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Acidophilic consortia and microbial interactions involved in securing mineral wastes and remediating mine waters

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Acidophilic consortia and microbial interactions involved in securing mineral wastes and remediating mine waters

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Abstract

Metal mining produces large volumes of unwanted materials that are dumped as waste rock or stored as fine-grain tailings. Both of these pose a potential environmental hazard, mostly associated with the microbiological oxidation of residual sulfide minerals (principally pyrite; FeS_2) in these waste materials which, in the absence of neutralising minerals or ongoing application of alkaline chemicals, results in the production of acidic mine drainage (AMD) waters. These are typically acidic, sometime extremely so, and contain elevated concentrations of iron and other soluble metals, metalloids (such as arsenic) and sulfate. The United States *Environmental Protection Agency*, categorises water contamination from mining as one of the top three ecological-security threats in the world. However, metal mining remains a basic industry underpinning economic growth and development, and so the question of how to minimise its environmental impact is perceived as an urgent issue.

In the majority of cases mine-impacted waters are conventionally remediated either using active chemical (aeration and lime addition) or passive biological (wetlands or compost bioreactors) treatment. Both of these approaches have major detractions including: (i) metals present in acid mine drainage are not recovered, and (ii) the mixed-metal sludges or metal-enriched spent composts generated need to be carefully disposed (usually in specially-designated landfill sites), and managed to prevent re-mobilisation of the metals and new cycles of environmental pollution. The concept of harnessing natural biological processes and systems to remediate AMD underpins passive bioreactors and wetland systems, and there are numerous examples of "natural attenuation" of mine waters, though in most cases these result in improved mine water chemistry though not to regulatory discharge levels. The current project has sought to understand the potential roles of acidophilic prokaryotic and eukaryotic microorganisms, in terms of their diversities and the interactions that occur between them, and to apply this knowledge to devise new approaches for securing mine wastes and remediating mine waters.

Extremely acidic habitats are inhabited by a range of microorganisms with contrasting metabolic life-styles. These include chemoautotrophic bacteria and archaea that catalyze the dissimilatory oxidation of iron, sulfur, and hydrogen, heterotrophic acidophilic bacteria, many of which can catalyse the dissimilatory reduction of ferric iron while some reduce sulfate (SRB), and phototrophic micro-algae. While chemolithotrophs are known to have a major role in AMD genesis,

heterotrophic prokaryotes and phototrophic eukaryotes can play important roles in mitigating AMD pollution.

While some heterotrophic acidophiles can generate alkalinity and immobilise metals, they require organic carbon to do this. Studies of the nature of organic materials produced by primary producers in extremely acidic environments (phototrophs and chemolithotrophs) were therefore carried out. *Chlorella protothecoides* var. *acidicola* and *Euglena mutabilis*, both isolated from abandoned copper mines were found to exude monosaccharides (glucose and fructose by the *Chlorella* isolate, and mannitol and glucose by the *Euglena* isolate). These exudates were shown to sustain the growth of iron-reducing heterotrophic bacteria commonly found in AMD. In contrast, three species of chemoautotrophic acidophiles (*Acidithiobacillus (At.) ferrooxidans*, *At. caldus* and *Leptospirillum ferriphilum*) were shown to secrete glycolic acid into their media. The ability to metabolize this compound was very restricted among acidophilic prokaryotes, with only species of *Firmicutes* (many of which catalyse both iron oxidation and iron reduction) being capable of utilising glycolic acid in pure culture.

Two continuous-flow bioreactors containing consortia of novel acidophilic SRB were established and tested over ~1 year for the selective precipitation of copper and zinc (as sulfide minerals) when fed with synthetic acidic mine waters containing mixtures of several transition metals and aluminium. The results showed selective precipitation of Zn (at pH 4.0) from a synthetic AMD containing Fe, Al and Zn and selective precipitation of Cu (at pH 2.2) from another synthetic AMD that contained Fe, Zn, Mn, Al and Cu. These experiments demonstrated the potential of pH-controlled microbial sulfidogenesis for the selective recovery (and recycling) of metals typically present in elevated concentrations in acidic mine drainage waters.

Finally, a mesocosm experiment was set up using pyritic mine tailings and test material and various combinations of microbial inocula. The results showed that that iron- and sulfate-reducing bacteria, sustained by organic carbon provided by acidophilic micro-algae, had significant impact on acid-generation and metal mobilisation in the tailings. This part of the study showed the importance of phototrophic algae in supporting the growth of "benign" bacteria that can help to minimize the oxidation of sulfide minerals and the consequent generation of acidity and solubilisation of transition metals, providing a long-term self-sustainable system.

Overall, the results of this study highlighted the importance of the diversity and interactions of acidophilic microbial consortia, and these might be used to develop new ecological and biotechnological approaches for reducing the generation of AMD from abandoned mines and to remediate mine waters.

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Abbreviations

16 S RNA	16S (small) ribosomal ribonucleic acid subunit
AAS	Atomic absorption spectrophotometry
AMD	Acid mine drainage
ARD	Acid rock drainage
BLAST	Basic logical alignment search tool
Bp	Base pair
BS	Plate basal salts
cfu	Colony forming units
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EPA	Environmental Protection Agency (U.S.A.)
EPS	Extracelullar polymeric substances
Fe	Iron (liquid medium)
Feo	Iron overlay (solid medium)
HBS	Heterotrophic basal salts
IC	Ion chromatography
iFeo	Inorganic iron overlay (solid medium)
nt	Nucleotide
PCR	Polymerase chain reaction
TE	Trace elements
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
U.K.	United Kingdom
v/v	Volume per volume
w/v	Weight per volume
XRD	x-ray diffraction
YE	yeast extract

Chapter 1

INTRODUCTION

1.1 Biogeochemistry of mineral tailings and discharge water

1.1.1 Mineral tailings

The earliest evidence of metal mining comes mainly from humans exploiting high-grade oxide ores. These were utilised because were easy to find and access at the land surface and also because they were possible to process using the resources that were available to our ancestors. As oxide ores of metals such as copper became more and more scarce and/or non-accessible, the exploitation of sulfide ores became increasingly significant. A major stimulus for this was the development of the flotation technique in the late 19^{th} century, which allowed the separation of metal sulfide minerals from gangue minerals that have no commercial value. This technique facilitates the selective flotation of minerals of economic interest (e.g. chalcopyrite) by making their surface hydrophobic by adsorption of a specific chemical reagent ("collectors"). The modified minerals float to the surfaces of suspensions when air bubbles are introduced, while minerals that are not targeted by the flotation chemicals sink to the bottom of the flotation tank and are ultimately disposed of as mineral "tailings". As a result of selective flotation, about 99% of the ground primary ores end up as fine-grain tailings, in the case of copper ores. The composition of tailings is directly dependent on that of the ore and therefore they are highly variable, though pyrite (FeS_2) is frequently the most reactive mineral present in tailings wastes (Dold, 2008; Johnson and Hallberg 2003; Johnson *et al.*, 2008a).

Although mine tailings have no economic value, they still frequently contain large quantities of leachable heavy metals, especially iron, and also microbial populations that are capable of mobilizing these metals. Tailings represents a strong enrichment of metals in relation to the earth's crust and may contain other sulfide minerals like arsenopyrite (FeAsS), enargite (Cu_3AsS_4), galena (PbS), sphalerite (ZnS) which can be source for future uncontrolled metal liberation (Dold, 2008).

Generally the rate of sulfide mineral oxidation is slow in unaltered (massive) rock assemblages, but is greatly accelerated as result of mining activities, due to disaggregation of rocks and coal strata causing greater sulfide surface areas being exposed to oxygen and water. Tailings are transported as mineral suspension for final deposition in impoundments, and sulfide mineral oxidation is limited as long as the tailings remain water-saturated. However, once mining operations cease, water levels in tailings impoundments will fall unless management practices are adopted to prevent this, and unsaturated zones containing both air and moisture will appear, facilitating the oxidation of sulfide minerals and resulting in the formation of acid mine drainage (AMD; Diaby *et al.*, 2007).

Mine tailings are usually stored under water in enclosed lagoons known as tailing dams, in order to reduce exposure of the reactive minerals present to oxygen and thereby to reduce their risk of oxidation. At the time of writing, thousands of tailings dams exist worldwide, containing billions of tonnes of waste material from mineral processing. Tailing dams are supposed to retain their integrity for hundreds if not thousands of years, but past experiences have shown that tailings lagoons pose a serious environmental threat that remains when mines close and, as is often the case, mine owners cease to bear responsibility for management of waste materials. Examples of tailing dams failure in operating and abandoned mines continue to accumulate, and in recent years have included major incidents at: (i) Kolontár (Hungary) in October, 2010; (ii) Huancavelica (Perú) in June, 2010; (iii) Baia Mare (Romania), where cyanide-contaminated water was released into major river systems in January 2000; and (iv) Baia Borsa (Romania), where a release of heavy-metal-rich tailings occurred in March 2000. One of the major infamous tailings disasters occurred on the 25th of April 1998, where breach of a retaining wall resulted in the release of a large scale sulfide tailings pond in Aznalcóllar, south-west Spain. Approximately five cubic hectometers of toxic mud were spread over 60 kilometres on both sides of Guadiamar River, coming very close to Donana National Park. About € 147 million were spent correcting the negative environmental and agricultural impacts of this incident (Guerrero *et al.*, 2008; Rico *et al.*, 2008; Salgueiro *et al.*, 2008; Wise Uranium project, 2011).

1.1.2 Genesis of acid mine drainage in mine spoils and tailings

Sulfide minerals are chemically (and biologically) stable in situations where both water and air are excluded. However, once sulfide minerals are exposed to both of these, oxidation of these minerals occurs spontaneously, either with molecular oxygen or ferric iron acting as the oxidant. Metals such as cobalt, copper, iron, nickel, lead, zinc, cadmium, etc. are released when sulfides of these metals are exposed to the weathering conditions found in mines, tailing impoundments and waste rocks (Johnson and Hallberg, 2003).

Sulfide minerals can be divided into those that are acid-soluble (e.g. sphalerite and chalcocite) and other which are acid-insoluble (e.g. pyrite and arsenopyrite). The mechanisms (s) of sulfide mineral dissolution have been the subject of debate and conjecture for many years. Acid-soluble sulfides are oxidized either by ferric iron or by protons (Equation 1) producing H_2S^+ that eventually form polythionates (Shippers & Sand 1999).

$$MS + Fe^{3+} + 2H^+ \rightarrow M^{2+} + Fe^{2+} + H_2S^+ \qquad (1)$$

In the case of acid-insoluble sulfidic minerals another mechanism has been proposed. According to Schippers and Sand (1999), once iron-oxidizing prokaryotic cells have attached to metal sulfide surfaces, the hexi-hydrated ferric iron, contained in the extracellular exopolymer can trigger the indirect attack of the metal sulfide following the following reaction:

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

The thiosulfate anion ($S_2O_3^{2-}$) is chemically unstable at low pH and thus prone to chemical oxidation in acidic solution, especially in the presence of Fe^{3+} (equation 3). The products of the reaction are protons as well as sulfate, elemental sulfur, and various other reduced inorganic sulfur compounds (RISCs) which are themselves susceptible to further chemical and biological oxidation.

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \qquad (3)$$

In tailings deposits, the oxidation of pyrite and other sulfide minerals depends on factors such as temperature, pH, humidity and the availability of oxygen, which is

mainly controlled by diffusion (Kock and Schipper, 2006). In addition, the regeneration of ferric iron, which is reduced to ferrous on reaction with pyrite, is the key reaction in promoting the ongoing oxidation of the mineral. At circum-neutral pH values, this may be mediated chemically or, at least in theory, biologically by neutrophilic iron-oxidizing bacteria such as *Gallionella ferruginea*, while below pH 4, the spontaneous chemical oxidation of ferrous iron is very slow, and the biological oxidation by acidophilic iron-oxidising bacteria has a pivotal role in the genesis of acid mine drainage (Johnson and Hallberg, 2005). The biological oxidation of ferrous iron can accelerate the kinetics of pyrite oxidation by several orders of magnitude. Measurements of the rates of sulfide mineral oxidation, using techniques such as micro-calorimetry (since this is an exothermic reaction) is useful in providing a means of predicting time periods of AMD formation, and also to assess remediation strategies (Schippers *et al.*, 2010).

1.1.3 The microbiology of sulfidic mine waste and tailing dumps

At low pH (<3) pyrite oxidation is 10-100 times faster when the oxidant is ferric iron rather than oxygen (Diaby *et al.*, 2007), although at near neutral pH values, oxygen is a more important pyrite oxidant. This is related to the solubility of ferric iron, which is highly pH dependent. The abiotic oxidation of ferrous iron is also highly pH-dependent, and proceeds very slowly (even in oxygen-saturated solutions) at pH < 3.5 (Stumm and Morgan, 1981). However, some species of acidophilic bacteria and archaea are able to catalyze the oxidation of ferrous iron in low pH environments, regenerating ferric iron, and some of these (and others) also generate sulfuric acid via oxidation of elemental sulfur and reduced inorganic sulfur compounds.

The microbial diversity of sulfidic mine dumps comprises aerobic and anaerobic species which are autotrophic or heterotrophic as well as lithotrophic or organotrophic. Several geomicrobiological investigations of sulfidic mine waste dumps and tailings located in different climatic zones have been undertaken to gather information about microbial processes and diversity in these extreme environments. At such sites microorganisms on the the genus level from the domains of life *Bacteria* and *Archaea* have been detected in mine dumps (Kock and Schippers, 2008; Schippers *et al.*, 2010).

Prokaryotic microorganisms that are metabolically active in extremely acidic environments have been categorized using a number of different criteria, such temperature (as mesophiles, moderate thermophiles and thermophiles) and pH optima, and also on the basis of cellular carbon acquisition: autotrophs assimilate CO_2, heterotrophs assimilate organic carbon and mixotrophs use both. Another useful subdivision of acidophiles differentiates those that can solubilize minerals and those that cannot.

Sulfide minerals such as pyrite are oxidized by ferric iron (as described above) and acidophilic iron-oxidizing prokaryotes (e.g. *Leptospirillum ferroxidans*, *Acidithiobacillus* ("*At*"), *ferrooxidans*, *Acidimicrobium* ("*Am*") *ferrooxidans*, and the archaeon *Sulfolobus metallicus*) have a central role in their dissolution and commercial contexts. Other sulfides, such as chalcocite (Cu_2S) are acid-soluble and can be oxidized by prokaryotes such as *Acidithiobacillus thiooxidans* and *Metallosphaera sedula* that generate sulfuric acid.

In contrast, iron-reducing bacteria have been shown to accelerate the reductive dissolution of ferric iron-containing minerals, such as jarosites and goethite. These include heterotrophic mesophiles, such as *Acidiphilium* spp. and *Acidobacterium* spp, which do not oxidize ferrous iron, and moderate thermophiles, such as *Sulfobacillus* spp., that can either oxidize or reduce iron (Johnson and Hallberg, 2003).

Iron- and sulfur-oxidizing acidophiles have been found in mine tailings (including samples taken below the water table) by a number of research groups (e.g. Diaby *et al.*, 2007; Kock and Schippers, 2008; Southam and Beveridge, 1992; Wielinga *et al.*, 1999). Also, heterotrophic acidophiles like *Acidiphilium* spp. and *Acidobacterium* spp. (Berthelot *et al.*, 1997; Diaby *et al.* 2007) and sulfate reducing bacteria (SRB) have been detected in mine tailings (Fortin *et al.*, 2002). SRB species related to *Desulfitobacterium* spp. have been detected in acidic tailings (Winch *et al.*, 2009). Interestingly, SRB were also found in oxygen-containing as well as anaerobic zones in the tailings and also in acid (pH 2.5) as well as circum-neutral pH samples (Fortin and Beveridge 1997). Dold *et al.* (2005) detected dissolved organic carbon in form of low molecular weight carboxylic acids (formic, acetic and pyruvic acids) which were thought to support the growth of the indigenous heterotrophic bacteria identified in the same tailings deposit.

1.1.4 Eukaryotic algae communities in acidic environments

Acidic mine waters are lethal to the majority of life forms, including most microorganisms. However, it is known that highly acidic environments are often inhabited by acid-tolerant algae that may be not directly involved in mineral oxidation (Aguilera *et al.*, 2007; Gonzalez-Toril *et al.*, 2011; Gross, 2000).

Although low pH and high metal concentration are restrictive to most aquatic life, bright-green algal mats composed of *Klebsormidium*, *Microspora*, *Mougeotia*, *Ulothrix* and *Stigeoclonium* species have been observed to thrive in AMD-impacted environments. Amaral Zetter *et al.* (2002) claimed that diverse photosyntethic eukaryotic microorganisms such as green algae, diatoms and euglenoids contributed more than 60% of the total of the biomass. Valente and Gomez (2007) proposed that the abundance and distribution of *Klebsormidium sp.* and *Euglena mutabilis* in AMD affected waters may be used as ecological indicators of pollution in these environments. Rowe *et al.* (2007) described massive growths of macroscopic "acid streamers" in an acidic (pH ~2.5) metal-rich stream draining an abandoned copper mine (Cantareras) located in the Iberian pyrite belt (IPB).The surfaces of the streamer/mat growths were heavily colonised with algae, including *Euglena* and *Chlamydomonas.*

Algae can influence mine water chemistry in various ways. Das *et al.* (2009) noted that assimilation of nitrate by algae can directly affect the acidity of the water by direct production of alkalinity, though this effect can only occur if the inorganic nitrogen present is predominantly nitrate, since assimilation of ammonium produces acidity. Also, algae scavenge metals and potentially decrease metal concentrations. However, there is little evidence that algae directly contribute to the improvement of water quality.

One of the possible roles of algae in AMD is that they serve as carbon providers for heterotrophic bacteria, such as SRB, which in turn generate alkalinity (Das *et al.*, 2009). In addition, Rowe *et al.* (2007) provided evidence that micro-algae can produce organic carbon that support the growth of species of iron-reducing heterotrophs which can encapsulate pyrite minerals tailings and thereby reduce the colonization by iron/pyrite-oxidizing bacteria (Johnson *et al.*, 2008a). In addition,

Molwantwa *et al.* (2000) showed that extracellular polysaccharide produced by algae can stimulate sulfate reduction activity by cultures of SRB.

It has been demonstrated that amino acids and peptides are liberated by algal cells (Forsberg and Taube, 1967). Also Tolbert and Zill (1956) found that actively growing cultures of *Chlorella* excreted 3-8 mg/l of glycolic acid. This small molecular weight organic acid is a potentially important source of carbon and energy for heterotrophic bacteria. Measurements of heterotrophic uptake by the indigenous planktonic microorganisms have indicated that they use glycolic acid at rates comparable to other common microbial substrates (Wright and Shah 1975). Glycolic acid has received much attention from plant physiologists because of its role in photosynthesis and photorespiration. Tolbert (1974) showed that glycolate formation involves oxidation of the CO_2-acceptor ribulose diphosphate in a competive reaction between O_2 and CO_2 for the common substrate, ribulose-diphosphate. After formation, glycolate may then be excreted or involved in other metabolic pathways.

1.2 Prevention and remediation of acid mine drainage

As with many other examples of environmental pollution abatement, AMD may be targeted either at the source of the problem (its genesis point) or in downstream treatment systems. Several methodologies have been applied to prevention and remediation of mine water pollution. Preventive measures include the following strategies: sealing and/or flooding of mine workings, storage of spoil and waste material in impermeable dumps, underwater spoil disposal, coating technologies and application of anionic surfactants (Johnson and Hallberg, 2005).

While the prevention of AMD formation is the ideal scenario, this is not often pragmatic and remediation of the polluted water is necessary. There are several strategies available for remediation AMD, one formed. These may be subdivided into:

(i) Active remediation technologies which uses either biological or abiotic (chemical) agent. The method most widespread used to mitigate acidic effluents is an active treatment process involving addition of a chemical agent to generate alkalinity and precipitate metals and require continuous provision of materials for the remediation process to operate. Various neutralising reagents have been used, including lime (calcium oxide), calcium carbonate, sodium hydroxide, and magnesium oxide and hydroxide (Johnson and Hallberg, 2005).

(ii) Passive technologies that require minimum maintenance once constructed. These systems utilize the biochemical and physical processes that often occur in the environment to modify the influent characteristics. Passive systems are often considered to be attractive due to their lower operational costs (though construction costs are often appreciable) and the fact that they can be installed in remote locations with minimal ongoing management.

