c-87
pCBSb2-13
Sphingomonas paucimobilis (D16144)
pCBSb2-17
pCBSB206-16
pCBPm1c-30
Nautilia lithotrophica (AJ404370)
Helicobacter felis (AY366428)
Hydrogenomonas thermophilus (AB113193)
Campylobacter hominis (AJ251584)
Desulfovibrio alcoholovorans (AF053751)
Desulfobacter halotolerans (Y14745)
Desulfuromonas alkaliphilus (DQ309326)
Desulfuromonas thiophila (Y11560)
Myxococcus flavescens (AB218220)
pCBPa0405-6
pCBCR2-1
pCBCR2-20
0.1
γ
(acidithiobacilli)
"β/γ"
(Xanthomonadales)
γ*
β
α
ε
δ

8.3.4 Other *Bacteria*

The resolution of recognised phyla in the phylogenetic tree (Figure 8.4) was not very good, presumably due to the size and complexity of the analysed data set. Some sequences known to be of the same phylum did not cluster together in monophyletic groups. However, in the context of elucidating any clustering of isolates and clones, by site or otherwise, this was not such a problem.

The vast majority of OTUs obtained during this study that did not fall into the "*Acidobacteria*", *Actinobacteria* or *Proteobacteria* phyla could not be classified using the RDP classifier. However, it was possible to place some of the unclassified OTUs into recognised phyla, and confirm the classification of others. Two closely related clones from the Parc mine sample were placed within the "*Verrucomibrobia*" phylum, with 100% bootstrap support. Three others were placed within the *Gemmatimonadetes* phylum, again with good bootstrap support. Two clones obtained from the Shilbottle Brass Heap were placed within the *Actinobacteria* phylum, but bootstrap support for this was poor and this phylum was not fully resolved in this analysis. Several OTUs from Mynydd Parys and Parc formed a group with the *Spirocaetes* and "*Acidobacteria*" phyla, but again, bootstrap scores were moderate, and their relationships could not be resolved.

Four isolates from Mynydd Parys, Parc and Sao Domingos formed a group with the genus *Alicyclobacillus* in the *Alicyclobacillaceae* family, with good bootstrap support. Six iron-oxidising isolates obtained from the Kennecott heap and São Domingos formed a distinct clade with the genus *Sulfobacillus*, with good bootstrap support. This group is referred to as "*Sulfobacillus*-like", and was not highly interrelated. It was most likely a member of the "*Firmicutes*" phylum, in agreement with the RDP classification of these OTUs. Three closely related clones obtained from Shilbottle formed another distinct group with good bootstrap support, and while they were classified as "*Firmicutes*" by the RDP Classifier, it was not possible to determine to which class therein, if any, they belonged. Several clones obtained from Parc were distributed throughout a clade that included the "*Chloroflexi*" phylum. A single clone obtained from Shilbottle was placed within

the "*Bacteroidetes*" phylum. Three clones obtained from Parc were placed within the *Cyanobacteria* phylum, with good bootstrap support. Three clones obtained from Shilbottle and a single clone obtained from Parc were placed within the deep-branching proposed class TM7 with good bootstrap support. This class is *incertae sedis*, and cannot be assigned to a particular phylum at present. Several clones from Cwm Rheidol, Mynydd Parys and Parc were placed within the "*Planctomycetales*" phylum, with good bootstrap support.

The remaining OTUs could not be classified using the RDP Classifier, but formed three distinct monophyletic groups, distributed throughout the bacterial kingdom. Each group occurred in 100% of the bootstrap replicates, and could not be easily ascribed to a particular phylum. OB (Other *Bacteria*) group I comprised a number of clones obtained from Cwm Rheidol, Mynydd Parys and Shilbottle. This group diverged at an early stage from an unclassified clone obtained from a forested wetland (Broft *et al.*, 2002). OB group II comprised mainly of clones obtained from Mynydd Parys, plus a single clone obtained from Shilbottle, but was not highly interrelated. OB group III comprised a diverse group of clones obtained from Mynydd Parys. It may be part of the "*Chloroflexi*" phylum, but the bootstrap support for this was only moderate.

OTUs obtained from the Parc mine and Shilbottle Brass Heap samples were the most widely distributed throughout the *Bacteria* kingdom. The Mynydd Parys OTUs, on the other hand, had a preponderance to form distinct monophyletic clusters, such as OB groups I, II and III.

Figure 8.4► Phylogenetic dendrogram of other *Bacteria*, as determined by maximum likelihood analysis of 689 nt of the 16S rRNA gene. Isolate SDE29, a *Ferroplasma acidiphilum*-like archaeon, served as the outgroup. Only bootstrap values for clusters discussed in the text are included. The scale bar represents changes per nucleotide. (Key: ●, Preceding branch has good bootstrap support; ●, Preceding branch has moderate bootstrap support; ●, Preceding branch has poor bootstrap support; pCB, 16S rRNA gene clone; CC, Cae Coch droplets; CR, Cwm Rheidol spoil; KB, Kennecott Bingham Canyon bioleach heap; Pa, Mynydd Parys spoil; Pm, Parc mine tailings; SD São Domingos spoil or tailings; SDE São Domingos enrichment cultures; SB, Shilbottle Brass Heap spoil; †, Could not be classified using the RDP Classifier above the 80% confidence threshold; *Phylum not fully resolved in this analysis.)