Although active chemical active treatment and passive biological treatment can provide effective remediation of AMD (though compost-based systems tend to have variable performance), a major drawback to both approaches is that the immobilised metals are contained in either a "sludge" (chemical treatment) or within spent compost (biological treatment) and need to be disposed of in specially-designated landfill sites. Changes in redox conditions during storage can lead to re-mobilisation of metals (and metalloids such as arsenic) in both sludges and spent composts. In addition, potentially useful and valuable metal resources are not recovered using conventional approaches for remediating mine waters. On the other hand, every discharge of mine water pollution is unique in terms of chemical composition and flow rate, complicating any standardization of treatment procedures (Johnson *et al.* 2006).

1.2.1 Remediation of AMD using biosulfidogenesis.

A radically different approach for remediating AMD which, like compost bioreactors, derives from the abilities of some microorganisms to generate alkalinity and to immobilize metals, is referred to generally as "active biological treatment". Microbiological processes that generate alkalinity are mostly reductive processes and include denitrification, methanogenesis, and dissimilatory reduction of sulfate, ferric iron and manganese (IV). However some of these processes tend to be limited in AMD. Considering that AMD usually contains elevated concentrations of both ferric iron and sulfate, the ability of some bacteria use these compounds as terminal electron acceptors suggests that these reactions can be highly useful for mine water remediation.

Acidic environments in which sulfur or sulfide minerals are subjected to (biologically)-accelerated oxidative dissolution characteristically contain large concentrations of soluble sulfate (Kimura *et al.*, 2006). Microbial sulfate reduction

might be anticipated to occur within anaerobic zones in these, as in non-acidic environments. Biosulfidogenesis, the generation of hydrogen sulfide as a result a reductive metabolic process, has the attraction of being a proton-consuming reaction, and also generates a waste product (HS^-/H_2S) that can give rise to the rapid and, in controlled situations, the selective mineral/immobilization of many potentially toxic metals (such as copper and zinc) often present in AMD at elevated concentrations (Jameson *et al.*, 2010).

There have been few successful applications of SRB-mediated active AMD treatment systems, even though the possibility of using SRB to remediate AMD has long been appreciated. On major reason for this is that, for a long time the accepted view was that SRB prefer an environment between pH 6 and 8 (Koschorreck, 2008), whereas AMD generally has a pH between 2 and 4 and commonly less than pH 3 (Jong and Parry, 2006). Under these circumstances, a neutralization step is necessary before AMD effluents are subjected to SRB treatment or, alternatively, "off-line" systems need to be used. The latter is necessitated by the fact that current systems use neutrophilic SRB or sulfur-reducing bacteria, and direct exposure to the inflowing acidic solution being treated would be lethal to these microorganisms. Therefore a separate vessel in which sulfide generated by the bacteria is contacted with the acidic, metal-laden waste water, is required (Jameson, *et al.*, 2010; Johnson *et al.*, 2006). Examples of this technology are the *Biosulfide* and *Thiopaq* processes operated under the auspices of two biotechnology companies, BioTeq (Canada) and Paques bv (the Netherlands) which are currently in operation various parts of the world. The *Biosulfide* process has two stages, one chemical and the other biological. Metals are removed from AMD in the chemical stage by precipitation with biogenic sulfide produced in the biological stage by SRB under anaerobic condition. In this system, hydrogen sulfide is generated by the reduction of elemental sulfur, or other sulfur source, in the presence of an electron donor, such as acetic acid, in an anaerobic bioreactor. The gas is passed to an anaerobic agitated contactor. In the latter, copper can be precipitated as a sulfide usually without pH adjustment and without amount of precipitation of other heavy metals present in the water, to generate a high copper value product, usually containing more than 50% of the metal. Other metals can also be recovered as separate high-grade sulfide products, although pH control using an alkali source is usually required to precipitate selectively the metal as a sulfide phase. The high-grade metal sulfide precipitate is then recovered by conventional clarification and filtration to produce a filter cake which can be shipped to a smelter (Adams *et al.*, 2008). The *Thiopaq* process uses

another system that involves the use of two biological reactors (a) an anaerobic upflow sludge blanket (UASB) reactor for the reduction of oxidized sulfur species. In this reactor, SRB using ethanol or hydrogen as electron donor produce sulfide (mostly HS^-) for the precipitation of metal sulfides (which can proceed in the same reactor depending of the toxicity of the wastewater), and (b) an aerobic submerged fixed film (SFF) reactor to oxidize excess sulfide produced to elemental sulfur, using sulfide-oxidising bacteria. In this process, metals such as zinc and cadmium can precipitated down to very low concentrations. The *Thiopaq* process has been successfully operating since 1992 at a zinc refinery Budelco bv, (located in the Netherlands) for the treatment of zinc-contaminated groundwater. The zinc sulfide produced is fed into the refinery and is incorporated into the metallic zinc product from the plant. Application of this process has also been demonstrated on a pilot-scale at the Kennecott Bingham Canyon copper mine in Utah, where >99% of copper present in a pH 2.6 waste stream was recovered (Boonstra *et al.*, 1999; Johnson and Hallberg, 2005; Pumpel and Paknikar, 2001).

Sulfate reduction activity has been reported in low pH ecosystems, for example in acidic lakes, wetlands, acid mine drainage (Alazard *et al.*, 2010; Gyure *et al.* 1990; Koschorreck, 2008); however, few acidophilic/tolerant SRB have been cultured (Alazard *et al.* 2010; Johnson *et al.* 2006; Senko *et al.* 2009). A major potential advantage of using acidophilic sulfidogens would be to allow simpler engineering design and reduce operational costs by using single on-line reactor vessels that could be used both to generate sulfide and selectively target metal(s). Precipitation and removal of many soluble transition metals, often present in AMD emanating from metal mines, may be readily biomineralized as their sulfides. These have different solubility products, so that these metals can be precipitated together or selectively by controlling concentrations of the key reactant S^{2-}, which may be achieved by controlling pH ($S^{2-} + H^+ \leftrightarrow HS^-$). Jameson *et al.* (2010) showed that it was possible to selectively precipitate and to recover copper from a solution also containing zinc and iron using an acidophilic sulfate-reducing enrichment culture and hydrogen derived from acid dissolution of zero-valent iron (ZVI) as electron donor, and yeast extract as carbon source, in an "on-line" bioreactor system.

1.3 Natural attenuation as an alternative approach for remediating AMD.

The term "natural attenuation" refers to the reliance on natural processes to achieve site-specific cleanup objectives within a reasonable time frame (USEPA, 1997). The attenuation processes involved in such a remediation approach include a variety of *in situ* physical, chemical, and biological processes that, under favorable conditions, are effective without human intervention, to reduce mass, toxicity, mobility, volume, or concentration of contaminants in soil and groundwater. The *in situ* processes may include biodegradation, dispersion, dilution, adsorption, and volatilization. Attenuation processes important at mining sites include pH buffering and acid neutralization, adsorption at mineral-water interfaces, mineral precipitation, and dilution/dispersion (USEPA, 1997).

Natural remediation of metal pollutants generally involves the catalytic action of microbial activities that can accelerate the reaction of precipitation of toxic compounds soluble and lead them to accumulate in precipitates (Bruneel *et al.* 2011). Natural attenuation of transition metals in AMD has been observed, for example at the Carnoulès mine in France (Bruneel *et al.* 2011) and the Iberian pyrite belt (IPB) in Spain (Sánchez Espaňa *et al.*, 2005). Biochemical processes such as oxidation and precipitation of iron consisting of a variety of iron (oxyhydr)oxides and hydroxysulfates such as jarosite, schwertmannite and the adsorption of other metals and metalloids to the ferric minerals formed, have been reported.

Rowe *et al.* (2007) described in detail such a natural process in a small site at the abandoned Cantareras copper mine (Fig. 1.1), which is located in the Tharsis mine district in the IPB. A survey of AMD-impacted waters at Cantareras was carried out to elucidate the roles of acidophilic microorganisms in the natural attenuation process. The acid stream showed very distinct stratification with depth. The benthic zone of the AMD channel was heavily populated by microorganisms that formed streamers and mat-like community structures. In the surface of the streamer growths micro-algae and prokaryotic acidophiles were identified. Lower layers yielded greater numbers of heterotrophic bacteria like *Acidiphilium* and *Acidobacterium* than chemolithotrophic acidophiles. Although it might be expected that oxygenation of the AMD would result in rapid bacterial ferrous iron oxidation, little net oxidation of ferrous iron was observed. This was explained for the presence of heterotrophic bacteria, which can catalyze the reduction of ferric iron to ferrous, and that dissolved

organic carbon produced by the algae supported the growth of the heterotrophic bacteria present. In addition, SRB were detected in and isolated from the bottom mat layer, which was grey/black in colour. The pH of AMD extracted from this bottom layer was extremely acidic (pH<3), and this dark grey coloration was due to the accumulation of copper sulfide, presumably as a result of biosulfidogenesis (Rowe *et al.*, 2007). No iron sulfides (e.g. hydrotroilite; $FeS.nH_2O$) were detected, presumably because of the low pH of the mine water even at depth. Because the solubility product of CuS (log *Ksp* at 25^0 C is -35.9) is much smaller than that of FeS (-18.8); this sulfide mineral precipitates in acidic waters whereas FeS does not. Selective immobilization of transition metals, apparently by the activities of acid-tolerant or acidophilic SRB, appeared therefore to be occurring in this post-mining environment.

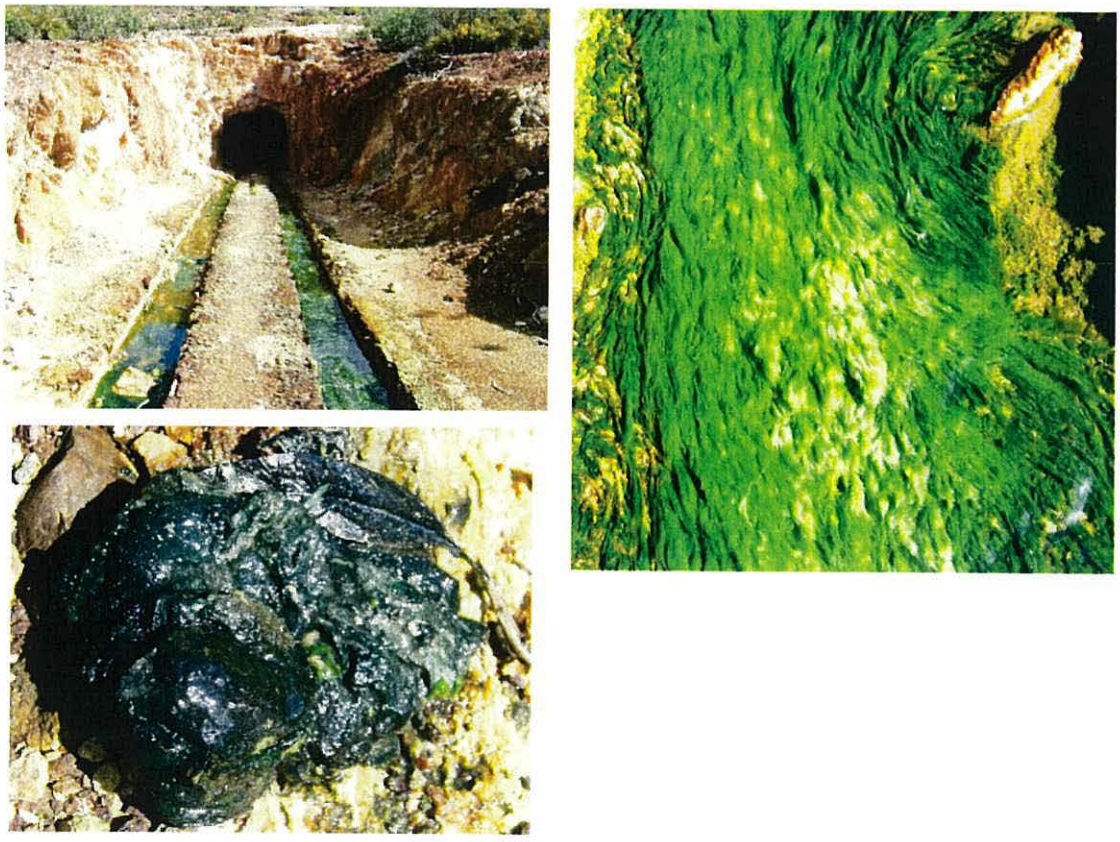


Fig. 1.1. The abandoned Cantareras copper mine in the Iberian pyrite belt (Tharsis, Spain). Top left, the drainage adit and drain channels; right algal-colonised surface streamer growths; bottom left, sulfide (CuS) accumulation in the lowest mat layer.

Rowe *et al.* (2007) proposed a model for the biochemical cycling of iron and sulfur at the abandoned Cantareras mine (Fig. 1.2). Dissolution of sulfide minerals in the exposed mine workings gives rise to a highly acidic, metal- and sulfate-rich

effluent. The anoxic water draining the mine is oxygenated by photosynthetic acidophilic algae in the surface (CL1) layer of the acid streamer growths that develop immediately outside of the adit, which facilitates oxidation of ferrous iron in the surface AMD (catalyzed primarily by *Acidithiobacillus ferrivorans*). DOC originated from photosynthetic and chemosynthetic primary producers serves as substrates for the (dominantly) heterotrophic bacteria in the deeper zone (CL2-4) streamer layers. Ferric iron is used as terminal electron acceptor in streamer layers (CL2 and CL3), while in the thick CL4 layer sulfate is also used by SRB, resulting in the deposition of copper sulfide.

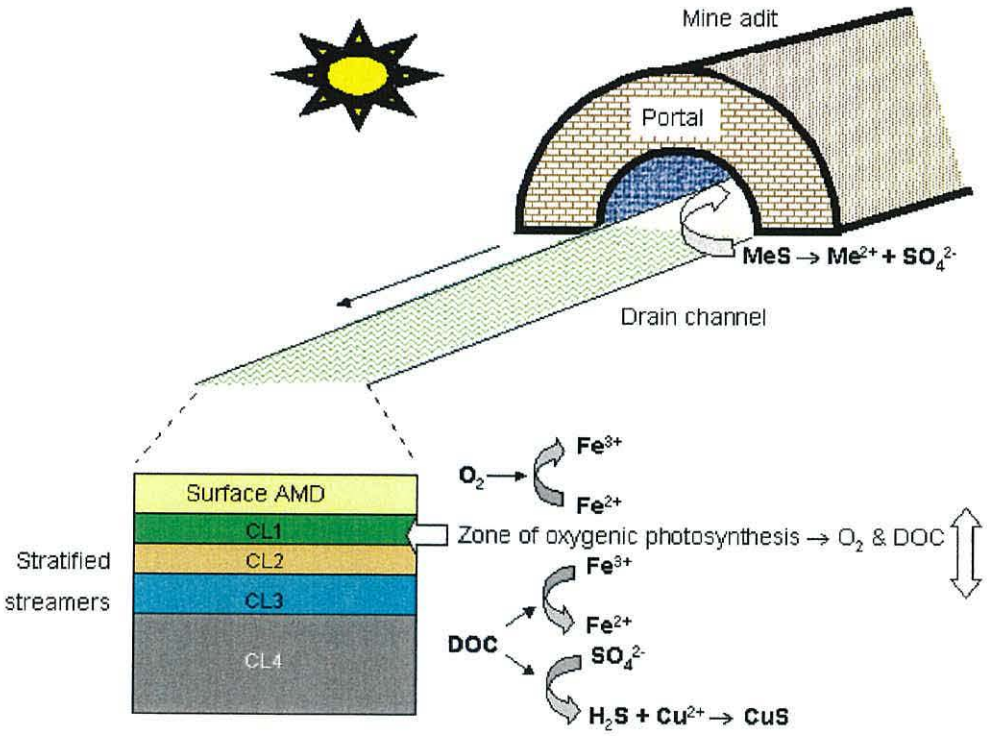
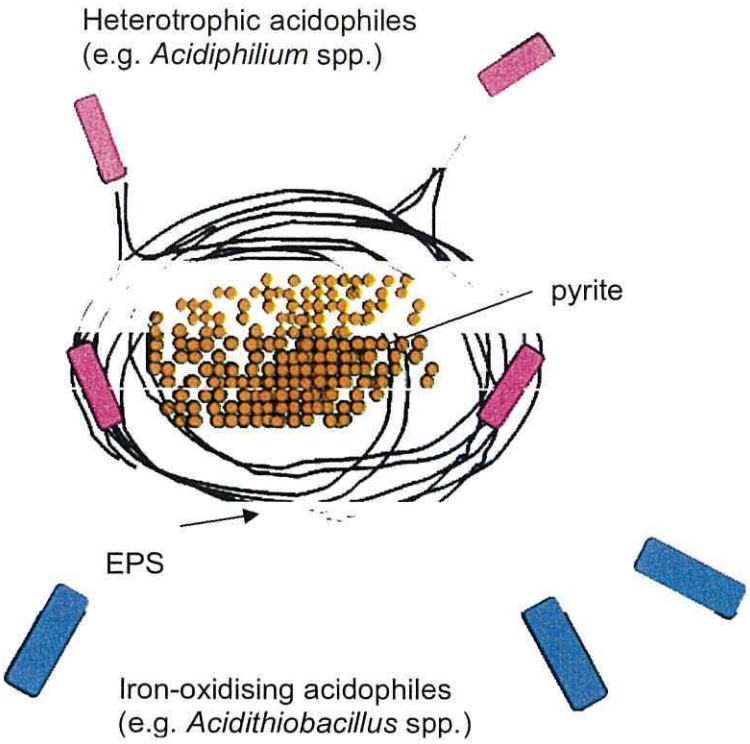


Fig. 1.2. Proposed model of the biogeochemical cycling of iron and sulfur at the abandoned Cantareras mine (Rowe *et al.*, 2007; see text for details).

1.4 The potential of "bioshrouding" for minimising AMD production in stored mine tailings

A novel ecological technique ("bioshrouding") has been proposed as a means to minimize the oxidative dissolution of sulfide minerals in tailings deposits and thereby reduce the rate of acid production and metal mobilisation from such sites. The principle is that, by restricting the exposed surface area of the highly reactive minerals in tailings that might be colonised and attacked by acidophilic chemolithoautotrophs, dissolution of these minerals will be retarded (Johnson *et al.*, 2008a). With this technique, freshly-milled tailings wastes are colonized by heterotrophic acidophilic bacteria that synthesise extracellular polymeric substances, and which are also capable of reducing Fe (III) to Fe(II). Like the chemolithotrophs, heterotrophic acidophiles microorganisms can colonize the reactive minerals present in tailings, and in so doing can impede the attachment of pyrite-degrading acidophiles (Fig. 1.3). For this to be effective, it is necessary to stimulate the growth of heterotrophic bacteria (e.g. by providing a suitable organic substrate) before the pyrite-degrading bacteria can establish biofilm growth on the surfaces of sulfide minerals (Fig 1.3). Initial experiments showed that the dissolution of pyrite could be reduced between 57 to 75% by "bioshrouding" the mineral with three different species of heterotrophic acidophiles (*Acidiphilium*, *Acidocella* and *Acidobacterium*), under conditions which optimised the microbial oxidative dissolution of pyrite. Bioshrouding of reactive mineral tailings could, in theory, be readily achieved in the field by addition of readily available and cost-effective substrates, but continued provision of organic carbon might be necessary to sustain the heterotrophs. One potential passive method of continued supply of organic carbon may be through the *in situ* production during the growth of algae, which due to the relatively harsh chemical conditions of mineral-oxidising liquors may be the only photosyntetic organisms capable of surviving in these environments (Senko *et al.*, 2011). By sustaining the "bioshrouding" bacteria by dissolved organic carbon derived from micro-algae in tailing ponds it would, in theory, be possible to devise a low-cost passive solution to minimising AMD genesis at tailings sites.



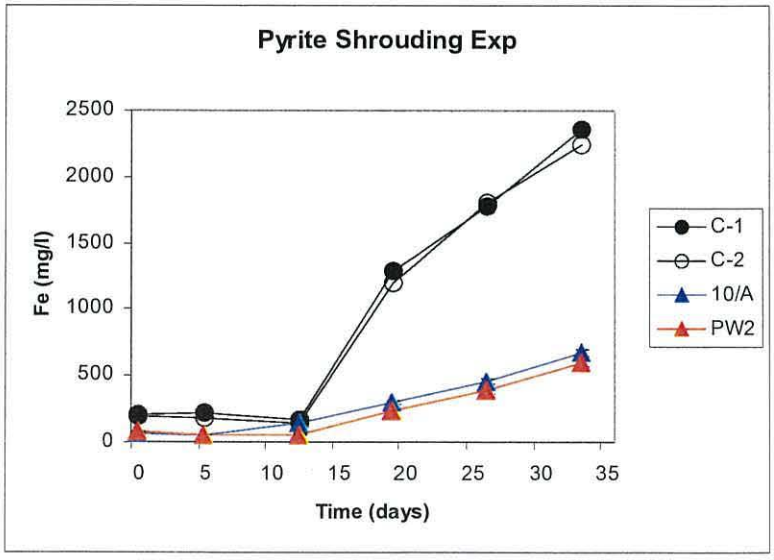


Fig. 1.3. "Bioshrouding" as a technology for securing mineral tailings. Top, schematic, showing extracellular polymeric substances produced by heterotrophic acidophlilic bacteria attach to and enshroud reactive sulfide minerals, such as pyrite. Biofilms of heterotrophic bacteria that form on mineral surfaces inhibit the attachment of mineral-oxidising bacteria. The lower graphic shows data illustrating reduced pyrite dissolution by *At. ferrooxidans* in "bioshrouded" pyrite (Johnson *et al.*, 2008a).

1.5 Scope of the current project

The present study set out to obtain a better understanding of the nature of the diversities and interactions of acidophilic microbial consortia that could be used to minimize the generation of acidic mine drainage in tailings and also to treat metal-contaminated mine waters. The project was structured into four main areas:

- Identification of organic exudates produced by three species of chemo-autotrophic acidophiles, and assessment of the potential of heterotrophic and mixotrophic acidophiles to metabolise the major exudate identified (glycolic acid).

- Characterization and identification of their organic exudates produced by two species of acidophilic micro-algae isolated from abandoned copper mines, and their utilisation by iron-reducing heterotrophic acidophiles.

- To understand the role of acid- and metal-tolerant micro-algae as carbon providers for acidophilic iron- and sulfate-reducing bacteria, using experimental mesocosms containing pyritic mine tailings, and to examine how "ecologically engineered" systems could be used to reduce the environmental impact of mine wastes.

- To develop an alternative approach for remediating metal-rich mine waters, using novel consortia of acidophilic SRB, which facilitates the recovery and ultimately the recycling of base metals, using on-line bioreactors.

This remaining chapters of this thesis comprises of five research papers, three of which at the time of submission have been published (all with peer review) while the other have been submitted to main-stream scientific journals for publication. The formats of these papers have been modified in order to provide a more uniform organisation of the thesis.

Chapter 2

Development of an integrated ecological engineering approach for securing metal mine wastes and remediating mine waters

Ivan Ñancucheo and D. Barrie Johnson

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http://www.proceedings-stfandicard-2009.com)

Abstract

Sulfidic mine wastes (rock dumps and tailings) are often populated by mineral-degrading microorganisms that give rise acid rock drainage pollution. However, the same environments host other bacteria that can generate alkalinity and immobilise metals. We are seeking to develop a low-cost, integrated biological engineering approach that uses these "mitigating" microorganisms both to minimize the generation of acid rock drainage in mine spoils and tailings, and to treat metal-contaminated mine waters. The scheme under development has three components, all of which have been successfully demonstrated at the laboratory-scale: (i) minimizing sulfide mineral dissolution in mine tailings; (ii) microbiological generation of alkalinity; (iii) biomineralization of soluble metals. The first of these uses "bioshrouding" of minerals whereby reactive sulfides are colonized and encased by heterotrophic iron-reducing bacteria that produce copious amounts of exopolymeric materials, thereby greatly reducing or eliminating attachment of mineral-oxidizing, acid-generating bacteria. Biological generation of alkalinity and selective biomineralization of metals such as copper and zinc is mediated by novel acidophilic species of sulfate-reducing bacteria. Acid- and metal-tolerant micro-algae can serve as both carbon- and energy-providers in the engineered ecosystems, to provide long-term sustainable systems that are underpinned by solar energy.

2.1 Introduction

Mining of metals that occur as, or associated with, sulfide minerals often has the legacy of long-term environmental degradation and pollution (Alpers and Nordstrom, 1999). The role of microorganisms in accelerating the oxidative dissolution of residual sulfide minerals (principally pyrite, FeS_2) in waste rocks and mine tailings, generating acidic drainage waters that typically contain highly elevated concentrations of soluble metals and arsenic, has been widely documented (e.g. Baker and Banfield, 2003). Knowledge of the mechanistic nature of microbiologically-accelerated sulfide mineral dissolution has greatly improved in recent times, including the recognition of the importance of cell attachment and the development of microbial biofilms in this process. Acidophilic iron-oxidizing bacteria and archaea, such as *Leptospirillum* spp., *Acidithiobacillus ferrooxidans* and *Ferroplasma*, have been shown to attach rapidly and preferentially to sulfide minerals, where their growth and production of exopolymeric substances (EPS) leads to the development of biofilms within which conditions for mineral degradation can be controlled and optimized (Sand and Gehrke, 2006).

As with many other examples of environmental pollution, abatement of acid rock drainage (ARD) may be targeted either at the source of the problem (its point of genesis) or in downstream treatment systems. The various options available for abatement and remediation of mine water pollution have been reviewed elsewhere (e.g. Johnson and Hallberg, 2005). Engineering options (restricting access of oxygen and/or water) to mine wastes are used most widely to control the genesis of mine water pollution at source, though alternatives such as "chemical coating technologies" (to make the surfaces of potentially reactive minerals inert) and inhibiting mineral-oxidizing bacteria have also been described. "Active" (chemical treatment) and "passive" (wetland-based systems) are used widely to treat mine waters, though both have significant drawbacks, particularly when used to remediate to acidic waters that contain significant concentrations of a variety of transition metals (Johnson and Hallberg, 2002 & 2005).

Natural attenuation is defined by the U.S. *Environmental Protection Agency* as the "reliance on natural processes (within the context of a carefully controlled and monitored site cleanup approach) to achieve site-specific remediation objectives within a time frame that is reasonable compared to that offered by other more active methods". There are numerous examples of natural attenuation occurring in metal-rich mine waters, examples of which are described below. By identifying the processes by

which natural attenuation of ARD occurs, and by isolating and studying the microorganisms that mediate these, we are devising new ecological engineering strategies for both source and downstream treatment of ARD. Here we describe the basic concepts that underpin these approaches, and how they may be applied in the field.

2.2 Examples of natural attenuation of ARD in the Iberian Pyrite Belt

The Iberian Pyrite Belt (IPB), located in southern Spain and Portugal, hosts the largest concentration of known massive sulfides on Earth, and is home to over 80 mines, including many (such as the Riotinto mine) of historic importance. The geochemistry and microbiology of one small derelict mine site (Cantareras) was described in detail by Rowe *et al.* (2007). This mine hosted a massive pyrite body (some 300 long by 30 m wide) and had contained an estimated six million tonnes of copper-rich mineralization. Water draining the abandoned mine (Fig. 2.1) at the time of sampling, was extremely acidic (pH 2.65) and contained highly elevated concentrations of soluble iron (ca. 1,100 mg/l) copper (ca. 160 mg/l) and zinc (ca. 25 mg/l) as a result of the microbially-accelerated oxidative dissolution of sulfide minerals on the exposed walls and fissures. A notable feature of this ARD stream was that it became heavily colonized by macroscopic streamer/mat growths immediately on exiting the mine adit. These displayed depth-related stratification in both texture and colour, and a combined biomolecular and cultivation approach revealed that was due to depth-related variations in indigenous microbial populations. Lower layers contained large numbers of heterotrophic bacteria (*Acidiphilium* and *Acidobacterium* spp.) that have known to catalyze the reduction of ferric iron to ferrous, while novel species and genera of sulfate-reducing bacteria (SRB) were also detected (and isolated) from the lowest depth of the microbial growths. The pH of ARD extracted from this bottom mat layer, which was grey/black in colour) was also extremely acidic (pH <3), and this dark colour was shown to be due to the formation and accumulation of copper sulfide (CuS) by the SRB. The low pH of the ARD precluded the mineralization of FeS and ZnS. The system therefore demonstrated how a microbiological community can bring about the selective removal of a transition metal from a waste stream that contained a mixture of chalcophilic metals.

Elsewhere in the IPB, the role of acidophilic iron-oxidizing bacteria in promoting the attenuation/mineralization of soluble iron from ARD streams has been reported (Sánchez-Espaňa *et al.*, 2005). In contrast to the biomineralization of metal sulfides,

this requires oxygen, though no extraneous energy source as ferrous iron oxidation is itself an energy-yielding reaction. However, while ferrous iron-oxidation is a proton-consuming reaction, hydrolysis of ferric iron, forming minerals such as schwertmannite (equation [1]) is proton-generating. The can limit the extent of iron mineralization as the solubility of ferric iron is greatly enhanced at pH < 2.5.

$$8Fe^{3+} + SO_4^{2-} + 14H_2O \rightarrow Fe_8O_8(OH)_6SO_4 + 22H^+ \qquad [1]$$

Fig. 2.1. The abandoned Cantareras copper mine, southern Spain, a mine drainage system displaying natural attenuation of soluble copper. (a) general view of the drainage adit and ARD stream; (b) view inside the adit, showing copper-rich acid-generating salts accumulating on the wall surface as a result of the oxidative dissolution (principally) of chalcopyrite; (c) the microbial streamer/mat community (15-20 cm depth) that colonizes the stream. The bottom mat layer comprises SRB (and other anaerobic bacteria) that promote the selective mineralization and accumulation of CuS.

2.3 Alkali-generating microbiological processes

While there are a number of metabolic processes carried out by microorganisms that are acid-generating (such as fermentation, nitrification and sulfur oxidation) other microbial activities consume protons or generate (bicarbonate of hydroxyl) alkalinity. This includes oxygenic photosynthesis, and a number of metabolic processes that occur under reducing conditions. Given the chemical compositions of ARD streams, biological reduction of ferric iron and of sulfate would appear to be the most potentially relevant to mine-impacted environments. The ability of some bacteria that inhabit metal-rich mine waters to use ferric iron rather than oxygen as terminal electron acceptor has been know for some time, though only recently has this particular trait been shown to be widespread among moderately and extremely acidophilic bacteria and archaea (Johnson and Hallberg, 2009; Coupland and Johnson, 2008). Reduction of soluble ferric iron is actually a net acid-producing reaction (equation [2]) though reductive dissolution of solid phase ferric compounds and minerals generates alkalinity, as in the case of goethite (equation [3]):

$$14Fe^{3+} + C_3H_8O_3^{*} + 3H_2O \rightarrow 14Fe^{2+} + 14H^{+} + 3CO_2 \quad [2]$$

$$14FeO.OH + C_3H_8O_3 + 3H_2O \rightarrow 14Fe^{2+} + 28OH^{-} + 3CO_2 \quad [3]$$

($^{*}C_3H_8O_3$ (glycerol) is used here a an example of an appropriate bacterial substrate)

Using reductive dissolution of ferric iron minerals as a source of alkalinity has its own inherent problems, one of which is that the ferrous iron produced is soluble and downstream biomineralization is required to remove it (e.g. as an oxidized phase, which regenerates acidity, or as a sulfide, which requires a relatively high pH and (indirectly) an input of energy. In addition, elements and compounds (such as arsenate) that co-precipitate with or adsorb onto ferric iron minerals are released when these minerals are solubilized, causing addition environmental hazard.

In contrast, microbiological reduction not only generates alkalinity (equation [4]) but also generates a waste product (sulfide) that can give rise to the rapid and, in controlled situations, the selective mineralization/immobilization of many potentially toxic metals present in ARD (equation [5]):

$$SO_4^{2-} + 4C_3H_8O_3 + 14H^{+} \rightarrow 7H_2S + 12CO_2 + 15H_2O \quad [4]$$

$$M^{2+} + H_2S \rightarrow MS + 2H^{+} \quad [5]$$

Although biological sulfidogenesis has been used to treat and recover metals from contaminated groundwater (Boonstra *et al.*, 1999) its application to mine waters has been restricted by the sensitivity of characterized SRB to even moderate acidity (pH <5.5) which constrains engineering designs for such applications either to "off-line" bioreactors, or by using large reactor volume to inflow volume ratios, where the incoming acidity of the ARD water can be instantly neutralized by alkalinity production by SRB housed in the reactors. A further drawback of the latter is that a mixed metal sludge is produced (as in lime treatment of ARD, though the mineral phase here is sulfidic) which requires disposal and precludes metal recovery and recycling.

Novel species of SRB that are active in low pH liquors, and which therefore have the potential to treat ARD directly, have recently been isolated and characterized (Johnson *et al.*, 2006, and unpublished data). The first of these ("*Desulfosporosinus acidophilus")* was isolated from a geothermal site on the Caribbean island of Montserrat and also from sediment in an ARD stream draining an abandoned copper mine (Sen and Johnson, 1999). "*D. acidophilus*" was shown to use carbon compounds (such as glycerol) or hydrogen as electron donor, but is an "incomplete oxidizer" of organic substrates, producing and excreting acetic acid as a waste product. The negative impact of this (acetic acid is highly biotoxic at low pH) could be eliminated by growing it in a mixed culture with an acetate-degrading acidophile (*Acidocella*). The mixed SRB/*Acidocella* culture was shown to be active at pH 3.8 and above (Kimura *et al.*, 2006). Even more acidophilic (and potentially useful) isolates have been obtained from the Cantareras streamer/mat growths, including the novel genus/species "*Desulfobacillus acidavidus*". Growth of pure cultures of "*Db. acidavidus*" has been demonstrated in bioreactor cultures where pH was maintained at pH 3.0 and above. In contrast to "*D. acidophilus*", "*Db. acidavidus*" appears to be a "complete oxidizer" of organic substrates, and does not require an partner bacterium to survive at low pH.

2.4 Biomineralization of metals in ARD streams

Precipitation and removal of soluble metals in ARD may be achieved chemically, as in lime treatment, or biologically, as occurs in aerobic wetlands and compost bioreactors. Controlled biomineralization of metals has a major advantage over both the former treatment systems in that it can result in metals being selectively recovered, thereby reducing costs of sludge disposal and producing in a saleable product. Given the solubility products of solid phases of the major metals (and the

metalloid, arsenic), different routes for biomineralization of elements often found in ARD are appropriate (Table 2.1).

Iron, the most abundant soluble metals in most ARD streams, may be removed as a hydroxide/hydrous oxide phase. In the case of schwertmannite (equation [1]) this also results in partial removal of sulfate, which is significant where this is also targeted. For effective mineralization of iron in aerated waters, it is essential that ferrous iron is first oxidized to ferric. While this can occur spontaneously, rate of chemical oxidation of iron are very small at pH < 4. In contrast, microbiological oxidation of ferrous iron can occur at pH values of < 1 and, since many of the acidophilic bacteria (and archaea) that oxidize iron also fix carbon dioxide, these can proliferate in organic carbon-free waters. However, to accumulate sufficient microbial biomass to obtain effective rates of iron oxidation for mine water treatment, it is necessary to immobilise the bacteria, e.g. by causing them to form biofilms on an inert (synthetic) support matrix. While many publications have reported how different supports can impact the effectiveness of iron-oxidation bioreactors, relatively little research has been carried out into the role of different species of iron-oxidizers in this context. Since the discovery of (*Acidi*)*thiobacillus ferrooxidans* in the mid 20th century, many different iron-oxidizing acidophiles have been described, each having distinct niches in terms of temperature and pH optima. Rowe and Johnson (2008) showed that bioreactors housing four distinct species of iron-oxidizing acidophiles performed very differently to each other when operated either as batch or continuous flow systems, highlighting the importance of matching the biological system used to the particular ARD being remediated.

Table 2.1. Solubility products (as K_{sp} values, calculated at 25°C) of hydroxides and sulfides of metals commonly found in ARD.

	Hydroxide	Sulfide
Fe (II)	-16.3	-18.8
Fe (III)	-38.6	-
Al	-32.4	-
Mn(II)	-12.7	-13.3
Cu	-19.8	-35.9
Zn	-16.1	-24.5
As*	-	-16.0

*The K_{sp} of ferric arsenate is -20.2

The chalcophilic metals, copper and zinc, are more readily biomineralized as their sulfides. Because of the different solubility products of their sulfide phases (Table 1) it is possible to precipitate these metals either separately, or together, in iron-rich ARD, by moderating concentrations of the reactant S^{2-}, e.g. by controlling pH. This has been demonstrated *in vitro* using the acidophilic SRB "*D. acidophilus*" and "*Db. acidavidus*", described above. Figure 2.2a shows a bioreactor culture operated in batch mode at a fixed pH of 4.0, in which the feed material contained ~300 mg/l of zinc and ~50 mg/l of ferrous iron. The pinkish-white precipitate accumulating on the walls of the vessel (EDAX analysis) is ZnS. Figure 2.2b, in contrast, shows another bioreactor containing "*Desulfobacillus*"-like SRB, operated at pH 3.0 and fed continuously with pH liquor containing ~60 mg/l each of copper and zinc. Hydrogen was generated in the bioreactor vessel from zero-valent iron (ZVI) and acted as an electron donor for the bacteria, causing ferrous iron to accumulate (500 to 1,000 mg/l) in the reactor vessel. Because of the low pH of operation, only CuS was precipitated within the bioreactor vessel itself (giving rise to the intense black coloration), further demonstrating the potential of biosulfidogenesis for controlled mineralization of transition metals from acidic, metal-rich wastewaters.

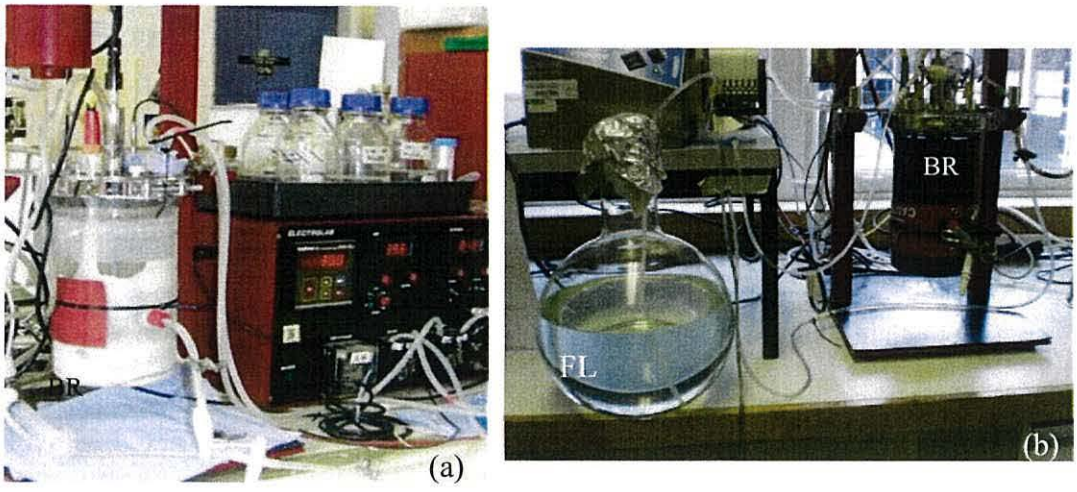

Fig. 2.2. Sulfidogenic bioreactors (BR) operated at (a) pH 4.0 (selectively precipitating ZnS) and (b) at pH 3.0, selectively precipitating CuS. The clear feed liquor (FL) is indicated in (b).

Arsenic frequently occurs in AMD in elevated and potentially toxic (or lethal) concentrations. Three effective routes are possible for its biomineralization: (i) as a sulfide, As_2S_3; (ii) as scorodite ($Fe(AsO_4).2H_2O$); (iii) by precipitation onto nascent positively-charged ferric oxy-hydroxide surfaces. Removal of As as a sulfide has been demonstrated *in vitro* (Newman *et al.*, 1997), while routes (ii) and (iii) and consequential on (biological) oxidation of ferrous iron in AMD.

Of the two other metals listed in Table 1, mineralization of aluminium (as gibbsite; $Al(OH)_3$ occurs as a result of increasing ARD pH, while manganese, like iron, is preferentially removed as oxidized minerals (such as pyrolusite; MnO_2). In contrast to iron, abiotic oxidation of manganese (from Mn(II) to Mn(IV)) proceeds slowly in all but alkaline waters, and consequently this metal, although less toxic than many others that occur in ARD, is often the most difficult and costly to remove from mine waters (Johnson and Hallberg, 2002). Again, microbiological systems may provide an answer to this problem. Biological oxidation of Mn(II), facilitating the precipitation of highly insoluble Mn(IV) can be far more effective than abiotic oxidation at neutral pH or even in slightly acidic waters (Mariner *et al.*, 2008).