SDE29
Uncultured candidate division OP11 bacterium clone 09D2Z47
Chlorobium chlorochromatii (AJ578461)
Leptospirillum ferriphilum (DQ343299)
Pa9
Pa8
Thermodesulfovibrio yellowstonii (AB231858)
Magnetobacterium bavaricum (X71838)
Candidatus Xiphinematobacter brevicolli (AF217462)
pCBPm1c-52
pCBPm1c-44
Prosthecobacter FC1 (U60012)
uncultured verrucomicrobium DEV003 (AJ401104)
"Verrucomicrobia"
Nitrospira sp. (AF035813)
Deinococcus radiodurans (Y11332)
Meiothermus silvanus (Y13599)
Vulcanithermus mediogtlanticus (AJ507298)
Oceanithermus desulfurans (AB107956)
Thermus aquaticus (L09663)
Marinithermus hydrothermalis (AB079382)
Thermovibrio ammoniificans (AY263403)
Desulfurobacterium thermolithotrophum (AJ001049)
Persephonella marina (AF188332)
Hydrogenothermus marinus (AJ292525)
Sulfurihydrogenibium yellowstonense (AY686713)
Thermocrinis sp. U223L3A (AJ320220)
Aquifex aeolicus (AJ309733)
Thermodesulfotobacterium sp. SRI-93 (AF255596)
Marinitoga hydrogenitolerans (AJ786363)
Petrotoga olearia (AJ311703)
Geotoga aestuarianus (AF509468)
Thermosipho sp. DSM 13256 (AJ272022)
Fervidobacterium islandicum (AF434670)
Uncultured bacterium clone FW68 (AF524022)
pCBCR2-58 †
pCBPa1b3 †
pCBSB206-32 †
pCBPa1b13 †
pCBPa1b6 †
pCBPa1b10 †
pCBCR2-17 †
OB Group I
pCBSB206-34 †
pCBSB206-33 †
Fibrobacter succinogenes (AJ505938)
pCBPm1c-43
pCBPm1c-26
Gemmatimonas aurantiaca (AB072735)
pCBPm1c-49
Gemmatimonadates
Bifidobacterium bifidum (AY694148)
Actinomyces georgiae (X80413)
pCBSB206-23 †
pCBSb2-2 †
Acidimicrobium ferrooxidans (U75647)
Actinobacteria*
Coriobacterium sp. CCUG 33917 (AJ131149)
Uncultured candidate division WS3 bacterium clone LD1-PB19 (AY114332)
Brachyspira aalborgi (Z22781)
Spirochaeta africana (X93928)
Treponema parvum (AF302937)
Borrelia sinica (AB022101)
Spirocaetes*
Acidobacterium capsulatum (D26171)
pCBPa0405-12 †
"Acidobacteria"
pCBPm1c-6 †
pCBPm1c-48 †
pCBPm1c-47 †
pCBPm1c-42 †
pCBPm1c-54 †
pCBPm1c-17 †
Leptonema illini (Z21632)
Leptospira kirschneri (AY996802)
Spirocaetes*
Chrysiogenes arsenatis (X81319)
Propionigenium maris (X84049)
Fusobacterium equorum (AJ295750)
Cetobacterium ceti (X78419)
Flexistipes sp. vp180 (AF220344)
Geovibrio ferrireductans (X95744)
Deferribacter desulfuricans (AB086060)
Desulfonosporus thiosulfogenes (Y18214)
Desulfosporosinus orientis (Y11571)
Desulfitobacterium frappieri (AJ404686)
Syntrophobotulus glycolicus (X99706)
Dehalobacter restrictus (U84497)
Thermoactinomyces kribbensis (AB049939)
Mycoplasma gallinarum (L24105)
Spiroplasma citri (AM157769)
Erysipelothrix tonsillarum (AB034201)
Acholeplasma sp. CODA 1G (AY785356)
Mesoplasma pleciae (AY257485)
Paenibacillus borealis (AJ011322)
Staphylococcus aureus (X68417)
Bacillus smithii (AY373319)
Caryophanon latum (AJ491302)
Listeria welshimeri (AJ535694)
Sporolactobacillus nakayamae (AJ698860)
Lactobacillus gasseri (DQ317605)
Pa19
Pm10
SDE2
SD5
Alicyclobacillaceae
Alicyclobacillus acidocaldarius (AY573796)
Sulfobacillus disulfidooxidans (AB089843)
Thermoterrabacterium ferrireducens (U76363)
Thermoanaerobacterium aotearoense (X93359)
Syntrophomonas flectens (AY290767)
Pelotomaculum isophthalicum (AB232785)
Desulfotomaculum geothermicum (Y11567)
KB46 †
SDE17 †
SDE13 †
Sulfobacillus sp. GG6/1 (AY140234)
Sulfobacillus thermosulfidooxidans (AY140233)
KB22 †
SD4 †
KB23 †
"Sulfobacillus-like"
"Firmicutes"*
pCBSB206-15
pCBSB206-14
pCBSb2-10
pCBSB206-17
Clostridium hiranonis (AB023970)
Eubacterium nodatum (Z36274)
Lachnospira pectinoschiza (AY699283)
Clostridium indolis (Y18184)
Halanaerobium aceloethylicum (X89071)
Rubrobacter radiotolerans (X98372)
Rickettsia aeschlimanni (U74757)
pCBPa0405-38 †
pCBSb2-14 †
pCBPa0405-16 †
pCBPa0405-39 †
pCBPa0405-28 †
pCBPa0405-31 †
pCBPa0405-17 †
pCBPa0405-11 †
pCBPa0405-44 †
pCBPa0405-5 †
pCBPa0405-2 †
pCBPa0405-43 †
pCBPa0405-3 †
pCBPa0405-7 †
OB Group II
pCBPm1c-59 †
pCBPm2b11 †
pCBPm1c-16 †
pCBPm1c-19 †
Sphaerobacter thermophilus (AJ420142)
Herpetosiphon geysericola (AF039293)
Roseiflexus castenholzii (AB041226)
"Chlorofexi"
Chloroflexus aurantiacus (AJ308501)
pCBPa0405-33 †
pCBPa0405-23 †
pCBPa0405-24 †
pCBPa0405-41 †
pCBPa0405-32 †
pCBPa0405-42 †
OB Group III
pCBPa0405-40 †
pCBPa0405-9 †
Salinibacter sp. 2Mb2 (AY987848)
Rhodothermus clarus (AB252420)
Thermonema rossianum (Y08956)
Uncultured Haliscomenobacter sp. clone SBRT303 (AF368190)
Chitinophaga sp. CC-SG1B (DQ062743)
Cytophaga arvensicola (AM237311)
Uncultured Lewinella sp. clone BJS81-142 (AB239037)
Saprospira grandis (AY527408)
Sporocytophaga myxococcoides (AJ310654)
Runella zeae (AF137381)
Flectobacillus speluncae (AY065626)
Microscilla sericea (AB078082)
Cyclobacterium marinum (AJ575266)
Pedobacter africanus (AJ438171)
pCBSB206-38 2
Sphingobacterium spiritivorum (AJ459411)
Anaerophaga sp. HS1 (DQ517535)
Dysgonomonas gadei (Y18530)
Bergeyella zoohelcum (AY827896)
Chryseobacterium joosteii (AJ271010)
Riemerella columbina (AF181448)
Blattabacterium relictus (AY631423)
Ornithobacterium rhinotracheale (U87105)
Coenonia anatina (AF118419)
Capnocytophaga haemolytica (X97247)
Salegentibacter flavus (AY682200)
Muricauda ruestringensis (AF218782)
Myroides odoratimimus (AJ854059)
Flavobacterium aquatile (AM230485)
Psychroserpens burtonensis (U62913)
Tenacibaculum mesophilum (AB032501)
Polaribacter filamentus (U73726)
Gelidibacter mesophilus (AJ344133)
Aequorivita ferruginea (AY027802)
Zobellia amurskyensis (AB121974)
Flexibacter aggregans (DQ514301)
Arenibacter latericius (AF052742)
Cytophaga fucicola (AJ005973)
Psychroflexus torquis (U85881)
Prevotella bryantii (AJ006457)
Bacteroides acidofaciens (AB021164)
"Bacteroidetes"
pCBPm2b17 †
pCBPm1c-90 †
Uncultured Gloeobacter sp. clone Sc65 (DQ058849)
Prochlorothrix hollandica (AJ007907)
Prochlorococcus marinus (AF311217)
Cylindrospermum sp. PCC 7417 (AJ133163)
Nodularia spumigena (AB039002)
Nostoc linckia (AB074503)
Cylindrospermopsis raciborskii (AB115466)
Anabaena cylindrica (AF247592)
Microcoleus glaciei (AF218374)
Oscillatoria sancta (AF132933)
Lyngbya sp. PCC 7419 (AJ000714)
Spirulina platensis (AB074508)
Arthrospira platensis PCC 9223 (DQ393285)
Chroococcidiopsis thermalis (AB039005)
Dactylococcopsis sp. PCC 8305 (AJ000711)
Cyanothece sp. PCC 7418 (AF296872)
Pleurocapsa sp. (X78681)
Xenococcus sp. PCC 7307 (AB074510)
Dermocarpella incrassata (AJ344559)
pCBPm1c-28
Synechococcus elongatus CCMP1630 (AY946243)
Cyanobacteria
pCBSB206-50 2
pCBSB206-56 2
pCBSB206-29 2
pCBPm1c-39
Metal-contaminated soil clone K20-12 (AF145815)
TM7
Dehalococcoides sp. JN18_A30_B (DQ168646)
Uncultured candidate division BRC1 bacterium clone LD1-PA21 (AY114315)
Acidithiobacillus ferrooxidans NO37 (AF376020)
Alcaligenes defragrans (AJ005447)
Criblamydia sequanensis (DQ124300)
Simkania negevensis (U68460)
Parachlamydia sp. Hall's coccus (AF366365)
Pirellula staleyi (AF399914)
Planctomyces brasiliensis (AJ231190)
pCBPa0405-10 †
pCBPm1c-14 †
pCBPm2b7 †
pCBPm1c-8 †
Gemmata obscuriglobus (X56305)
pCBPm1c-81
pCBCR2-34
pCBPm1c-71
pCBPm1c-32
pCBPm1c-40
pCBPm1c-25
pCBPm1c-64
"Planctomycetales"
0.1