2.5 Safeguarding mine tailings by "bioshrouding" of reactive minerals

Ecological engineering can also be used to target the problem of mine water pollution at its source. One suggested method of achieving this is to control or eliminate colonization of reactive sulfides by mineral-oxidizing/acid-generating bacteria, by promoting prior colonization by benign bacteria, an approach described as "bioshrouding" (Johnson *et al.*, 2008a). The concept is that freshly-generated tailings are inoculated with heterotrophic bacteria that attach to and develop biofilms on sulfide minerals. The bacteria used, like the mineral-degraders, need to be tolerant of acidic conditions and also produce large amounts of EPS to facilitate sufficient encapsulation ("shrouding") of the reactive minerals. Initial experiments used two species of extremely acidophilic heterotrophic bacteria (*Acidiphilium* and *Acidocella*). More recently, experiments with moderately acidophilic *Acidobacterium*-like bacteria have indicated the potential of other bioshrouding microorganisms. *Acidobacterium* spp. could prove to be superior to both *Acidiphilium* and *Acidocella* spp. as: (i) many strains produce copious amounts of EPS; (ii) they tend to have a broader pH range for growth than the "extreme acidophiles"; (iii) they are highly tolerant of a wide range of oxygen concentrations; (iv) they are often found in large numbers in ARD-impacted environments, such as the Cantareras mine. Many *Acidobacterium* isolates (like

Acidiphilium and *Acidocella*) have been shown to catalyze ferric iron reduction, an attribute that could compliment their "bioshrouding" roles in restricting sulfide mineral dissolution.

2.6 Integration and application of low cost, self-sustaining bioremediation systems

Ecological engineering solutions focus on developing and optimizing environmental conditions to allow natural attenuation processes to be effective for remediating mine waters. Keys to the success of any new technology are both its cost effectiveness relative to existing remediation technologies and its short- and long-term environmental impacts. One of the major problems in controlling the activities of microorganisms that generate ARD is that they have minimal requirements for growth, and these are generally met in rock dumps and mineral tailings, where residual sulfide minerals act as their sources of energy. In contrast, bacteria that can be used to remediate mine waters (with the exception of iron-mineralizing acidophiles) require extraneous energy sources, and some require oxygen-free conditions to be active. Energy sources for these can be provided as organic carbon, though the form of carbon provided is critical (e.g. many SRB use only relatively simple, small molecular weight organic compounds). A need to a provide continuous supply of a carbon-based or other energy source would, however, be a potential detraction to using bioremediative strategies for safeguarding mine wastes and remediating mine waters. The abandoned Cantareras mine, however, points to how a self-sustaining attenuation system can develop naturally in ARD. Much of the organic carbon that fuels the growth and activities of the iron-reducing bacteria and SRB in the lower depths of the streamer/mat communities derives from acidophilic filamentous and unicellular micro-algae that comprise the bulk of the surface growths (Rowe *et al.*, 2007; Fig. 2.1). There is considerable merit and attraction in using photosynthesis to underpin bioremediative strategies, and there is a wide diversity of acidophilic and metal-tolerant algae that could, potentially, be used in such a context (Gross, 2000). Surface growths of micro-algae could, for example, sustain "bioshrouding" bacteria in tailings ponds, thereby providing a long-term passive solution for safeguarding reactive minerals (Fig. 2.3).

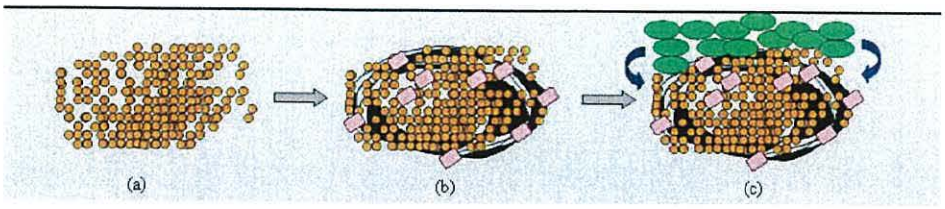

Fig. 2.3. Proposed evolution of mine tailings safeguarded by "bioshrouding": (a) freshly deposit reactive tailings; (b) minerals enshrouded with EPS following inoculation with heterotrophic bacteria (indicated as magenta rods); (c) surface colonization by acid-tolerant micro-algae (shown as green oval objects), which provide organic carbon to the bioshrouding bacteria, developing a self-sustaining ecosystem.

Engineering systems for removing metals from ARD by biomineralization also need to utilize low-cost and sustainable energy sources to be competitive with other remediation options. For biomineralization of iron, energy derives from the oxidation of ferrous iron itself though, as noted, additional alkalinity may be require to counterbalance proton production from ferric iron hydrolysis. While SRB are heterotrophic microorganisms (they use organic carbon as energy sources) many strains, including the novel acidophilic isolates, can also use hydrogen. In acidic environments, such as ARD, this may be derived conveniently from the dissolution of zero-valent iron. An example of how a biomineralization system could be engineered and integrated is shown in Fig. 2.4, where the target ARD (pH 2.2, and containing ca. 500 mg/l iron, 60 mg/l zinc and 50 mg/l copper) is ARD at the former Parys copper mine in North Wales. The sulfidogenic reactors are placed ahead of the iron-oxidation reactor to take advantage of the fact that water draining this mine (as with many others) is essentially anoxic at source. Sulfidogenesis provides the alkalinity required for comprehensive ferric iron hydrolysis in the third in-line reactor. The proposed system operates as a lime-free system, and the treated mine water could drain directly into the nearby (1 km) Irish Sea where it would pose no environmental risk. At present, the Parys ARD is not remediated, and an estimated 130 tonnes of iron, 16 tonnes of zinc and 13 tonnes of copper enter the sea each year from the untreated drainage stream.

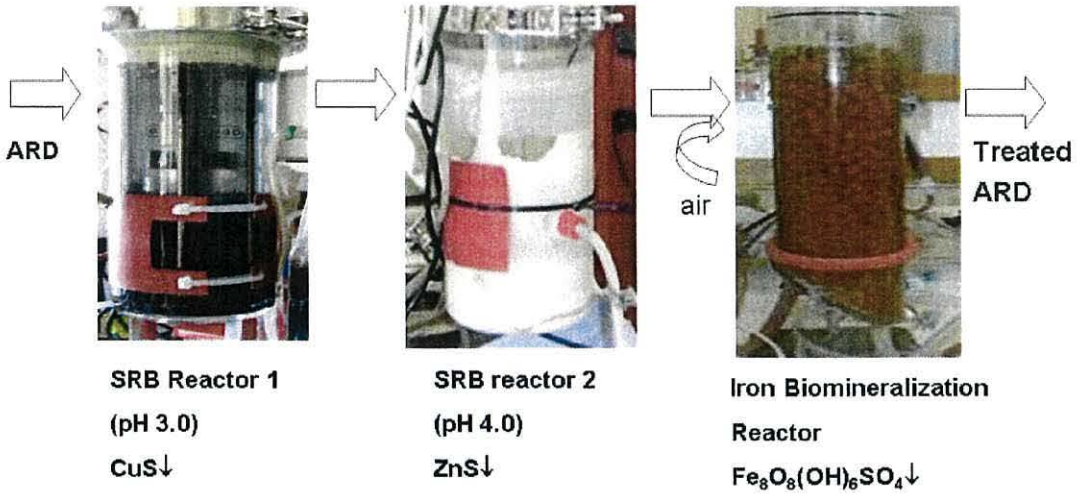

Fig. 2.4. Proposed bioremediation system for sequential removal of metals from Parys ARD.

2.7 General conclusions

Ecological engineering-based solutions offer new, sustainable and environmentally-benign solutions to the problem of mine water pollution. The potentials of novel microorganisms to secure mine wastes and to treat ARD have been demonstrated in the laboratory, and await validation as pilot- and full-scale operations.

Chapter 3

Production of Glycolic Acid by Chemolithotrophic Iron- and Sulfur-Oxidizing Bacteria and its Role in Delineating and Sustaining Acidophilic Sulfide Mineral-Oxidizing Consortia

Ivan Ňancucheo and D. Barrie Johnson

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Abstract

Glycolic acid was detected as an exudate in actively growing cultures of three chemolithotrophic acidophiles that are important in biomining operations: *Leptospirillum ferriphilum*, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus caldus*. Although similar concentrations of glycolic acid were found in all cases, this corresponded to ca. 24% of total dissolved organic carbon (DOC) in cultures of *L. ferriphilum*, but only ca. 5% in those of the two *Acidithiobacillus* spp.. Rapid acidification (to pH 1.0) of the culture medium of *At. caldus* resulted in a large increase in DOC, though the concentration of glycolic acid did not change in proportion. The archaeon *Ferroplasma acidiphilum* grew in the cell-free spent medium of *At. caldus*; glycolic acid was not metabolized though other unidentified compounds in the DOC pool were. Glycolic acid displayed similar levels of toxicity to twenty-one strains and species of acidophiles screened as acetic acid. The most sensitive species were chemolithotrophs (*L. ferriphilum* and *Acidithiobacillus ferrivorans*) while the most tolerant were chemo-organotrophs (*Acidocella*, *Acidobacterium* and *Ferroplasma*), and the ability to metabolize glycolic acid appeared to be restricted (among acidophiles) to *Firmicutes* (chiefly *Sulfobacillus* spp.). Results from this study help explain why *Sulfobacillus* spp. rather than other acidophiles are found as the main organic carbon-degrading bacteria in continuously-fed stirred tanks used to bioprocess sulfide mineral concentrates, and also why temporary cessation of pH control in these systems, resulting in rapid acidification, often results in a plume of the archaeon *Ferroplasma*.

3.1 Introduction

Extremely acidic environments (generally considered as those that have a pH of <3) are unusual in that chemo-autotrophs rather than photo-autotrophs are often the dominant (and sometimes the exclusive) agents of primary production (Johnson, 2009). There are two major reasons for this: (i) inorganic electron donors (ferrous iron, and reduced forms of sulfur) are often very abundant, as many of the most extremely acidic environments are found in sulfur-rich (e.g. solfatara springs) or sulfide-mineral-rich (e.g. metal mine wastes) locations, and (ii) in general, cyanobacteria, algae and higher plants are more sensitive than chemotrophic bacteria and archaea to the elevated concentrations of soluble transition metals and other solutes often present in acidic waters. Lithotrophy-based primary production may be evident in subterranean chambers (caves and worked-out mines) in the form of slimes and massive "acid streamer" growths (e.g. references Bond *et al.*, 2000, Johnson *et al.*, 1979; Macalady *et al.*, 2007). In lower temperature (<40°C) environments, micro-organisms that use energy derived from the oxidation of ferrous iron and/or reduced sulfur to fix CO_2 are predominantly bacteria, and include a number of species (e.g. bacteria of the genera *Acidithiobacillus* and *Leptospirillum,* and the archaeon *Ferroplasma*) that are known to be of fundamental importance in biomining operations (Rawlings and Johnson, 2007) and in the generation of acidic, metal-rich mine effluents (Nordstrom, 2000).

Commercial mineral bioprocessing operations that use large (often >1,000 m^3) stirred tanks operate under conditions of fixed temperature (generally 35-45°C) and pH (often ~1.5). The main primary producers identified in these systems are the iron-oxidizer *Leptospirillum ferriphilum* and the sulfur-oxidizer *Acidithiobacillus* (*At.*) *caldus*. The chemo-mixotrophic bacterium *Sulfobacillus* and the chemo-heterotrophic archaeon *Ferroplasma* are also often present, though in smaller numbers (Rawlings and Johnson, 2007).The acid-generating nature of pyrite oxidation means that continuous addition of an alkaline material (such as lime) is necessary to maintain the required pH of the leach liquor. When, on occasions, the pH of the liquors have decreased due to temporary failure of the control mechanisms, the microbial communities within the tanks have been observed to change markedly, with greatly increased numbers of *Ferroplasma* relative to the iron- and sulfur-oxidizing autotrophic bacteria (D.E. Rawlings, Stellenbosch University, personal

communication). The same trends have been observed in pilot-scale (Okibe *et al.*, 2003) and laboratory-scale (Okibe and Johnson, 2004) systems.

Acidophilic chemolithotrophic bacteria, like other autotrophs, lose significant amounts of the carbon that they fix as small molecular weight compounds during active growth (exudates) as well due to lysis of dead and dying cells. Schnaitman and Lundgren (1965) estimated that 9.6% of labelled carbon ($^{14}CO_2$) was leaked by *Acidithiobacillus ferrooxidans* into its growth medium, and identified pyruvic acid as one of the small molecular weight exudates. Borichewski (1967) reported that, in cultures of *At. thiooxidans* grown on elemental sulphur, keto acids accumulated to concentrations which inhibited growth of the bacterium. Oxaloacetic acid and, again, pyruvic acid, were identified in cell-free culture media, and removal of these organic acids by dialysis eliminated growth inhibition. Okibe and Johnson (2004) found that dissolved organic carbon (DOC) accumulated to 88 +/- 17 mg l^{-1} in cultures of the thermo-tolerant iron-oxidizer *L. ferriphilum* grown on pyrite, and to > 100 mg L^{-1} when this acidophile was grown in co-culture with the sulfur-oxidizer *At. caldus*. Inclusion of organic carbon-degrading acidophiles (*Acidimicrobium ferrooxidans* or a *Ferroplasma* sp.) resulted not only in far smaller concentrations of DOC being detected in cultures but also, in most cases, in more rapid dissolution of pyrite. Heterotrophic and/or mixotrophic acidophiles are inevitably found growing alongside chemo-autotrophic primary producers in natural (Johnson *et al.*, 1979) and anthropogenic (Okibe *et al.*, 2003; Rawlings and Johnson, 2007) environments.

Chemo-autotrophic acidophilic bacteria use different pathways for assimilating carbon dioxide (Johnson and Hallberg, 2009; Levican *et al.*, 2008). *At. ferrooxidans* and *At. thiooxidans* (both mesophiles) use the Calvin-Benson-Bassham (CBB) pathway, while *L. ferriphilum* is thought to utilize the reductive tricarboxylic acid (rTCA) cycle. Genes coding for enzymes involved in the CBB cycle have also been found in the moderate thermophile, *At. caldus* (Valdes *et al.*, 2008). A key enzyme in the CBB cycle is ribulose bisphosphate carboxylase oxygenase (RuBisCO). Besides combining carbon dioxide and ribulose bisphosphate (RUBP), RuBisCO also oxidizes RUBP to phosphoglyceric acid and phosphoglycolate. Enzymic hydrolysis of the latter produces glycolate, much of which is exported out of actively-growing cells. Glycolate has been detected as an exudate in cultures of marine and freshwater algae (Wright and Shah, 1975), photosynthetic bacteria such as *Rhodospirillum rubrum* (Storro and Mcfadden, 1981), the facultative chemolithotroph *Alcaligenes eutrophus* (Codd *et al.*, 1976), and the neutrophilic

sulfur-oxidizing bacterium *Thiobacillus neapolitanus* (Cohen *et al.*, 1979). Glycolic acid ($CH_2OHCOOH$) has not been reported previously as an exudate in cultures of acidophilic chemo-autotrophic bacteria, yet it could be of considerable significance not only in sustaining organic carbon-utilizing acidophiles but also because of its potential biotoxic effect. The pK_a of glycolic acid is 3.83, which means that at the pH of extremely acidic environments it exists almost exclusively as the more toxic undissociated form, rather than the glycolate anion.

Here we report the production and excretion of glycolic acid by three species of chemo-autotrophic acidophiles that are important in biomining operations, the relative sensitivities of acidophilic microorganisms to this acid, and the varying abilities of mixotrophic and heterotrophic acidophiles to metabolize this compound.

3.2 Materials and Methods

3.2.1 ***Microorganisms and growth media.*** Twenty-one strains (nineteen different species) of acidophilic microorganisms, comprising chemo-autotrophic bacteria, mixotrophic bacteria and obligately heterotrophic acidophiles, and one acidophilic archaeon (*Ferroplasma acidiphilum*), were used in the present study (Table 3.1). The growth media used varied in composition depending on the nutritional requirements of the different acidophiles, and a common basal salts and trace elements mix (Wakeman *et al.*, 2008) was used throughout (trace elements were not included when yeast extract was added to the culture media). Iron-oxidizing chemo-autotrophs were grown routinely in media containing 20 mM ferrous iron (pH 1.7 -2.2, depending on pH growth optima), and sulfur-oxidizers that do not oxidize iron (*At. thiooxidans* and *At. caldus*) in a similar medium in which elemental sulfur (at 5%, w/v) replaced ferrous sulfate. Mixotrophic and heterotrophic iron-oxidizers were grown routinely in a liquid medium containing 10-20 mM ferrous iron supplemented with 0.02% (w/v) yeast extract (at pH 1.8 – 2.0). Heterotrophic iron-reducing acidophiles were grown in basal salts/trace elements supplemented with 5 mM glucose plus 0.001% yeast extract (*Acidiphilum* SJH and *Acidobacterium capsulatum*) or 5 mM fructose (*Acidocella* PFBC). The pH of these media were adjusted to either 2.5 (*Acidiphilium* SJH and *Acidocella* PFBC) or 3.0 (*Ab. capsulatum*). Finally, the archaeon *Fp. acidiphilum*, which does not possess a cell wall, was grown in a higher osmotic potential growth medium containing 50 mM

ferrous sulfate, 50 mM potassium sulfate, 0.02% (w/v) yeast extract, and basal salts (pH 1.5).

TABLE 3.1. Acidophilic bacteria and archaea used in the present study.

Species	Strain	Reference
Iron-Oxidizing Autotrophic Bacteria		
Leptospirillum ferrooxidans	Type	(Hippe, 2000)
*Leptospirillum ferriphilum**	Type	(Coram and Rawlings,2002)
*"Leptospirillum ferrodiazotrophum"**	UBA1	(Tyson *et al.*, 2005)
Iron- and Sulfur-Oxidizing Autotrophic Bacteria		
Acidithobacillus ferrooxidans	Type	(Kelly and Wood, 2000)
Acidithiobacillus ferrivorans	Type	(Hallberg *et al.*,2010)
Sulfur-Oxidizing Autotrophic Bacteria		
*Acidithiobacillus caldus**	Type	(Hallberg and Lindstrom, 1994)
Acidithiobacillus thiooxidans	Type	(Kelly and Wood, 2000)
Iron-Oxidizing Mixotrophic Bacteria		
*Acidimicrobium ferrooxidans**	TH3	(Clark and Norris, 1996)
Iron- and Sulfur-Oxidizing Mixotrophic Bacteria		
*Sulfobacillus thermosulfidooxidans**	Type	(Karavaiko *et al.*, 2000)
*Sb. thermosulfidooxidans**	TH1	(Brierley *et al.*,1978)
*Sulfobacillus acidophilus**	ALV	(Norris *et al.*, 1996)
*Sb. acidophilus**	YTF1	(Johnson *et al.*,2001)
*Sulfobacillus benefaciens**	Type	(Johnson *et al.*,2008b)
*Sulfobacillus thermotolerans**	L15	(Johnson *et al.*,2001)
Firmicute sp.*	G1	(Johnson *et al.*,2005)
Iron-Oxidizing Heterotrophic Bacteria		
Ferrimicrobium acidiphilum	Type	(Johnson *et al.*,2009)
Firmicute sp.	SLC1	(Johnson *et al.*,2001)
Iron-Reducing Heterotrophic Bacteria		
Acidiphilium sp.	SJH	(Bridge and Johnson, 2000)
Acidocella sp.	PFBC	(Kimura *et al.*, 2006)
Acidobacterium capsulatum	Type	(Kishimoto *et al.*, 1991)
Iron-Oxidizing Archaeon		
*Ferroplasma acidiphilum**	BRGM4	Johnson *et al.* (unpublished)

Cultures were incubated at 30° (or 37°C, indicated by *)

3.2.2. *Bioreactor cultures.* *L. ferriphilum, At. ferrooxidans* and *At. caldus* were each grown in 2 liter bioreactors (with 1.0 liter working volumes; Electrolab Ltd., U.K.) in which pH and temperature were maintained at pre-determined levels. In the case of *L. ferriphilum*, the growth medium was 5% (w/v) pyrite (Strem Chemicals,

Newburyport, MA) and pH and temperature were maintained at 1.7 and 37°C, respectively. Both *At. ferrooxidans* and *At. caldus* were grown on 5% (w/v) elemental sulfur (VWR, UK) at pH 2.5 and 30°C (*At. ferrooxidans*) or 45°C (*At. caldus*). All bioreactors were aerated (1 liter min^{-1}) and stirred at 150 rpm. Samples were withdrawn at regular intervals to measure concentrations of ferrous and total iron (pyrite culture) or sulfate (sulfur cultures), dissolved organic carbon (DOC), glycolic acid and numbers of planktonic-phase cells. After the bioreactor cultures had been running for between 10 and 13 days, they were subjected to a "pH shock", which entailed a rapid lowering of culture pH (to pH 1.0, completed within 10 mins.) by automated addition of 1 M sulfuric acid. This was designed to mimic the occasional failure of pH control in stirred tank biomining operations. Samples were withdrawn either side of subjecting cultures to this pH shock to measure the parameters listed above, and also numbers of total and viable bacteria.