8.3.5 Clone library richness estimates

The number of observed OTUs, estimated total OTUs and reliability of this estimate for each clone library, are summarised in Table 8.1. Only the CR2 (Cwm Rheidol spoil) and Pm1c (Parc mine tailings sample obtained in June 2003) clone libraries produced stable total OTU estimates. Therefore, it is not possible to comment on how well the other clone libraries represented biodiversity within their relative samples. However, if they were too small to allow for a stable richness estimate, then they were most likely too small to adequately represent OTU richness.

The total richness estimate for the Cwm Rheidol library was 68 OTUs, which implies 47% of the total OTUs were identified in this sample. The total richness estimate for the Parc mine Pm1c clone library was 146 OTUs, which implies that 32%. of the total OTUs from this sample were identified. Total richness estimates will be underestimated when based on libraries that are too small (Kemp and Aller, 2004). Therefore, the implication is that the total OTU estimates for the Shilbottle SB206 and the Mynydd Parys Pa0405 libraries were minimum estimates, and so the clone library analysis revealed less than 15% and 23% of the total OTUs, respectively.

Table 8.1 Observed and estimated richness values, defined by the number of OTUs, for each clone library from each site.

Clone library[a]	Total clones[b]	Observed OTUs[c]	Estimated total OTUs (S_{chao1} value)	Stable estimate	OTUs detected[d]
CR2	57	32	68	Yes	47%
Pa1[e]	6	5	8	No	63%
Pa1b	17	13	28	No	46%
Pa0405	43	39	255	No	15%
Pm2b	14	12	28	No[f]	43%
Pm1c	54	47	146	Yes	32%
Sb2	19	13	42	No	31%
SB206	53	40	175	No	23%

[a]CR = Cwm Rheidol, Pa = Mynydd Parys, Pm = Parc mine, Sb = Shilbottle Brass Heap; [b]Excluding Chimerae; [c]Differentiated on the basis of RFLP analysis; [d]The number of OTUs detected as a percentage of the total estimate; [f]The results of this clone library were not included in the original analysis as the spoil had been stored at 4°C for several weeks prior to DNA extraction. [f]Reached an asymptote, but library deemed too small to give a reliable estimate.

8.3.6 Site biodiversity

Microbial complexity is defined as the number of OTUs obtained from a sample, whereas phylogenetic diversity is the total phylogenetic distances between these OTUs. Biodiversity reflects the number of different phylogenetic groups formed by the OTUs obtained from a sample. A qualitative assessment of site biodiversity was based on the formation of phylogenetic groups by OTUs from each sample. These groups were based on the phylogenetic analyses carried out, but did not necessarily represent whole genera or families. These groups could be further classified by whether or not the closest relatives of any of the OTUs within the group had been obtained from sulfide mineral- or mining-impacted environments. Table 8.2 shows the occurrence of these phylogenetic groups at each site. It is important to differentiate between the biodiversity observed in a sample and the level of complexity, or OTU-richness, given in Table 8.1. In this table, based on richness estimates, Mynydd Parys appears to be the most complex site studied. However, many of the Mynydd Parys OTUs formed moderately interrelated phylogenetic clusters and so site biodiversity, although still high, was not as high as perhaps initially suggested. Biodiversity, as implied here, is based on a subjective, qualitative assessment of the phylogenetic profiles of each site.

Parc mine appeared to have the greatest biodiversity, and was the least likely to contain OTUs related to organisms previously obtained from sulfide mineral- or mine-impacted environments. It was also the least likely to contain OTUs that grouped with organisms from the other sites studied. Shilbottle and Mynydd Parys appeared to have quite similar levels of biodiversity, although there was not a great deal of overlap between the groups detected at each site. Cwm Rheidol had less biodiversity, and most of the groups detected at this site were also detected at Mynydd Parys. Cae Coch, São Domingos and Kennecott had the least biodiversity, although of the three, biodiversity was greatest at Kennecott. Cae Coch was represented in just two groups of OTUs. Every group detected at São Domingos (including the archaeon *Ferroplasma acidiphilum*, not shown) except the iron-oxidising

Alicyclobacillaceae isolate were also detected at Kennecott. The acidithiobacilli were the only group detected at every site.

Table 8.2 Site biodiversity, indicating the distribution of organisms belonging to groups that are associated with mine-impacted or sulfide mineral-impacted environments.

Site	"Acidobacteria"			Actinobacteria																
				Actinobacteridae									*Acidimicrobidae*				*Rubrobacteridae*			
													TM Group III	TM Group II	†	"TM Group IV"	TM Group I		misc.	
CC																				
CR	+		+											+		+				
KB															+					
Pa	+	+										+		+		+	+			
Pm					+	+	+	+	+	+	+			+				+	+	
SD																				
SB				+									+	+	+	+	+			+

Site	*Proteobacteria*													
	γ	β/γ	*Xanthomonodales*			β			α			δ	ε	misc.
	U-mine waste group†	acidithiobacilli†	WJ2-group†	*Frateuria*-group	misc.				*Acetobacteraceae*†	misc.				
CC		+												
CR	+	+	+						+			+		
KB	+	+							+					
Pa	+	+	+			+			+			+		+
Pm		+			+	+	+	+	+	+	+		+	
SD		+												
SB		+	+	+	+					+	+			

Site	Other *Bacteria*																						
	Nitrospira	"*Verrucomicrobia*"	"OB Group I"	*Gemmatimonadates*	"*Firmicutes*"				"OB Group II"	"OB Group III"	"*Bacteroidetes*"	*Cyanobacteria*	TM7	"*Planctomycetales*"		misc.							
	†				*Alicyclobacillaceae*†	*Sulfobacillus*-like†	misc.																
CC	+																						
CR			+												+								
KB						+																	
Pa	+		+		+				+	+				+				+					+
Pm		+		+	+							+	+	+	+				+	+	+	+	
SD					+	+																	
SB			+				+	+	+		+		+			+	+						

Gray-shaded groups are related to organisms associated with mine-impacted or sulfide mineral-impacted environments; Green-shaded groups represent novel lineages that have no close relatives in GenBank; †Group contains organisms known to accelerate mineral dissolution via the oxidation of iron and/or sulfur; CC, Cae Coch; CR, Cwm Rheidol; KB, Kennecott Bingham Canyon Bioleach Heap; Pa, Mynydd Parys; Pm, Parc mine; SD, São Domingos; SB, Shilbottle Brass Heap.

8.4 DISCUSSION

These analyses show that there is a high degree of phylogenetic diversity within, and between, the sites. As many of the clone libraries were too small to fully represent the true richness of a sample, and no molecular ecology data were obtained from the Kennecott Bingham Canyon or São Domingos samples, quantitative evaluation of diversity between, or even within, sites could not be made. However, a qualitative assessment of the overall results is presented.

The distribution of the isolated organisms between just a few monophyletic groups clearly indicates the limitations of cultivation-based methods in elucidating microbial population complexity. The isolates formed three groups within the *Proteobacteria*, a single group within the "*Acidobacteria*", and two groups within the "*Firmicutes*". Many of these groups, such as the *Acidithiobacillus* isolates, the *Acidiphilium*-like isolates and the iron-oxidising "*Firmicutes*", are often reported in studies of acid mine drainage and biomining operations, and many are well-studied as a result. This demonstrates that a reliance on just cultivation methods alone would, on the whole, lead to the erroneous conclusion that the microbial populations of the abandoned and often weathered mine wastes in this study are almost identical to those found in other acid mine environments, and to each other.

The organisms isolated during this study were rarely represented in the clone libraries; the only exception was the "*Acidobacteria*". This is somewhat ironic as the "*Acidobacteria*" are typically grossly under-represented in culture-based analyses, and are difficult to isolate using conventional media. Yet, most of the isolates appeared to be fairly closely related to most of the clones, forming a single monophyletic group within group 1. The majority of the RCP clones also belong to this group, as does *Ab. capsulatum*. This could suggest that acidophily within the "*Acidobacteria*" may be limited to this group.

Most of the clones from the Parc mine tailings samples, and many of those from the Shilbottle spoil heap were well distributed throughout the *Bacteria* kingdom. On the other hand, clones from the Mynydd Parys spoil had a tendency to form very distinct monophyletic clusters, some of which included clones from the Cwm Rheidol tailings, and less frequently with those

from Shilbottle. The Cwm Rheidol clone library was the least complex, and was the most representative of the total estimated richness (see Table 8.1). Where these clones formed monophyletic clusters, this was always with clones from Mynydd Parys. This implies that these two sites are the most similar in terms of the composition of their microflora, although Mynydd Parys had far greater overall complexity and biodiversity. In other words, the majority of organisms observed in the Cwm Rheidol spoil were also found at Mynydd Parys.

The Parc mine spoil was, in terms of *p*H, the least extreme environment of all those examined, and this may be the reason for the greater biodiversity observed at this site. The most extreme sites in terms of *p*H, Cae Coch, Kennecott and São Domingos, had the lowest biodiversity, suggesting that *p*H may have the greatest effect on site biodiversity. It appears unlikely that concentrations of readily soluble metals had such a great effect as concentrations at Kennecott, Shilbottle and Parc were quite similar, yet biodiversity was highly variable between these sites.

Analysis of the Mynydd Parys clones was interesting in that many of these could not be classified taxonomically, and many formed distinct monophyletic clusters. These clusters appeared entirely novel, and not strongly associated with any characterised taxonomic divisions. These groups (Figure 8.4) were most closely related to clones obtained from a study of microbial succession in volcanic deposits on a Hawaiian island (Gomez-Alvarez and Nuesslein, unpublished), although sequence identity ranged from less than 80% up to approximately 95%. Therefore, it may be that these organisms are unique to environments which are in the stages of transition from sulfide mineral spoil to soil. The depth at which these groups branch in the phylogenetic tree suggests that they diverged a relatively long time ago and perhaps represent ancient divisions of organisms responsible for the early colonisation of the planet. However, it must be noted that the length of the sequences used in the phylogenetic analysis (<700 nt) may preclude accurate positioning of these groups, especially as they are so distantly related to taxonomically characterised organisms.

Many of the clone libraries were too small to allow an accurate estimate of total richness, let alone whether this richness had been adequately

sampled. As Kemp and Aller (2004) suggest, future clone libraries should be analysed during the selection of clones. Sufficient OTUs should be determined to allow a stable total estimate. It would then be possible to make an informed decision about how many more OTUs need to be obtained in total so as to ensure the clone library is truly representative of richness within the sample. Exhaustive sampling of total OTU richness from each site, and the determination of nearly complete 16S rRNA gene sequences would allow quantitative comparisons of biodiversity within and between samples, and would also allow more accurate placing of OTUs within the phylogenetic tree.

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

The microbial oxidation of exposed sulfide minerals is one of the most important components of the global iron and sulfur cycles. For the past two centuries at least, the most common route for the exposure of sulfide minerals has been as a result of extractive process, such as coal and metal mining. The microbially-accelerated oxidative dissolution of these minerals has important implications for industry and the environment. On the one hand, the uncontrolled and unmanaged release of the highly polluting mine drainage waters has a serious and detrimental effect on the environment. On the other hand, the efficiency of the microbial processes involved makes them an attractive alternative to pyrometallurgical processing for extracting metals from low-grade sulfide ores. Importantly, the wastes produced by these biomining operations pose less of an environmental hazard than those produced using conventional technologies. As sources of high-grade ores are becoming increasingly scarce, the processing of low-grade ores to meet growing demands for raw metals is increasingly a necessity. Additionally, the mining industry is coming under increasing legislative and public pressure to reduce its effect on the environment. Reprocessing polluting waste heaps with biomining technology could lessen their environmental impact whilst providing an additional source of metals that would otherwise be lost. Such a scenario is the case with the Kasese biomining operation in Uganda. This plant processes a huge tailings stockpile for the recovery of cobalt (as discussed in section 1.2).