3.2.3 *Growth of Fp. acidiphilum on cell-free medium of At. caldus*. Four days after the pH of the *At. caldus* bioreactor culture was lowered to 1.0, the reactor was drained, and bacterial cells and residual sulfur were removed by centrifugation (15,000 RCF, 15 minutes) followed by filtration through 0.2 μm (pore size) cellulose nitrate membrane filters (Whatman, U.K.). Four hundred milliliter aliquots were dispensed into three sterile 1 liter conical flasks, and filter-sterilized ferrous sulfate (1 M, pH 2.0) added to a final concentration of 20 mM. Two of the flasks were inoculated with 10 ml of an actively growing culture of *Fp. acidiphilum* (strain BRGM4), with the third acted as a non-inoculated control. The cultures were incubated with shaking (150 rpm) at 37°C, and samples withdrawn every two days to determine concentrations of DOC, glycolic acid, ferrous iron, and to enumerate total numbers of cells.

3.2.4 *Screening acidophilic prokaryotes for sensitivities to glycolic acid.* The acidophilic prokaryotes listed in Table 1 were tested for growth in the presence of different concentrations of glycolic acid. Small volume (5 ml) cultures were set up in 20 ml universal bottles using the liquid media described previously. Glycolic acid was added to these to give final concentrations of 0.1, 0.5, 1.0, 2.5 or 5.0 mM. In order to assess the relative sensitivities of these acidophiles to acetic acid, cultures containing the same concentrations of acetic acid were set up simultaneously, and organic acid-free cultures were prepared as controls. These were incubated at 30° or 37°C for up to 10 days, and positive or negative growth scored on the basis of

changes in cell numbers and, in the case of iron-oxidizing prokaryotes, by the production of ferric iron.

3.2.5 ***Metabolism of glycolic acid by acidophilic prokaryotes.*** The mixotrophic and heterotrophic acidophiles listed in Table 1 were assessed for their abilities to metabolize glycolic acid. Positive cultures from the toxicity screening experiment that contained 0.1 mM glycolic acid were used as inocula in each case. Mixotrophic acidophiles were subcultured into a liquid medium (10 ml aliquots in universal bottles) containing 0.1 mM glycolic acid, 10 mM ferrous iron (plus basal salts and trace elements) at pH 1.9, and the iron-reducing heterotrophic bacteria into the same medium but with no added iron (pH 2.5 for *Acidocella* PFBC and *Acidiphilium* SJH, and 3.0 for *Ab. capsulatum*). Cultures were incubated at 30° or 37°C for up to 20 days, and samples removed at regular intervals to measure concentrations of glycolic acid. Non-inoculated controls were also set up in order to account for any glycolic acid lost via evaporation or by abiotic means.

For those acidophiles that showed an apparent ability to metabolize glycolic acid, a second experiment was set up in which the time course of glycolic acid removal was recorded. Cultures were inoculated into 20 ml of growth medium (as previous) in duplicate 100 ml conical flasks, which were incubated (at 30° or 37°C) and shaken at 150 rpm. Samples were removed every two days to measure concentrations of glycolic acid.

3.2.6 ***Analytical techniques.*** Glycolic acid was determined using ion chromatography (IC) using a Dionex-IC25 ion chromatograph fitted with an IonPac® AS-11 column equipped with a conductivity detector (Dionex Inc., U.S.A.), and occasionally using a colorimetric technique (Calkins, 1943). The IC protocol used enabled separation of glycolic and pyruvic acids, and was used routinely except where excessively high concentrations of sulfate (from microbial oxidation of elemental sulfur in bioreactors) precluded adequate separation of glycolate and sulfate. Dissolved organic carbon was measured using a LABTOC DOC analyser (Pollution and Process Monitoring, U.K.). Concentrations of ferrous iron were measured using the ferrozine colorimetric assay (Lovley and Phillips, 1987) and sulfate using a turbidometric technique (Kolmert *et al.*, 2000). Total iron was determined by ion chromatography, using a Dionex-320 ion chromatograph fitted with an IonPac® CS5A column and an AD25 absorbance detector). pH was measured

using a pHase combined electrode (VWR, U.K.) coupled to an Accumet pH meter50 (Cole-Palmer, Vernon Hills, IL). Microbial cells were enumerated using a Helber counting chamber marked with Thoma ruling (Hawksley, U.K.) and viewed with a Leitz Labolux phase contrast microscope (at x400 magnification). Viable bacteria were enumerated by plating onto selective solid media (Johnson, 1995b).

3.3 Results

In bioreactor cultures of *L. ferriphilum*, *At. ferrooxidans* and *At. caldus,* there was a general increase in the concentrations of both total DOC and glycolic acid as oxidation of pyrite or elemental sulfur progressed, though concentrations of DOC were much greater in the bioreactor containing *At. caldus* bioreactor than in the other two (Fig. 3.1). Pyruvic acid was not detected by IC analysis. Numbers of planktonic cells did not parallel DOC and glycolic acid concentrations, presumably because of the attachment of the bacteria to the solid substrates present. There was no obvious formation of biofilms on the bioreactor vessel walls. The pH shock induced by rapid acidification of the bioreactors caused different perturbations to the three bacterial cultures. With *L. ferriphilum* and *At. ferrooxidans*, concentrations of both DOC and glycolic acid increased after the pH had been lowered to 1.0, but at a similar rate to that before this change was imposed. The pH shock caused the oxidation of pyrite by *L. ferriphilum* and of sulfur by *At. ferrooxidans* to come to a halt, as evidenced by no net increases in concentration of soluble iron and sulfate being detected for the following two days. Sulfur oxidation was also arrested by the pH shock event in the bioreactor culture of *At. caldus*, though with this acidophile the sudden fall in pH also resulted in a large (80%) increase in total DOC concentration within 48 hours, though there was only a 25% increase in the concentration of glycolic acid within the same time period.

In order to estimate the net contribution of glycolic acid to the total DOC pool in the bioreactor cultures, concentrations of glycolic acid were converted to carbon equivalents (as mg l^{-1}). Data, listed in Table 3.2, show that, although concentrations of glycolic acid were similar in all three cultures prior to the acidification of the bioreactors, this aliphatic acid accounted for a far higher percentage of the total DOC

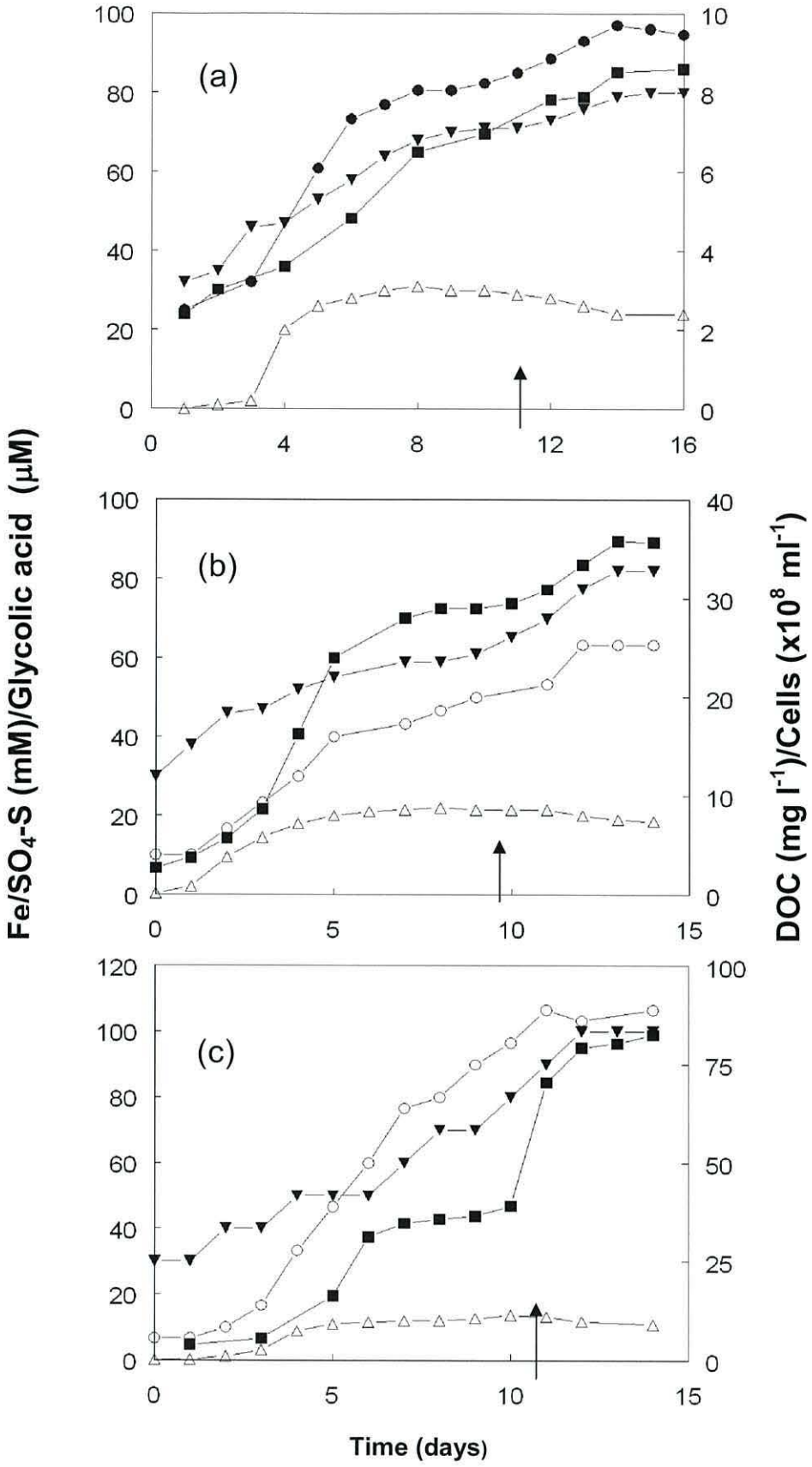

Fig. 3.1. Changes in concentrations of dissolved organic carbon (DOC) and glycolic acid in bioreactor cultures of (a) *L. ferriphilum*, (b) *At. ferrooxidans* and (c) *At. caldus*. Key: •, total soluble iron (*L. ferriphilum*); o, sulfate-S (*At. ferrooxidans* and *At. caldus*); ■, DOC; ▼, glycolic acid (colorimetric assay); Δ, total cell numbers. The arrows indicate the time at which the "pH shock" (rapid lowering of bioreactor pH to 1.0) was imposed.

present in the *L. ferriphilum* culture than in either of the *Acidithiobacillus* spp. cultures. It was also found that, with both *L. ferriphilum* and *At. ferrooxidans*, there were only minor changes in glycolic acid/DOC ratios immediately before and subsequent to the pH shock event, whereas in the case of *At. caldus* the percentage of total DOC present as glycolic acid fell by 41% following the acidification of the reactor.

TABLE 3.2. Percentages of total dissolved organic carbon present as glycolic acid carbon in bioreactor cultures of *L. ferriphilum, At. ferrooxidans* and *At. caldus*, before and after the "pH shock" event.

Bacterium (bioreactor pH)	Glycolic acid (mg C l^{-1})	DOC (mg l^{-1})	% total DOC present as glycolic acid
L. ferriphilum			
- pH 1.7	1.70	7.0	24
- pH 1.0	1.92	8.6	22
At. ferrooxidans			
- pH 2.5	1.46	29	5.0
- pH 1.0	1.97	36	5.5
At. caldus			
- pH 2.5	1.92	39	4.9
- pH 1.0	2.40	82.5	2.9

Rapid acidification of the bioreactors also impacted the viabilities of the three chemoautotrophic bacteria to different extents, as evidenced by their abilities to grow on solid media. While total numbers of the three bacterial species remained fairly similar for up to two days following adjustment of pH of the bioreactors to 1.0, plate counts declined far more severely in the case of *At. caldus* than with *L. ferriphilum* or *At. ferrooxidans* (Fig. 3.2). Numbers of cultivatable *At. caldus* fell by >99.8% within one day of culture acidification, and continued to decline subsequently, though at a slower rate (data not shown).

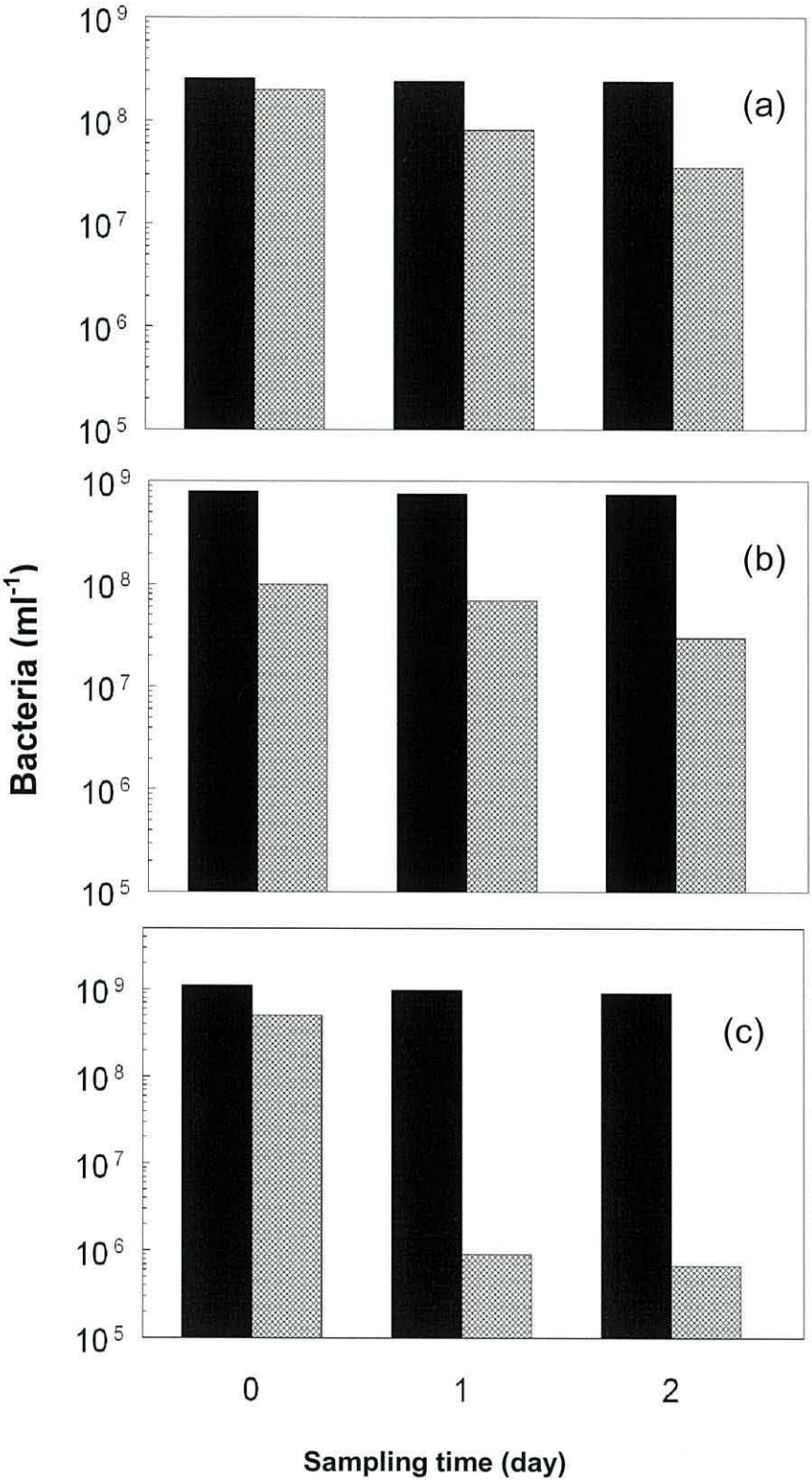

Fig. 3.2. Changes in total numbers (solid bars) and viable counts (hatched bars) of (a) *L. ferriphilum*, (b) *At. ferrooxidans,* and (c) *At. caldus*, immediately before (day 0) and 1 and 2 days following acidification of the bioreactors to pH 1.0.

The archaeon *Fp. acidiphilum* was able to grow on the spent cell-free bioreactor medium of *At. caldus*. Data in Fig. 3.3 show that cell numbers of *Fp. acidiphilum* increased and all of the available ferrous iron was oxidized within 9 days in amended spent medium from the *At. caldus* bioreactor. However, only about 25% of the total DOC was metabolized over this period and there was no significant change in the concentration of glycolic acid. The cessation of growth of *Fp. acidiphilum* after day 9 suggests that the residual DOC (including glycolic acid) could not be metabolized by this archaeon. No changes in concentrations of DOC, glycolic acid or ferrous iron were observed in control cultures containing sterile spent medium of *At. caldus*.

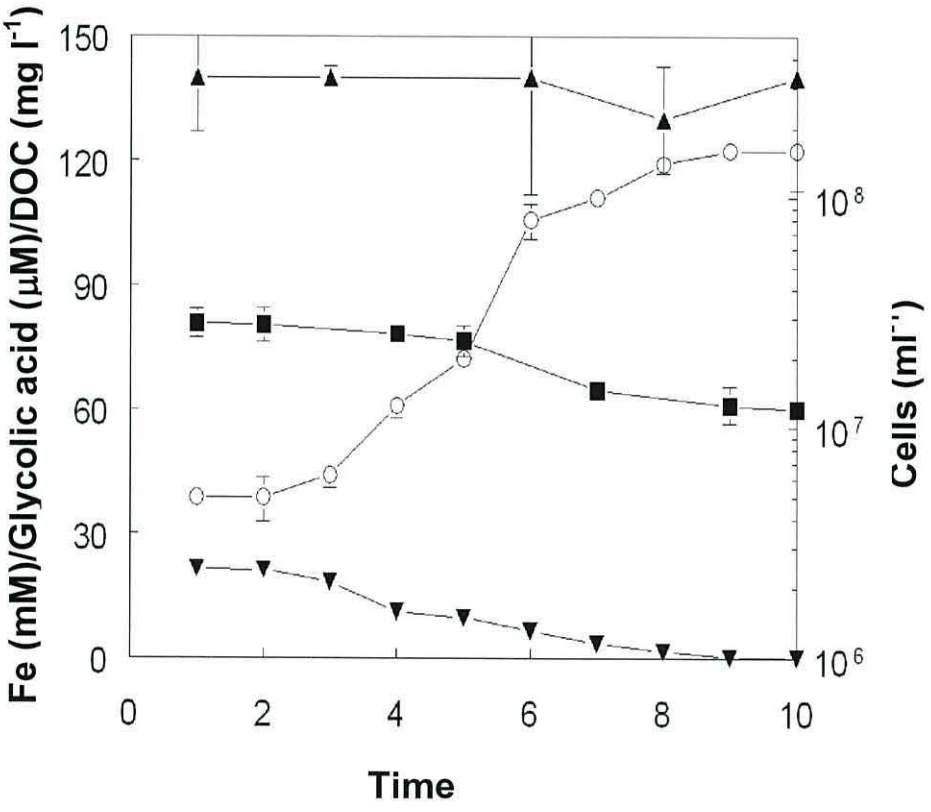

Fig. 3.3. Growth of *Fp. acidiphilum* on the spent medium of *At. caldus*. Key: ▼, Fe^{2+} (mM); ■, DOC (mg l^{-1}); ▲, glycolic acid (μM); ○, cell numbers. The symbols indicate means for duplicate cultures, and the error bars indicate ranges.

Glycolic acid displayed similar levels of toxicity to the 21 species and strains of acidophilic prokaryotes tested to acetic acid (data not shown). While all of the acidophiles tested could grow in the presence of 100 μM glycolic acid, progressively fewer species were able to grow at higher concentrations (Table 3.3). The most sensitive of the acidophiles tested to both acids were the obligate chemoautotrophs *L. ferriphilum* and *At. ferrivorans*, while the least sensitive of the acidophiles tested were all chemo-organotrophs (*Fp. acidiphilum, Acidocella* PFBC and *Ab. capsulatum*).

The sensitivity of *At. ferrooxidans* (which can grow in both sulfur-containing and ferrous iron media) to glycolic and acetic acids was similar regardless of energy source (data not shown) indicating that the trends observed were not related to the different energy sources used.

TABLE 3.3. Comparison of the relative toxicities of glycolic acid to 21 strains (19 different species) of acidophilic microorganisms.