Improvement in biomining technologies, particularly with regard to reprocessing of mines wastes, is highly desirable. Critical to this is a better understanding of the microbial communities that are associated with sulfide mineral dissolution. While the microbial populations associated with acid mine drainage are relatively well studied, those of unmanaged mine wastes are not. The aim of this research project was to address this; by better understanding the microbial populations of mine wastes it should be possible to better understand aspects of AMD genesis in mine waste heaps, such as the longevity of the problem, and the effects of climate and mineralogy. Another

potential benefit is that novel microorganisms that are of potential importance to the biomining industry could be identified.

In this context, materials from seven mine sites from locations in the U.K., mainland Europe and the U.S.A. were investigated. These provided a wide diversity in terms of age of the heaps, mineralogy and climate, as illustrated in Table 9.1. At six of these sites, solid samples were analysed. In the case of Cae Coch, it was possible to take liquid samples directly from the mineral (pyrite) surface, which facilitated the identification of those microorganisms that were directly involved with the oxidative dissolution of pyrite at that particular site. With the exception of the Kennecott Bingham Canyon mine, all of these mines are currently abandoned. However, the bioleach heap at Kennecott has itself been inactive for six years.

Table 9.1 Summary of geochemical data obtained from the sites sampled.

Site	Age[a] (years)	*p*H[b]	Readily extractable metals (μg g^{-1})			Total extractable metals (μg g^{-1})		Total bioleached[c]	
			Fe	Cu	Zn	Fe	Cu	Fe	Cu
Cwm Rheidol spoil	~90	3.40	65.85	1.52	7.93	n/d	n/d	n/d	n/d
Mynydd Parys spoil	>120	3.04	369	8.86	3.07	n/d	n/d	n/d	n/d
Parc mine tailings	50-100	5.38	1,017	23.1	1,697	n/d	n/d	n/d	n/d
Shilbottle Brass Heap spoil	20-80	3.66	1,237	3.51	7.81	n/d	n/d	n/d	n/d
Kennecott bioleach heap ore	~11	2.82	1,405	130	n/d	56,033	2,800	55%	30%
São Domingos spoil	~50	2.06	15,190	450	690	104,000	1240	-[d]	-[d]
São Domingos tailings	~50	2.29	2,630	5,650	240	192,400	8,820	97%	74%
Cae Coch; pyrite liquor	~85[e]	2.00	31,535[f]	37[f]	118[f]	N/A	N/A	N/A	N/A

[a]Approximate age of the heap sampled, based on information about its deposition or the working lifespan of the mine. [b]1:2.5 sample to RO water. [c]Total metal leached by the most effective bioleaching culture, as a % of total extractable metal concentrations; [d]This culture was ineffective at bioleaching, and final metal concentrations differed little from readily extractable concentrations; [e]The time this mine was last worked; [f]mg L^{-1}; n/d, no data.

As biomolecular data were obtained from enrichment cultures from the São Domingos and Kennecott samples, but not directly from the spoil materials, it is difficult to make like-for-like comparisons between all the sites

studied. However, based on the data obtained, some general comparisons can be made.

Of the solid materials, the São Domingos site was the most extreme, in terms of acidity, readily extractable metal concentrations and seasonal variations in temperature and rainfall, and the microbiology reflected this. The indigenous microflora appeared to be of limited complexity and did not change during bioleaching. In contrast, the microbial population of the Kennecott heap was more diverse and more dynamic in response to changing conditions, as could be seen following the enrichment and bioleaching processes. The biodiversity was considered to be greater with the Kennecott ore due to the slightly less extreme conditions: higher *p*H, cooler temperatures and the previous irrigation with inorganic nutrients. However, both of these sites were characterised by the dominance of iron- and sulfur-oxidising "*Firmicutes*", and the occurrence of *Ferroplasma acidiphilum*-like *Archaea*.

In contrast, the Cwm Rheidol and Mynydd Parys samples were much more weathered, not as acidic, contained lower concentrations of readily extractable metals and, being located in a temperate climate, were less affected by drought or high ambient temperature and other seasonal affects. Subsequently, they provided more stable conditions for the microbiota and the biodiversity of both sites was much greater than that found at São Domingos or Kennecott. The Cwm Rheidol and Mynydd Parys spoil samples were also the most similar of all the materials worked with, in terms of geochemistry and microbiology, although the Mynydd Parys population was generally much more complex. This is interesting as the Mynydd Parys samples had, on average, the highest concentrations of readily extractable metals and the lowest *p*H of the two. However, the greater complexity of the Mynydd Parys samples is probably due to the greater age of the spoil heap.

The Shilbottle spoil and Parc mine tailings heaps were unique in that they had been subjected to extensive reclamation, and were associated with a significant macrobiological population in the form of plants, trees and possibly other organisms. Microbial biodiversity was greatest at these two sites of all those examined, and this probably reflected the impact of the macrobiota and subsequent influx of exogenous organic carbon. However, it was interesting that *At. ferrooxidans*-like organisms appeared to be the dominant known

mineral-oxidising microorganisms at both sites. It may be that there are sufficient quantities of sulfide minerals at these two sites to allow the establishment of these autotrophs. At the same time, acidophilic heterotrophs, such as WJ2, may benefit from the additional organic carbon at Shilbottle, but are possibly unable to compete with larger populations of neutrophilic heterotrophs that may be present at the Parc site. While it is likely that these iron- and sulfur-oxidising microorganisms occupied, and dominated, their own microniches within the environment, as the characterisation of isolate Pa33 has shown, it is probable that iron-oxidation is more widespread throughout the *Bacteria* than previously considered. Therefore, the contribution of novel, as yet undescribed, mineral-oxidising organisms cannot be discounted.

The level of biodiversity observed at each site was, in general, most congruent with the *p*H data obtained. Biodiversity was lowest in the most acidic materials, and appeared to increase with increasing *p*H. This implies that the *p*H of the waste is the biggest single limiting factor to biodiversity. Plant-microorganism interactions and the influx of organic carbon also appear to have a substantial effect on indigenous microbial populations, allowing the establishment of microorganisms that may otherwise be precluded from such environments. Ore type, and concentrations of readily extractable (and, by inference, more bioavailable) metals did not appear to have so great an effect. It is difficult to say what affect climate may have on the microbial populations as the two non-temperate sites were highly acidic, and generally very different to the other sites studied. It is likely that the conditions of relatively low acidity and readily extractable metal concentrations observed at Cwm Rheidol and Mynydd Parys are a result of depleted sources of sulfide minerals, rather than the result of a less active mineral-oxidising population.

The results of this study suggest that Iron- and sulfur-oxidising "*Firmicutes*" are very important members of the microbial population in non-irrigated systems. Their adaptability, flexible metabolism and ability to form highly resistant endospores probably allows them to survive in conditions that are prone to fluctuations, e.g. in moisture contents, which more adversely impact Gram-negative mineral-oxidisers such as *At. ferrooxidans* and *Leptospirillum* spp.. However, they appear unable to compete with these organisms where conditions are more homogeneous and abundant in water,

such as was observed in the Kennecott pyrite enrichment culture, and the Cae Coch droplets. Therefore, it seems that the effects of extreme *p*H in limiting biodiversity at the São Domingos and Kennecott samples was compounded by their typically arid nature. In the less extreme, well-weathered sites, moderately acidophilic heterotrophs, some of which also oxidise iron and/or sulfur, were seen to be more dominant. This represents a gradual transition away from dependence on sulfide mineral-based metabolism towards heterotrophy as a site ages. Despite this, extremely acidophilic mineral-oxidising acidophiles were isolated from all of the sites studied. As such, providing there are sufficient quantities of sulfide minerals remaining, enhanced mineral-oxidation could be achieved by stimulation of this section of the microbial population, as these are likely to be more efficient mineral-oxidising organisms.

The range of solid media used in this study proved to be effective for isolating a broad spectrum of acidophiles. These included commonly reported autotrophs such as *Leptospirillum ferrooxidans*, *Acidithiobacillus ferrooxidans* and *At. thiooxidans* as well as iron- and sulfur-oxidising *Alicyclobacillaceae* and *Sulfobacillus*-like organisms. Several heterotrophic prokaryotes, including *Ferroplasma acidiphilum*-like *Archaea* were readily isolated. Additionally, rarely cultured organisms such as *Acidobacterium* spp., and the iron-oxidising isolate m-1 and hitherto uncultured organisms, such as the iron-oxidising *Rubrobacterales* isolate Pa33 were successfully isolated and grown *in vitro*. However, the media used supported the growth of a small fraction of the total population only and much of the microbial biodiversity was not elucidated. The microbial populations studied might have included substantial numbers of neutrophiles, for which the media used are not appropriate. Given that most of organisms grow in close association with the solid phase rather than as planktonic individuals, there may be issues associated with the successful detachment of viable cells from the matrix such as the loss of cell viability during the process, or the more ready detachment of some organisms than others.

PCR-based methods were able to reveal a much greater level of biodiversity in many of the samples. The extraction of DNA directly from the solid-phase eliminated the need to detach the microorganisms from the

sample matrix. However, PCR-based methods are subject to several problems. Each step in community analysis is open to error or bias, such as cell lysis, DNA extraction and purification, choice of primers, PCR conditions, and cloning (Farrelly *et al.*, 1995). Fundamentally, PCR can only be classed as a semi-quantitative method as significant bias can be introduced as a result of differing 16S rRNA gene copy numbers in individual microbial species. This would affect the observed relative abundances of individual organisms or groups of organisms (Farrelly *et al.*, 1995). The limit of PCR resolution is considered to be around 1% of the total population (Forney *et al.*, 2004), which is in agreement with the empirical evidence presented from this study. This is compounded by the often low numbers of organisms in the samples studied, and other aspects such as soluble metal concentrations and acidity. This meant that there was often a low concentration of DNA template in the DNA extracts, and that PCR was sometimes inhibited altogether. Therefore it is necessary to develop a DNA extraction technique which works equally well with all the sample types studied, dealing with low cell numbers and other inhibitory agents.

Molecular profiling methods, such as T-RFLP and denaturing gradient gel electrophoresis (DGGE), are commonly used tools in the elucidation of microbial assemblages. T-RFLP has been shown to give slightly higher resolution than DGGE and detects more OTUs, while providing consistent results. It also has the advantage that it is relatively rapid (Moeseneder *et al.*, 1999; Osborn *et al.*, 2000). However, the available T-RFLP database was limited in its applicability to this project as this largely comprised acidophilic organisms cultured by members of the *Bangor Acidophile Research Team* at UWB. These particular prokaryotes were mostly not found as dominant members of the mine spoil microbial communities. Therefore, many of the T-RFs could not be identified in the profiles that were obtained, certainly not above the genus or even class level. Given the unexpected complexity of many of these sites, and the applicability of statistical analyses to data obtained by T-RFLP, future experimental regimes should be designed to allow statistical comparisons of different microbial communities. In general, T-RFLP performed very well in depicting overall population complexity in the samples studies, rather than identifying dominant OTUs. Such designs will have to

account for temporal and spatial variation within a site, and carefully monitor variables such as climate, nutrient availability, *p*H, predominant mineral type, and age of the material studied. However, it is still necessary to determine the major metabolic processes occurring in different environments and how these may affect sulfide mineral dissolution.