Maximum glycolic acid concentration permitting growth (mM)	Acidophile species (strain)
0.1	*L. ferriphilum*^{T}, *At. ferrivorans*^{T}
0.5	*L. ferrooxidans*^{T}, *At. caldus*^{T}
1.0	"*L. ferrodiazotrophum*" (UBA1), *Am. ferrooxidans*^{T}, *Sb. thermosulfidooxidans*^{T}, *Sb. thermosulfidooxidans* (TH1), *Sb. acidophilus* (ALV), *Sb. acidophilus* (YTF1), *Sb. benefaciens*^{T}, *Sb. thermotolerans* (L15), *Firmicute* (G1), *Fm. acidiphilum*^{T}, *Firmicute* (SLC1)
2.5	*At. ferrooxidans*^{T}, *At. thiooxidans*^{T}, *Acidiphilium* (SJH)
5.0	*Acidocella* (PFBC), *Ab. capsulatum*, *Fp. acidiphilum* (BRGM4)

Of the fourteen (mixotrophic and heterotrophic) acidophiles tested for their abilities to metabolize glycolic acid, five were confirmed as positive and all of these were *Firmicutes* (*Sb. benefaciens*^{T}, *Sb. acidophilus* (both strains), *Sb. thermosulfidooxidans* (strain TH1) and the unclassified isolate SLC1 (Fig. 3.4). The type strain of *Sb. thermosulfidooxidans* showed only limited (30%) degradation of glycolic acid over 14 days, which was similar to the unclassified *Firmicute* isolate G1.

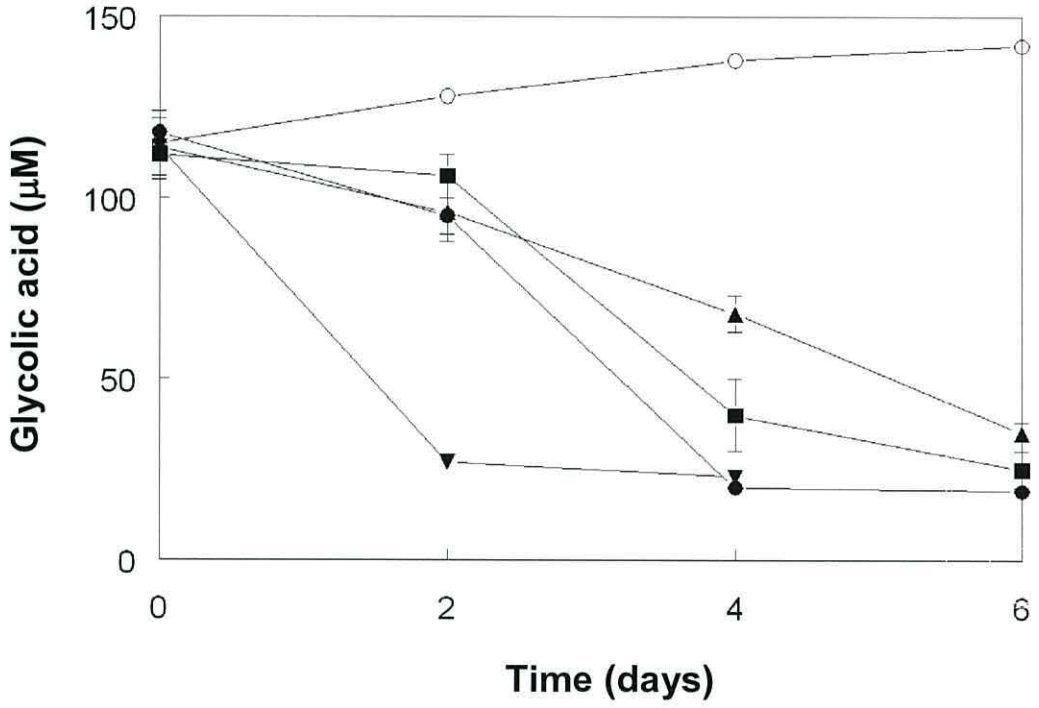

Fig. 3.4. Degradation of glycolic acid by acidophilic *Firmicutes*. Key: ▲, *Sb. thermosulfidooxidans* strain TH1; ■, *Sb. acidophilus* strain YTF1; ▼, *Sb. benefaciens*^{T}; ●, isolate SLC1; o, non-inoculated control. Data points show means of duplicate cultures and error bars (where visible) range of glycolic acid concentrations.

3.4 Discussion

Glycolic acid was detected in cell-free culture liquors of three chemolithotrophic bacteria (*L. ferriphilum, At. ferrooxidans* and *At. caldus*) that are considered to have major roles in the oxidative dissolution of sulfide minerals in acidic environments and biomining operations (Rawlings and Johnson, 2007). The identity of glycolic acid was confirmed using both ion chromatography and the colorimetric technique used. The latter was claimed by Calkins (1943) to be free from interference from commonly-encountered small molecular weight organic acids, as was found (with acetic and pyruvic acids) in the present study. In contrast to previous reports, we did not detect pyruvic acid as a microbial exudate. The fact that concentrations of glycolic acid paralleled those of DOC during culture growth indicated that this organic acid was an exudate of actively growing cells rather than a lysate product of dead bacteria, This was confirmed in the case of *At. caldus* where the pH-shock event led to mass mortality of the cells and a rapid and large increase in DOC but only a relatively small increase in glycolic acid. While DOC values (prior to the pH-shock) varied between 7 and 39 mg l^{-1} for the three bioreactor cultures, this was mostly related to differences in cell numbers, and plots of total bacteria versus DOC concentrations for the three cultures showed a linear fit with a regression coefficient of 0.999 (data not shown).

The presumed origin of the glycolic acid in cultures of the two *Acidithiobacillus* spp. is considered to be due to RuBisCO acting as an oxygenase, and both bacteria use the CBB pathway for carbon assimilation. In the case of *L. ferriphilum*, which is thought (from genomic analysis) to use the rTCA cycle, the biochemical pathway which might have over-produced glycolic acid resulting in its excretion from viable cells is unknown. A gene encoding for phosphoglycolate phosphatase, an enzyme that produces glycolate from phosphoglycolate, has been annotated in the genome of "*Leptospirillum rubarum*", a proposed novel species that is closely related to *L. ferriphilum* (Aliaga *et al.*, 2009). The origin of the phosphoglycolate itself as an intermediate in the central metabolism of *Leptospirillum* spp. is currently unclear. This apparent conundrum is accentuated by the fact that the percentage of DOC present as glycolic acid was far greater (24%) in the *L. ferriphilum* culture than in those of the *Acidithiobacillus* spp., where it accounted for about 5% of total DOC.

The effect of rapid acidification of the bioreactor cultures ("pH shock") was far more lethal to *At. caldus* than to the other two bacteria investigated. The percentage

of viable *At. caldus* cells (determined from plate counts relative to total counts) fell from 45% to <1% within one hour of lowering the bioreactor pH to 1.0; corresponding figures for the *L. ferriphillum* and *At. ferrooxidans* cultures were from 77 to 33%, and from 13 to 9%, respectively. These different responses do not directly correlate with the pH growth optima and minima described for these acidophiles (2.0-2.5 and ~1.0 for *At. caldus*^{T}, 1.5 and 0.8 for *L. ferriphilum* (strain MT6) and 2.0-2.5 and ~1.4 for *At. ferrooxidans*^{T}) and are probably more related to the different degrees of stress induced by the rapidity of the acidification of the cultures.

In general, the toxicity of glycolic acid to the acidophiles tested was similar to that of acetic acid, though both of these acids appear to be less toxic to these extremophiles than pyruvic acid, which was reported to completely inhibit the growth of *At. thiooxidans* when added at between 20 and 40 μM (Borichewski, 1967). The most glycolic acid-tolerant acidophiles were all heterotrophic. Chemo-autotrophic acidophiles in general, and *Leptospirillum* spp. in particular, tend to be more sensitive to small molecular weight organic compounds than their mixotrophic and heterotrophic counterparts (Johnson, 1995b). The concentration of glycolic acid required to inhibit the growth of *L. ferriphilum* (the most important primary mineral-oxidizer in stirred tank biomining operations) was 500 μM, which was 5-6 times greater than concentrations of the acid recorded in the three bioreactor cultures. However, concentrations of DOC have been reported to increase to 88+/-17 mg l^{-1} in pure cultures of *L. ferriphilum* (Okibe and Johnson, 2004). Assuming that, as in the present study, about 24% of DOC was present as glycolic acid-carbon, this gives an equivalent concentration of about 870 μM glycolic acid, a concentration that would be predicted to be inhibitory to the iron-oxidizer.

One of the more intriguing findings was that, of all the organic carbon-degrading acidophiles investigated, the ability to metabolize glycolic acid was only observed for *Firmicutes*, and it appeared to be a widespread trait within the eight species and strains (mostly *Sulfobacillus*) tested. It was particularly surprising to find that *Acidocella* PFBC, a strain of the proposed species "*Acidocella aromatica*", which has been found to be adept at growing on small molecular weight organic acids, including acetic acid (Gemmell and Knowles, 2000), was not able to metabolize glycolic acid.

The results of this study help explain the compositions of microbial consortia in biomining operations, particularly those using continuously-fed stirred tanks (Rawlings and Johnson, 2007). In the majority of these, the numerically-dominant acidophiles present are the iron-oxidizer *L. ferriphilum*, and the sulfur-oxidizer *At. caldus*, both of which fix inorganic carbon. Other bacteria are also found, but these tend to be exclusively *Sulfobacillus* spp. (often *Sb. benefaciens*, though *Sb. thermosulfidooxidans* is more common when bioleaching copper-containing mineral concentrates). *Sulfobacillus* spp. are iron- and sulfur-oxidizing mixotrophs (they preferentially utilize organic carbon, though they are able to fix CO_2) but they probably have only a minor impact in net mineral oxidation, and their role in degrading organic carbon compounds, thereby preventing accumulation and potential toxicity to the "organic sensitive" chemo-autotrophs, is probably more important to the efficient operation of stirred tank systems. Other mixotrophic or heterotrophic bacteria, such as the iron-oxidizer *Acidimicrobium ferrooxidans*, have not been detected in commercial stirred tank operations, even though physico-chemical conditions within them are conducive to their growth. Part of the reason for this might be that the ability of *Sulfobacillus* spp. to metabolize glycolic acid, which is provided on a continuous basis by the primary producers in the tanks, gives the *Firmicutes* a competitive advantage. On the other hand, iron-oxidizing heterotrophic *Ferroplasma* spp. are often also detected in stirred tanks, even though (on the basis of the single isolate used in the current study, which was isolated from a tank system bioleaching cobaltiferous pyrite) these archaea appear not able to metabolize glycolic acid. *Ferroplasma* appears adept at scavenging organic carbon released from dead and dying cells, as supported here by their growth in cell-free medium from the *At. caldus* bioreactor collected after the pH shock event. In a previous study (Okibe *et al.*, 2003) of an stirred tank bioleaching pilot-scale system, comprising of three in-line tanks, *Ferroplasma* was not detected in the first of tanks, though it outnumbered the bacteria (*L. ferriphilum, At. caldus* and a *Sulfobacillus* sp.) in tanks 2 and 3. Its emergence in tank 2 and dominance in tank 3 coincided with the demise and death of a large proportion of the bacterial population. Data from the present study strongly suggests that this is due to a rapid input of DOC into the mineral leachate from killed *At. caldus*, rather than from *L. ferriphilum.*

This study has highlighted the importance of glycolic acid in acidophile microbiology. While its excretion by chemo-autotrophic iron- and sulfur-oxidizing bacteria has the potential to inhibit the bacteria that are key players in commercial bioprocessing of minerals, glycolic acid-metabolizing *Sulfobacillus* spp. can eliminate this, highlighting further the importance of microbial consortia in biomining operations.

Chapter 4

Dissolved Organic Carbon in Cultures of Acidophilic Algae and its Role in Sustaining Heterotrophic Acidophilic Bacteria

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Abstract

Two acidophilic algae, identified as strains of *Chlorella protothecoides* var. *acidicola* and *Euglena mutabilis*, were isolated in pure culture from abandoned copper mines in Spain and Wales and grown in pH- and temperature-controlled bioreactors. The *Chlorella* isolate grew optimally at pH 2.5 and 30 °C, with a corresponding culture doubling time of 9 hours. The isolates displayed similar tolerance (10-50 mM) to four transition metals tested. Growth of the algae in liquid media was paralleled with increasing concentrations of dissolved organic carbon (DOC). Glycolic acid was identified as a significant component (12- 14%) of total DOC. Protracted incubation resulted in concentrations of glycolic acid declining in both cases, and glycolic acid added to a culture of *Chlorella* incubated in the dark was taken up by the alga (~100% within three days). Two monosaccharides were identified in cell-free liquors of each algal isolate: fructose and glucose (*Chlorella*), and mannitol and glucose (*Euglena*). These were rapidly metabolised by acidophilic heterotrophic bacteria (*Acidiphilium* and *Acidobacterium* spp.) though only fructose was utilised by the more fastidious heterotroph "*Acidocella aromatica*". The significance of algae in promoting the growth of iron- (and sulfate-) reducing heterotrophic acidophiles that are important in remediating mine-impacted waters is discussed.

4.1 Introduction

Mining of metals and coal impacts the environment in many ways. One of the most widely documented is the generation of mine-impacted water bodies (MIWs; drainage streams and pit lakes) that are characteristically acidic (sometimes extremely so) and which contain elevated concentrations of iron and other transition metals, aluminium and sulfate. These constitute an "extreme" environment, which is hostile to most life forms (Johnson, 2009). In the most severe cases, indigenous organisms are exclusively microbial and predominantly prokaryotic. Eukaryotic microorganisms, including acidophilic and acid-tolerant species of microalgae, fungi and yeasts, protozoa and rotifera, have, however, been reported in MIWs on a number of occasions (Das *et al.*, 2009; Aguilera *et al.*, 2007; Baker *et al.*, 2004).

Primary production in MIWs is mediated by autotrophic microorganisms that use either solar or chemical energy to fuel carbon dioxide fixation. In underground locations, chemolithotrophic acidophilic bacteria and archaea that use ferrous iron, reduced sulfur (and possibly hydrogen) as electron donors are the sole agents of primary production (Bond *et al.*, 2000; Kimura *et al.*, 2011). Mechanisms of carbon fixation and other physiological characteristics of bacteria such as *Acidithiobacillus* spp. and *Leptospirillum* spp. have been well studied, not only because these mineral-oxidising acidophiles are considered to be the most important micro-organisms involved in the genesis of MIWs, but also because the same bacteria are considered to be the most significant agents of metal extraction in commercial "biomining" operations (Rawlings & Johnson, 2007). However, where solar energy is available, the net contribution of chemolithotrophic bacteria to net carbon fixation in MIWs can be far less than that by acidophilic algae, since the most abundant chemical energy source in the majority of mine waters (ferrous iron) is a relatively poor electron donor in terms of its free energy (ΔF_{298} of -73 kJ; Kelly, 1978).

The biodiversity of known acidophilic and acid-tolerant algae is relatively limited (Novis & Harding, 2007). Micro-algae reported to be metabolically active in metal-rich, highly acidic environments include species of Chlorophyta, such as *Chlamydomonas acidophila* and *Dunaliella acidophila*; Chrysophyta, such as *Ochromonas* sp.; and Euglenophyta, such as *Euglena mutabilis*. Some diatoms, including several *Eunotia* spp., have also been found to colonize extremely acidic waters. Filamentous algae, identified as *Zygnema circumcarinatum* and *Klebsormidium acidophilum*, have been found in extremely acidic (pH <3) mine

waters in Spain and New Zealand, respectively (Rowe *et al.*, 2007; Novis & Harding, 2007). Species of moderately thermophilic and acidophilic Rhodophyta (*Cyanidium caldarium*, *Galdieria sulfuraria* and *Galdieria maxima*) are frequently encountered in acidic waters in geothermal areas (Toplin *et al.*, 2008). In contrast to acidophilic autotrophic bacteria, there have been few reports of laboratory studies of axenic cultures of acidophilic microalgae, presumably due to difficulties in obtaining cultures of these eukaryotes that are free of bacteria. Axenic cultures are necessary, for example, to identify and quantify the amounts of organic carbon released into the environment by acidophilic algae, a phenomenon that has previously been reported for chemolithotrophic acidophiles (Ňancucheo & Johnson, 2010).

Macroscopic growths of acidophilic algae have been reported in streams and rivers in the Iberian Pyrite Belt (IBP) in south-western Spain, including the Rio Tinto. Aguilera *et al.* (2007) found that the diversity of indigenous algae in biofilm growths was related to the acidity and metal contents of sites within the Rio Tinto, and that algal populations displayed seasonal trends. Initially, biofilm growths were dominated by flagellated green algae (*Dunaliella* or *Chlamydomonas*), and *Euglena*. Later sessile species of algae such as *Chlorella* and *Cyanidium* appeared, followed lastly by filamentous algae (*Zygnemopsis* and *Klebsormidium*). Elsewhere in the IBP, Rowe *et al.* (2007) reported that an open drainage channel at an abandoned copper mine (Cantareras) was ramified contained a thick (~12 cm) microbial mat, the upper layer (~2.5 mm) of which was green coloured and contained both unicellular (*Euglena* and *Chlamydomonas*) and filamentous (*Zygnema*) micro-algae. The mat under the algal layer was bacterial, and dominated by heterotrophic acidophiles (including species of ferric iron- and sulfate-reducing bacteria). With increasing distance from the mine adit, algae were rare or absent, and this correlated with a much less thick (or absent) microbial mat within the drain channel. The inference was made that the autotrophic algae were providing the organic materials that sustained the heterotrophic acidophiles in the microbial mat, as the concentration of dissolved organic carbon (DOC) in the mine water an its point of discharge was very low (~ 1 mg/L).

Here we describe the isolation, in pure culture, of strains of two acidophilic algae (*Chlorella protothecoides* var. *acidicola* and *Euglena mutabilis*) from MIWs in Spain and Wales, the nature of their organic exudates and lysates, and the significance of the latter in sustaining heterotrophic bacteria in mine waters.

4.2 Materials and Methods

4.2.1 ***Origins, isolation and cultivation of acidophilic algae.*** The two acidophilic algae investigated in the present study were isolated from extremely acidic (pH 2.5 - 2.6) MIWs draining abandoned copper mines in Europe. *Chlorella protothecoides* var. *acidicola* was isolated from surface growths of a microbial mat at Cantareras, Spain (Rowe *et al.*, 2007; supplementary Fig. S4.1) while *Euglena mutabilis* was isolated from green streamer-like growths at Mynydd Parys, Wales (Coupland & Johnson, 2004; supplementary Fig. S4.1). In both cases, small algal growths were removed using sterile tweezers, suspended in acidic (pH 2.5) basal salts and dispersed by vortexing. Cell suspensions were then streaked onto a solid medium containing acidified (pH 2.5) basal salts and trace elements (Wakeman *et al.*, 2008) supplemented with 100 µM ferrous sulfate (aBS/TE;), and gelled with 0.5% (w/v) agarose (Sigma type I). The pH of the gelled medium, measured with a flat bottomed combination pH electrode (Russell pH, U.K.) was ~2.8. Plates were incubated at 22 °C under a light bank with constant illumination (70 µmol of photons m^{-2} s^{-1}). After ~20 days, small green-coloured colonies were apparent. These were re-streaked onto fresh plates, and single colonies from these placed into aBS/TE liquid medium (25 mL in 100 mL conical flasks) and incubated under light. Microscopic examination showed that bacteria, as well as single eukaryotic cell morphologies, were present, in grown cultures. To eliminate the bacteria, the micro-algae were cultivated in aBS/TE medium containing various mixtures of antibiotics. Each algal isolate was sub-cultured in medium containing different concentrations of ampicillin (100, 300 and 500 µg/mL) supplemented (or not) with 100 µg/mL of streptomycin, and incubated under the light bank for 20 days. This procedure was repeated until no bacteria were observed by phase contrast microscopy. Aliquots of purified liquid cultures were streak-inoculated onto solid medium, and single colonies from these placed into liquid aBS/TE medium containing no antibiotics. Cultures were checked routinely for the presence of bacteria throughout the course of the experiments.

The identities of the algal isolates were confirmed by amplification and sequencing of their 18S rRNA genes. Genes were amplified by PCR (30 cycles; denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and elongation at 72 °C for 90 s, followed by 10 min period at 72 °C). The primers used, EukF (ACCTGGTTGATCCTGCAG) and EukR (TGATCCTTCYGCAGGTTCAC), were modified versions of those described by Medlin *et al.* (1988). Gene sequences were

determined using a Beckman Coulter dye terminator cycle sequencing kit and a CEQ8000 Genetic Analysis System (Beckman Coulter, UK) and were compared with those in public databases using BLAST.

4.2.2 *Bioreactor cultures of acidophilic algae.* Purified cultures of the two acidophilic algal isolates were grown in aBS/TE liquid medium in a 2 L (working volume) FerMac 200 modular bioreactor (Electrolab, U.K.) under constant illumination. For routine cultivation, the *Chlorella* isolate was grown at pH 2.5 and at 25 °C, and cultures were aerated at 200 mL/min and stirred at 75 rpm. The *Euglena* isolate was grown under the same conditions, but without stirring. To determine the effects of pH and temperature on the growth of the *Chlorella* isolate, cultures were grown: (i) at a fixed temperature of 25 °C and varying (and fixed) pH (2.0 to 3.5), and (ii) at a fixed pH of 2.5 and temperature ranging from 22 to 35 °C. Samples were removed from the reactor at regular intervals and biomass determined initially both by enumerating cells (using a Helber counting chamber marked with Thoma ruling (Hawksley, U.K.) and viewed with a Leitz Labolux phase contrast microscope, at a magnification of x400) and by measuring the optical densities (OD) of cultures at 600 nm. Since the two measurements were found to be highly correlated, only OD measurements were made in later experiments. Culture doubling times were evaluated from semi-logarithmic plots of biomass increase against time. Attempts to carry out parallel growth response experiments with the *Euglena* isolate were not successful as mechanical agitation of the bioreactor was found to impede the growth of this alga (possibly due to physical damage to the cells), and consequently this isolate did not grow as dispersed planktonic phase cells, precluding accurate determination of biomass.

4.2.3 *Effect of some transition metals on the growth of algal isolates.* Isolates were grown in aBS/TE liquid medium (5 mL aliquots in universal bottles) containing different concentrations (0, 10, 50, 100 and 200 mM) of transition metals (Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} or Zn^{2+}). The cultures were set at pH 2.5 and were incubated at 22 °C, shaken (100 rpm) for 30 days. Growth of the micro-algae was assessed by microscopic examination of the cultures.

4.2.4 *Dissolved organic carbon in cultures of algal isolates.* Isolates were grown in a bioreactor for 60 days (the *Chlorella* isolate) or 90 days (the *Euglena* isolate), the cultures removed and cell-free liquors obtained by centrifugation (10,000

x g; 15 min) followed by filtration through 0.2 µm (pore size) cellulose nitrate membrane filters (Whatman, U.K.). Concentrations of total dissolved organic carbon (DOC) in cell-free culture liquors were measured using a LABTOC DOC analyzer (Pollution and Process Monitoring, U.K.). Glycolic acid and other aliphatic acids were determined using a combination of ion chromatography and colorimetry (Ňancucheo & Johnson, 2010). Carbohydrates and amino acids were determined using a Dionex ICS 3000 ion chromatograph fitted with an ED amperometric detector. Separation of sugars was carried out on a Dionex CarboPac MA1 column with a CarboPac MA1 guard column, eluted with 0.25 mM sodium hydroxide (0.4 mL/min), and amino acids were separated on a Dionex AminoPAC PA10 column.

4.2.5 *Metabolism of glycolic acid by the Chlorella and Euglena isolates.* The *Chlorella* isolate was grown in a bioreactor, as described above, for 20 days. On day 20, the light source was removed, and on day 22 glycolic acid was added to give a concentration of 0.5 mM in the reactor vessel. The light source was reinstated on day 25. Samples were withdrawn at regular intervals to measure the concentrations of total DOC and glycolic acid, and to determine optical densities.

4.2.6 *Metabolism of algal DOC by acidophilic heterotrophic bacteria.* The acidophilic, iron-reducing heterotrophic bacteria *Acidiphilium* SJH (Bridge & Johnson, 2000), *"Acidocella* (*Ac.*) *aromatica*" strain PFBC (Coupland & Johnson, 2008) and the type strain of *Acidobacterium (Ab.) capsulatum* (Kishimoto *et al.*, 1991) were sourced from the *Acidophile Culture Collection* maintained at Bangor University, and assessed for their abilities to metabolize DOC present in the algal cultures. Cell-free culture liquors were prepared as above, adjusted to pH 3.0 with 1 M NaOH, and 20 mL aliquots dispensed into 100 mL conical flasks. These were inoculated (duplicate cultures) with each of the three heterotrophic bacteria, and a fourth set used as a control. Cultures were incubated with shaking (150 rpm) at 30 °C for up 6 days, and samples were withdrawn at days 0, 3 and 6 to enumerate bacterial cells (total counts, as above) and concentrations of total DOC and monosaccharides.

4.3 Results

Green-pigmented colonies were observed after 10-14 days on gelled "inorganic" solid media inoculated with surface streamer growths from both Cantareras and Mynydd Parys, and incubated in the light. After a single transfer on

solid medium and inoculation into liquid media, the cultures of micro-algae obtained were found to be contaminated with bacteria. These were eliminated by subculturing in the presence of both ampicillin (500 µg/mL) and streptomycin (100 µg/mL). Subsequently, axenic cultures of two algal isolates were maintained in liquid medium. One of these grew as single or small groups of round to oval-shaped cells, ~ 3-5 µm diameter, while the other occurred as aggregating worm-like cells, ~ 30-50 µm long, that displayed gliding motility. The identities of these isolates was confirmed by analysis of their 18S rRNA genes. The smaller algae shared 99% gene similarity with *Chlorella protothecoides* var. *acidicola* (strain 124) which was isolated from acidic (pH 2) water at Pisciarelli, Italy (AJ439399; Huss *et al.*, 2002), while the other shared 99% gene similarity with a strain of *Euglena mutabilis* (ELC 1) isolated from Lake Caviahue, an acidic water body in Argentina (EU090196; Brankatschk *et al.*, unpublished). The 18S rRNA gene sequences of the current isolates have been deposited in GenBank, and have the accession numbers JF694006 (the *Chlorella* isolate) and JF694007 (the *Euglena* isolate).

4.3.1 ***Effect of pH and temperature on the growth of Chlorella isolate.*** The *Chlorella* isolate grew between pH 2 and 3.5, and optimally at ~ pH 2.5 (Fig. 4.1a). No growth was obtained at pH 1.8, and growth above pH 3.5 was not tested. The optimum temperature for growth of this alga was ~ 30 °C (Fig. 4.1b), and growth was strongly inhibited at 40 °C. Under optimum conditions of pH and temperature, the *Chlorella* isolate had a culture doubling time of 9 hours. The temperature and pH characteristics of the *Euglena* isolate could not be determined, though von Dach (1943) had earlier reported growth of a strain of *E. mutabilis* between pH 2.1 and 7.7, and optimum growth between pH 3.4 and 5.4.

4.3.2 ***Effects of transition metals on growth.*** The *Chlorella* and *Euglena* isolates showed similar tolerances to the transition metals tested. Both isolates grew in the presence of 10 and 50 mM, but not 100 mM, of ferrous or ferric iron, though fewer cells were observed in the presence of 10 mM than 50 mM iron. Both isolates were more sensitive to copper, zinc and nickel, with growth being observed in media containing 10 mM but not in 50 mM (or higher concentrations) of these transition metals.

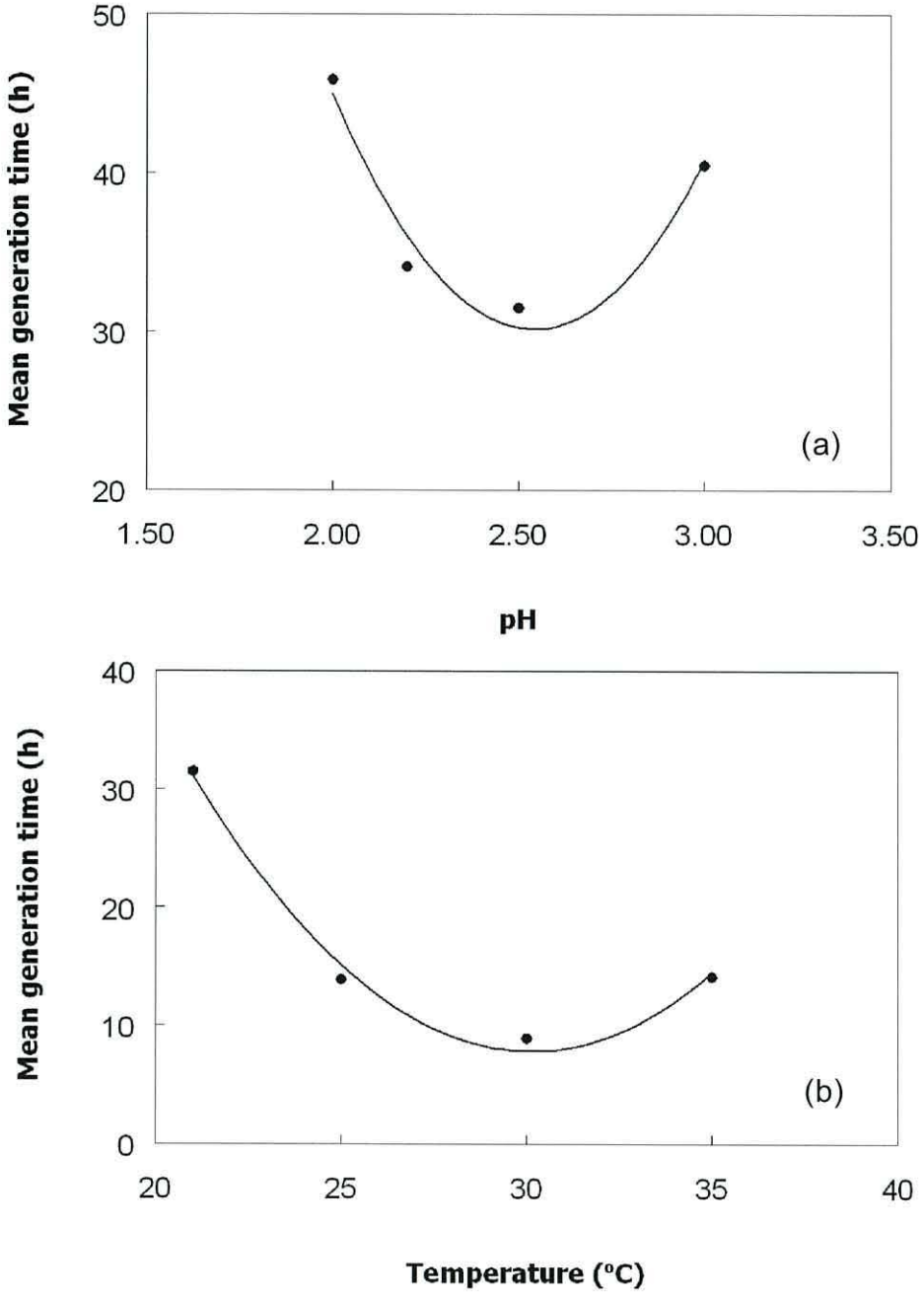

Fig. 4.1. Effect of (a) pH, and (b) temperature, on growth of the *Chlorella* isolate. To determine the pH optimum, cultures were grown at 25 °C, and to determined temperature optimum, cultures were grown at a constant pH (2.5).

4.3.3 ***Organic carbon in batch cultures of Chlorella.*** Growth of the *Chlorella* isolate at pH 2.5 and 30 ˚C was paralleled by increasing concentrations of DOC, from 4 mg/L at day 0 to 30 mg/L at day 19 (Fig. 4.2a). During the first 13 days of culture growth, concentrations of glycolic acid also increased, reaching a maximum value of 120 µM (corresponding to ~12% of total DOC in the cell-free culture liquor). From day 13-15, glycolic acid concentrations fell by ~60%, though this was not reflected by a lower DOC values. From day 15, the glycolic acid concentrations remained fairly stable, at a level corresponding to about 4% of total DOC.

A repeat bioreactor experiment was carried out for a more protracted (29 day) period (Fig. 4.2b). The *Chlorella* biomass increased more rapidly than over the first 15 days than in the first experiment, and the DOC concentration was ~60% greater at day 15 than in the previous experiment. After day 15, growth of the alga slowed down, but the rate of increase of DOC during this period was greater than previously observed. Removing the light source at day 20 resulted in the immediate cessation of both algal growth (followed by a decline in culture optical density) and accumulation of DOC. Glycolic acid, added to the bioreactor culture at day 22, was found to be quickly metabolised, with concentrations of both glycolic acid and DOC returning to levels similar to those immediately prior to addition of extraneous glycolic acid by day 25 (Fig. 4.2b). At day 25, the light source was reinstated, which resulted in DOC concentrations again increasing and also, to more limited degree, *Chlorella* biomass.

4.3.4 ***Organic carbon in batch cultures of the Euglena isolate.*** As with the *Chlorella* isolate, concentrations of DOC and glycolic acid also increased in bioreactor cultures of the *Euglena* isolate (Fig. 4.3) though, due to extensive biofilm formation in the growth vessel, these changes could not be directly correlated with growth of this acidophilic alga. As with *Chlorella*, glycolic acid concentrations increased initially and then stabilised before declining a little, though not to the same extent as in the *Chlorella* cultures. At day 27, the concentration of glycolic acid in the

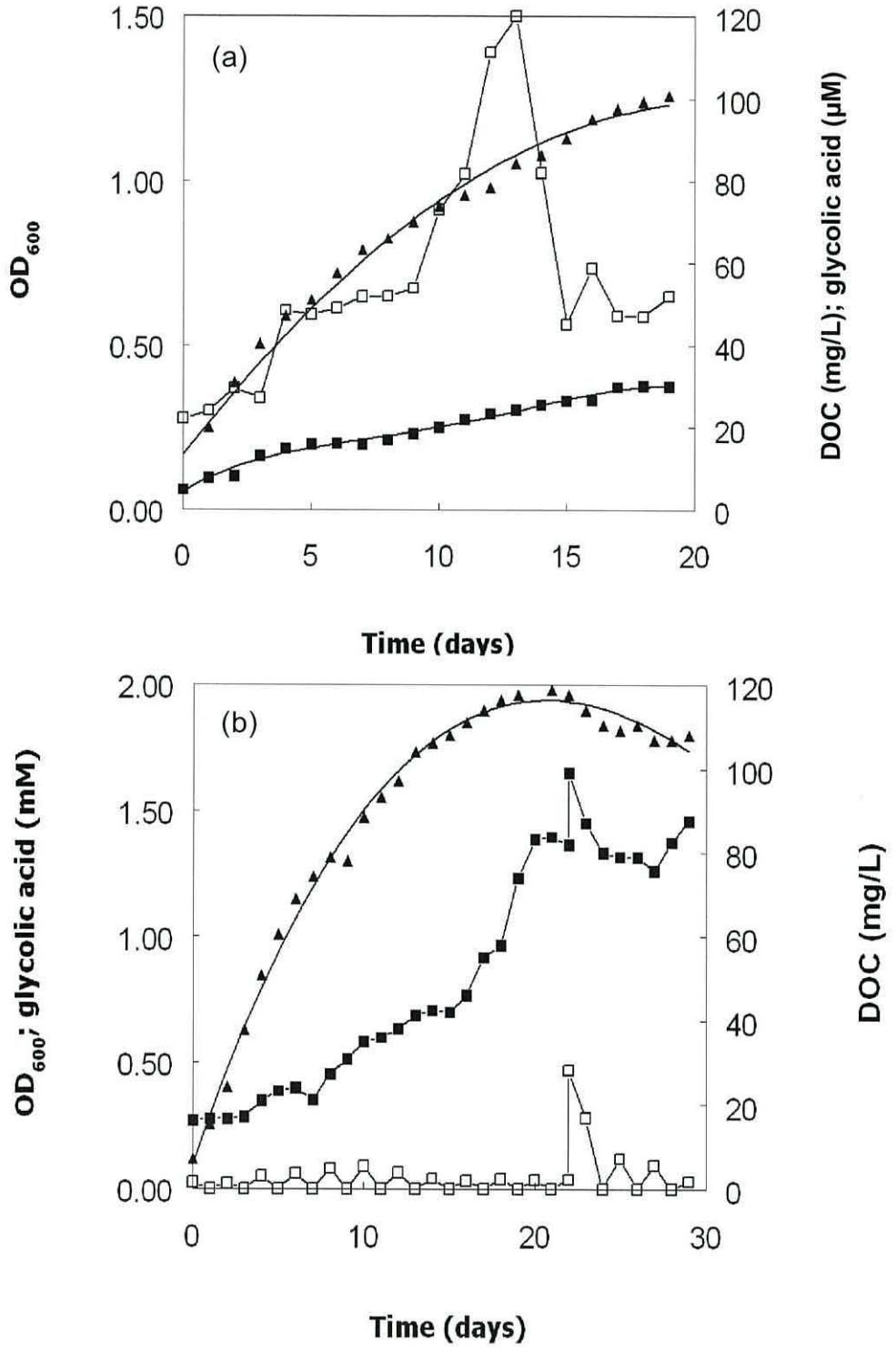

Fig. 4.2. Changes in biomass and concentrations of DOC in glycolic acid in cultures of the *Chlorella* isolate, grown at pH 2.5 and 30°C over (a) 19 days, and (b) 29 days. In the second experiment, the light source was cut off at day 20 and reinstated at day 25 (indicated by the downward and upward pointing arrows, respectively) and glycolic acid added (to 0.5 mM) at day 22 (indicated by the asterisk). Key: (▲), culture optical densities at 600 nm; (■) total DOC; (□) glycolic acid.

cell-free culture liquor was equivalent to 14% of the DOC, but this figure fell to ~7% by day 45.

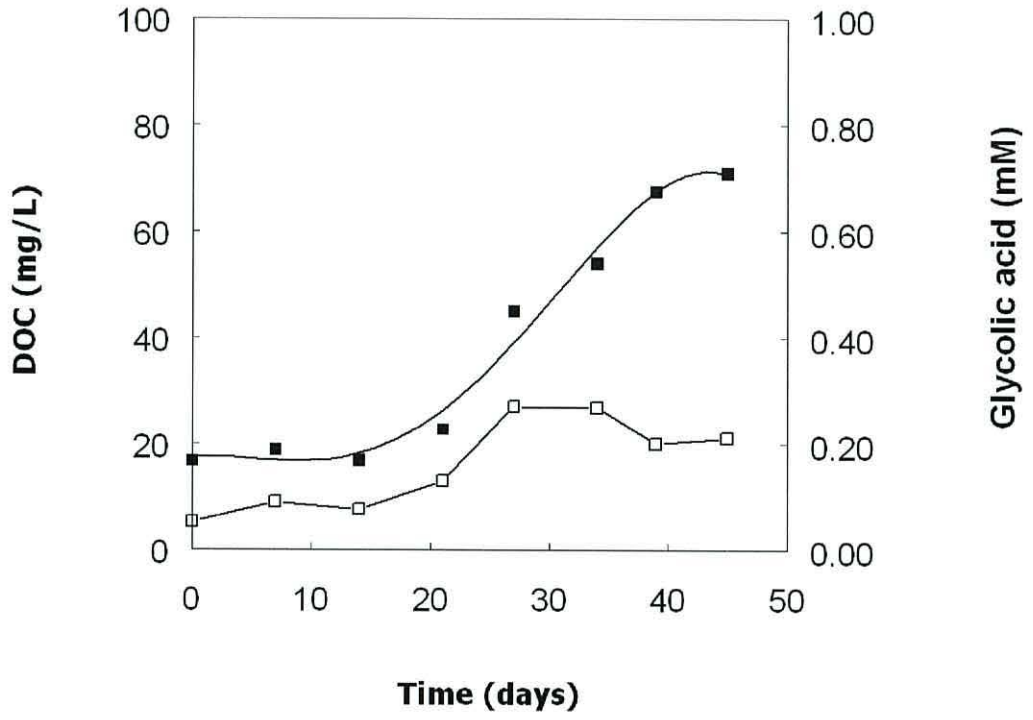

Fig. 4.3. Changes in concentrations of DOC (■) and glycolic acid (□) in a culture of the *Euglena mutabilis* isolate grown in a bioreactor at pH 2.5 and 30°C.

4.3.5 ***Identification of other organic compounds in DOC of acidophilic algal cultures.*** Besides glycolic acid, the only organic compounds identified in cultures of both the *Chlorella* and *Euglena* isolates were monosaccharides. Extensive analysis failed to detect amino acids and other aliphatic acids. Two monosaccharides were detected in each of the two algal cultures – fructose and smaller concentrations of glucose in cultures of *Chlorella*, and mannitol and glucose in cultures of *Euglena*. In both cases, these accounted for smaller amounts of total DOC than the glycolic acid component (Table 4.1).