The use of whole-cell *in situ* probing methods, such as FISH, would provide the most quantifiable assessment of the microbial compositions of the sites studied. However, attempts to use the DNA-staining dye DAPI were often unsuccessful, presumably due to the high concentrations of metals and acidity and (often) high background fluorescence. These attempts also used only the cell suspensions obtained from the solid materials. However, these problems would likely be overcome with improvements in experimental protocols, and should allow the probing of cells *in situ*, without the need to detach them from the matrix. The utilisation of enhanced methods such as CARD-FISH has been successfully demonstrated on similar materials (Demergasso *et al.*, 2005). However, the development of probes specific to certain groups of organisms requires extensive DNA sequence datasets of the organisms likely to be in the samples studied. The utilisation of any new probes also involves substantial adaptation and optimisation of experimental protocols to ensure probe specificity and maximum detection.

9.1 GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Mine waste heaps are highly heterogeneous environments, and conditions vary widely from site to site. The *p*H of mine spoil and tailings is probably the biggest factor in determining their microbial biodiversities, while heap age and availability of water are also important. However, while the most extreme conditions may cause indigenous microbial communities to be restricted in composition, these populations can still be capable of extensive mineral oxidation.

Mineral-oxidising populations were present at every site examined, but the size and metabolic capacities of these populations varied greatly and was probably determined by *p*H and the relative abundance of sulfide minerals. Biodiversity was enhanced as a result of plant-microbial interactions, but this

did not appear to eliminate extremely acidophilic mineral-oxidising bacteria. In most cases only a fraction of the total microbial population was isolated with the media used. The majority of organisms detected by biomolecular methods could not be identified, and very little could be inferred about their metabolic activities *in situ*.

Superficial re-grading/reclamation probably does little to protect the environment by reducing AMD-genesis. However, mineral oxidation is likely to be very protracted, possibly lasting for hundreds of years, in many unmanaged mineral mine spoil waste heaps and mineral tailings. Therefore, it may well be more desirable to accelerate the process, and to remediate the AMD produced in a relatively short timeframe. As mineral-oxidising extremophiles were isolated from every site, in varying numbers and relative abundances, these organisms could probably be augmented to accelerate the mineral oxidation process.

The combination of culture-based and molecular techniques is essential to elucidate not just the complexity of microbial populations and how these vary in different conditions, but also what their different roles are.

This research project has highlighted several areas that warrant further investigation.

- It is important to develop a universal method of sample processing and DNA extraction that allows the molecular profiling of populations *in situ* rather than relying on the use of enrichment cultures.
- This will allow the statistical comparison of sites through the use of profiling methods such as T-RFLP, and the analysis of sufficiently large 16S rRNA gene clone libraries.
- More sites need to be studied, and these should include the study of similar heaps in different climates. Sites need to be sampled more intensively to take into account the heterogeneity, and so allow more robust conclusions to be drawn.
- The development of different media may allow the isolation of groups of organisms that have been shown to be common to many sites, and so elucidate their metabolic capacities. Of particular interest is the distribution of iron- and/or sulfur-oxidising organisms, especially within the *Actinobacteria* phylum and the roles of the novel lineages discovered.
- Further characterisation of novel and interesting isolates should be undertaken to elucidate their roles in the environment and their potential for use in biomining processes. This includes the iron-oxidising acidophile m-1-like organism and the moderately thermophilic *Acidisphaera*-like heterotroph isolated from Kennecott, the novel "*Firmicutes*" isolated from both Kennecott and São Domingos and the potentially sulfur-oxidising *Acidobacterium* isolate from Cwm Rheidol.
- The adaptation of current FISH protocols to study microorganisms *in situ* will overcome the issues of DNA extraction and PCR-bias, and the limitations of culture-based methods. This will require the development of an extensive sequence database in order to develop probes for the relevant groups of organisms.

Thus, in revisiting the aims of the project, the following outcomes become apparent:

Aim:	Outcome:
1. To identify a range of accessible sites that varied in terms of mineralogy, age, climate and geographic location;	Seven mine sites identified and sampled, which varied in terms of mineralogy, age, climate and location. However, a wider range needs to be studied.
2. To ascertain whether the sites were still active in terms of sulfide mineral oxidation;	Every site studied presented elevated concentrations of readily extractable metals, indicative of continuing mineral oxidation. Known and presumed mineral-oxidising microorganisms were detected at every site, albeit in varying numbers, confirming the microbiological potential for mineral oxidation.
3. To determine the biodiversity of metal-mobilising and other acidophilic microorganisms in spoil and tailings samples, using a combined cultivation-based and cultivation-independent approach;	The elucidation of biodiversity was good when these approaches were combined, but poor when used in isolation. However, larger clone libraries need to be employed, better media need to be developed and a robust sampling method should be used.
4. To examine the possibilities of accelerating sulfide mineral dissolution in spoil and tailings samples by stimulating indigenous microflora;	Extensive mineral oxidation was shown by the São Domingos and Kennecott microbial populations in shake flasks. The availability of inorganic nutrients appeared to be the limiting factor. Biostimulation of identified and unknown mineral-oxidising microorganisms detected at all the sites should therefore be possible.
5. To construct a working hypothesis on the evolution of acidophilic microbial populations in abandoned mine sites;	Microbial population size and diversity is primarily affected by *p*H, and aridity. In general, biodiversity increases with the age of the waste. The availability of inorganic nutrients may limit microbial activity *in situ*. Mineral-oxidising microorganisms can not be entirely displaced by the introduction of a heterotrophic population, and are ubiquitous in all sulfide mineral-impacted environments.

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