Table 4.1. Monosaccharides identified in cell-free culture liquors of the acidophilic *Chlorella* and *Euglena* isolates, determined after 60 and 90 days of culture incubation, respectively.

	Concentration (μM)	Concentration (C equivalent; μM)	Total DOC (mg/L)	Contribution to DOC (%)
Chlorella isolate				
Glucose	5	0.36	167	0.22
Fructose	38.5	2.8	167	1.7
Euglena isolate				
Glucose	65	4.7	192	2.5
Mannitol	43	3.1	192	1.6

4.3.6 *Growth of acidophilic heterotrophic bacteria in cell-free culture liquors of Chlorella and Euglena.* Dissolved organic materials originating from both the *Chlorella* and *Euglena* isolates were able to support the growth of representative species of two genera of acidophilic heterotrophic bacteria frequently found in MIWs – *Acidiphilium* and *Acidobacterium*, though the species of *Acidocella* used ("*Ac. aromatica*" strain PFBC) was only found to grow in cell-free culture liquor of *Chlorella* (Fig. 4.4). Growth of heterotrophic acidophiles was accompanied by corresponding decreases in concentrations of DOC and monosaccharides (Table 4.2). In the case of "*Ac. aromatica*" PFBC, fructose declined to less than detectable concentrations, though those of other sugars were similar after six days incubation to initial values. Growth of the different species of heterotrophic acidophiles appeared to correlate with net changes in DOC, with *Acidiphilium* SJH showing the largest increase in cell numbers

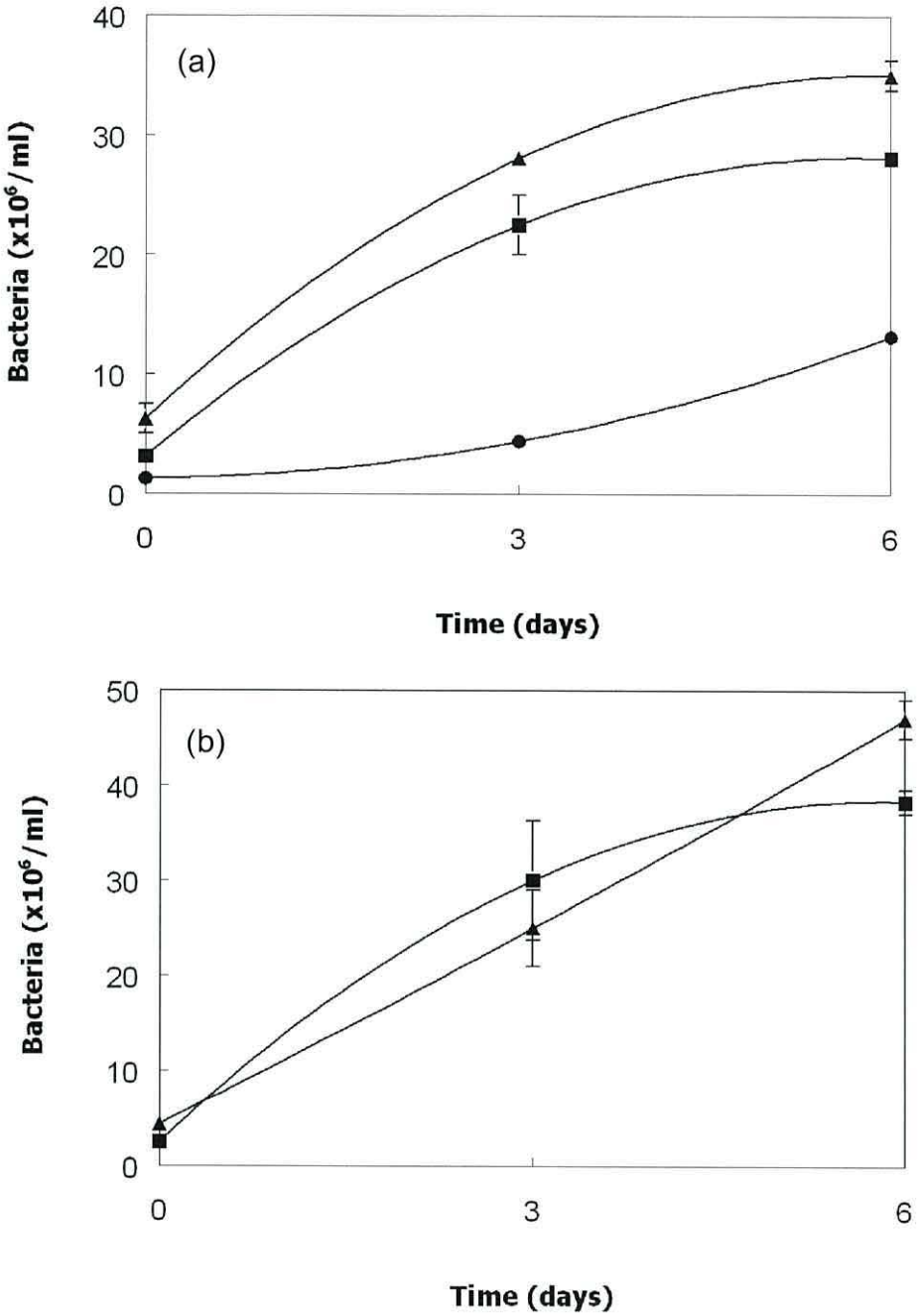

Fig. 4.4. Growth of acidophilic heterotrophic bacteria in cell-free culture media of (a) the *Chlorella* isolate, and (b) the *Euglena* isolate. Key: (▲) *Acidiphilium* sp. SJH; (■) *Acidobacterium capsulatum*^{T}; (●) "*Acidocella aromatica*" strain PFBC. Growth of "*Ac. aromatica*" was not observed in cell-free culture media of the *Euglena* isolate.

Table 4.2. Changes in concentrations of DOC and monosaccharides in cell-free culture liquors from the *Chlorella* and *Euglena* isolates, inoculated with acidophilic heterotrophic bacteria

	Acidiphilium SJH	"*Ac. aromatica*" PFBC	*Ab. capsulatum*^{T}
Chlorella cell-free culture liquor			
DOC (mg/L)			
Day 0	167	167	167
Day 6	155 +/- 4	162 +/- 2	158 +/- 3
Glucose (µM)			
Day 0	5.0	5.0	5.0
Day 6	<1	5.0 +/- 0.1	<1
Fructose (µM)			
Day 0	38.5	38.5	38.5
Day 6	<1	<1	<1
Euglena cell-free culture liquor			
DOC (mg/L)			
Day 0	192	192	192
Day 6	170 +/- 1	190 +/- 3.0	175 +/- 4
Glucose (µM)			
Day 0	65	65	65
Day 6	<1	64.5 +/- 1.3	<1
Mannitol (µM)			
Day 0	43	43	43
Day 6	<1	42.4 +/- 0.3	1.9 +/- 0.2

and greatest change in DOC concentrations during incubation, and "*Ac aromatica*" PFBC the opposite trend. DOC concentrations in cell-free culture liquors of *Chlorella* declined by between 5 mg/L (for "*Ac. aromatica*" PFBC) and 12 mg/L (for *Acidiphilium* SJH), which was greater than the combined carbon equivalents (3.2 mg/L) of the two monosaccarides (glucose and fructose) that were analysed. Corresponding data for the *Euglena* cell-free culture liquors were 17 mg/L for *Ab. capsulatum* and 22 mg/L for *Acidiphilium* SJH, compared with a combined carbon equivalent of 7.8 mg/L for glucose and mannitol (both of which were >95% metabolised). These data indicate that other components of the DOC were also utilised by the heterotrophic acidophiles, though this was not the case with "*Ac. aromatica*" PFBC cultivated in cell-free culture liquor of the *Euglena* isolate.

4.4 Discussion

Acidophilic micro- algae have widespread distribution in MIWs (Novis & Harding, 2007; Das *et al.*, 2009), though they have been the focus of far less research than indigenous prokaryotes. One reason for this is that eukaryotes are not directly involved in the geochemical transformations of iron and sulfur that result in the dissolution of sulfide minerals and which give rise to the formation of acid mine drainage waters, as is the case with chemolitho-autotrophic and chemolitho-heterotrophic acidophiles (Johnson & Hallberg, 2009). However, their indirect impact on these transformations can be considerable. Brake *et al.* (2001) reported that prolific growth of *E. mutabilis* can lead to MIWs being over-saturated (by up to 200%) with dissolved oxygen. Waters draining underground mines and adits are frequently devoid of dissolved oxygen, and soluble iron tends to be present predominantly as reduced ferrous iron (Johnson, 2003). Oxygenation of MIWs by acidophilic algae facilitates the oxidation of ferrous iron and reduced sulfur compounds present in MIWs by bacteria such as *Acidithiobacilllus* and *Leptospirillum* spp., in reactions that are net proton-generating and which can result in further acidification of MIWs downstream of the point of discharge. Such a scenario was reported for the Cantareras mine in Spain (Rowe *et al.*, 2007). A second way in which algae can impact geochemical transformations in MIWs is by stimulating the growth of iron- and sulfur-reducing heterotrophic acidophiles *via* their organic exudates and lysates. Mine waters are generally oligotrophic environments (Johnson, 2003; Das *et al.*, 2009). This phenomenon has received very little attention, though it is probably highly

significant for both natural attenuation of MIWs and engineered bioremediation of mine waters.

There have been many reports describing the occurrence of *E. mutabilis* in MIWs throughout the world, and this organism has frequently been considered as an "indicator species" of acid mine drainage (Valente & Gomez, 2007). This member of the Protista is a unicellular protozoan, and commonly referred to as an alga (Brake *et al.*, 2001). In addition to being able to grow over a wide pH range (2.1 – 7.7; von Dach, 1943), *E. mutabilis* is also tolerant of elevated concentrations of total dissolved solids, including some transition metals and aluminium. The site from which the current isolate was obtained contains about 500 mg iron (ferrous plus ferric)/L and 50 mg copper/L (~0.8 mM), which are concentrations well below those which inhibited its growth i*n vitro*. In contrast, there are very few reports of *Chlorella* spp. in MIWs, and this alga has been reported to be more prevalent in acidic soils (Huss *et al.*, 2002). Little is known about the detailed physiology of *C. protothecoides* var.*acidicola*, though Huss *et al.* (2002) reported that its lower pH and upper temperature limits for growth are 2.0 and 34 °C, respectively, which are similar to those found in the present study.

Concentrations of DOC increased to similar extents during growth *in vitro* of both the *Euglena* and *Chlorella* isolates. Values obtained were similar to those found in sulfur-grown cultures of the chemolitho-autotrophic bacteria *Acidithiobacillus ferrooxidans* and *Acidithiobacillus caldus* (Ňancucheo & Johnson, 2010), though glycolic acid represented a greater proportion of DOC in *Chlorella* and *Euglena* cultures (12-14%) than in cultures of these two bacteria (both ~5%). The presumed origin of the glycolic acid in all these acidophiles is due to the activity of RuBisCO which, besides combining carbon dioxide and ribulose bisphosphate (RUBP) also oxidises RUBP to phosphoglyceric acid and phosphoglycolate. Enzymatic hydrolysis of the latter compound produces glycolate, which is exported out of actively growing cells. In low pH liquors, glycolate is present as undisocciated glycolic acid which, in common acetic and with many other small molecular weight aliphatic acids, is highly toxic to most acidophiles (Johnson & Hallberg, 2009). Production and excretion of glycolic acid has previously been reported for neutrophilic micro-algae (Miller *et al.*, 1963; Fogg & Watt, 1965).The ability to metabolize glycolic acid appears to be restricted, among acidophilic prokaryotes, to *Firmicutes* (e.g.*Sulfobacillus* spp), and none of the three species of heterotrophic acidophiles used in the present study were found to be able to grow on this organic acid (Ňancucheo and Johnson, 2010).

Other organic compounds (monosaccharides) identified in cell-free culture liquors of the two algae have more widespread use by heterotrophic acidophiles (Johnson & Hallberg, 2009). *Acidiphilium* SJH and *Ab. capsulatum* metabolized the fructose and glucose originating from *Chlorella,* and the mannitol and glucose present in the *Euglena* culture, within 6 days. Mass balance calculations of DOC and sugar concentrations confirmed that other (unidentified) materials were also catabolised by the bacteria during this period. The situation with "*Ac. aromatica*" PFBC was very different. In contrast to other *Acidocella* spp, this candidate species grows on a restricted range of substrates, including fructose and some aliphatic acids, but not on glucose or mannitol (Gemmell & Knowles, 2000; Johnson & Hallberg, 2009). While it grew in cell-free culture liquors of *Chlorella*, only fructose (of the compounds analysed) was metabolized, and its failure to grow in cell-free liquor from *Euglena* was apparently due to neither mannitol nor glucose being suitable substrates for this acidophile.

One of the characteristics of the acidophilic algae studied was their abilities to take up (and presumably to metabolise) glycolic acid that they had previously excreted. This was more closely studied with the *Chlorella* isolate, where it was shown that extraneous glycolic acid added to a non-photosynthesising culture was quantitatively removed within three days. The ability of some micro-algae to grow as heterotrophs has been widely documented, e.g. Miller *et al.* (1963) reported that a neutrophilic *Chlorella* (*C. pyrenoidosa*) also excreted glycolic acid into its growth medium when cultures were illuminated and that this was taken up rapidly by the algal cells in the absence of light.

The impact of algae in sustaining populations of acidophilic heterotrophic bacteria may be understood by comparing streamer/mat growths which occur above and below ground in acidic (pH ~2.5) streams draining abandoned mines. Cae Coch in north Wales is an abandoned pyrite mine which contains massive growths (~100 m^3) of acid streamers, microbial stalactites and slimes (Kimura *et al.*, 2011). Because of its underground location, all primary production *in situ* is via chemolithotrophy. While the macroscopic growths present contain some heterotrophic bacteria, these represent a minority of cells present and the streamers and slimes are primarily composed of autotrophic bacteria. Some *Firmicutes*, presumably metabolizing the glycolic acid produced by the chemolithotrophs, are also present. In contrast, the algal-covered microbial mats at Cantareras in Spain are composed mostly of

heterotrophic bacteria, including *Acidiphilium* and *Acidobacterium* spp., and novel species and genera of sulfate-reducing bacteria (Rowe *et al.*, 2007). The reason for this is thought to be the greater input of DOC into the ecosystem from phototrophic microorganisms, which includes monosaccharides (and other unidentified compounds) that are widely used by acidophilic heterotrophic bacteria. Elsewhere, there have been reports that organic carbon originating from acidophilic algae can support the growth of sulfate-reducing bacteria present in MIWs (reviewed in Das *et al.*, 2009) and mannitol (one of the exudates in the *Euglena* cultures) has been identified as a substrate for the acid-tolerant SRB "*Desulfosporosinus acidophilus*" (Johnson *et al.*, unpublished data) . In contrast, monosaccharides were not detected in cell-free media of three species of acidophilic iron- and sulfur-oxidizing bacteria (Ňancucheo & Johnson, 2010).

Acidophilic heterotrophic bacteria have considerable potential for remediating mine waters and securing mine wastes. Reductive dissolution of ferric iron minerals is a highly alkali-generating process (Coupland & Johnson, 2008) while sulfidogenesis in acidic environments not only generates alkalinity but also promotes the immobilisation of many transition metals as insoluble sulfides (Jameson *et al.*, 2010). In addition, "bioshrouding" (enveloping sulfide minerals with biofilms of heterotrophic iron-reducing acidophiles) has been proposed as a technique for securing reactive mine tailings (Johnson *et al.*, 2008a). By producing and exporting soluble organic materials into the environment, acidophilic and acid-tolerant micro-algae have the potential for sustaining populations of benign heterotrophic acidophiles in engineered remediation systems at abandoned mines in systems that are essentially passive, requiring little or no ongoing maintenance (Ňancucheo and Johnson, 2009).

Supplementary Material: Chapter 4

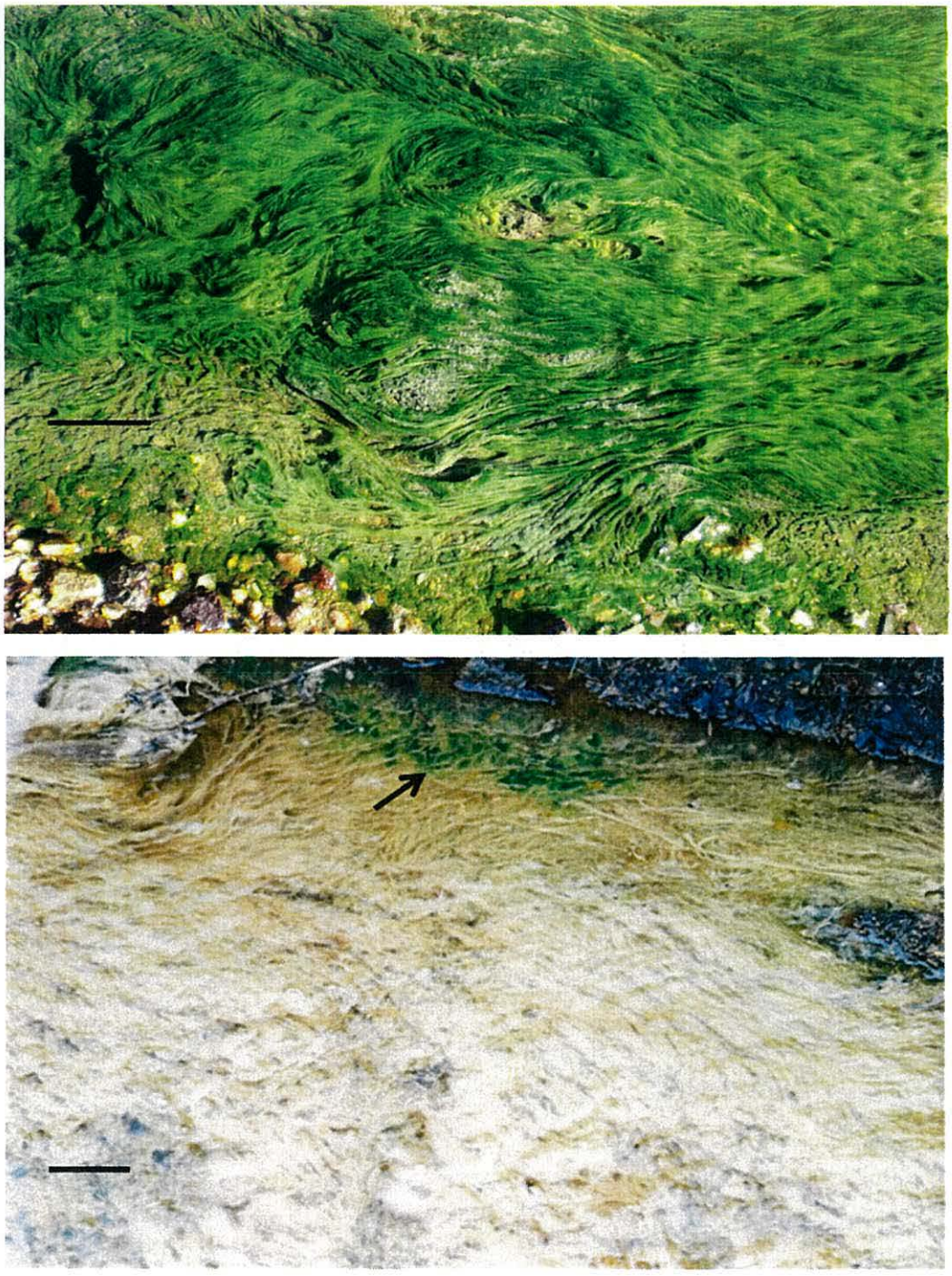

Fig. S4.1. Surface algal growths on a microbial mat in a stream draining the abandoned Cantareras copper mine, Spain (top); acid streamers colonised with *Euglena mutabilis* (arrowed) in a stream draining the abandoned Mynydd Parys mine, Wales (bottom). The solid bar represents ~10 cm in both pictures.

Chapter 5

Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria

Ivan Ňancucheo and D. Barrie Johnson

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Abstract

Two continuous-flow bench-scale bioreactor systems populated by mixed communities of acidophilic sulfate-reducing bacteria were constructed and tested for their abilities to promote the selective precipitation of transition metals (as sulfides) present in synthetic mine waters, using glycerol as electron donor. The objective with the first system (selective precipitation of copper from acidic mine water containing a variety of soluble metals) was achieved by maintaining a bioreactor pH of ~ 2.2 to 2.5. The second system was fed with acidic (pH 2.5) synthetic mine water containing 3 mM of both zinc and ferrous iron, and varying concentrations (0.5 to 30 mM) of aluminium. Selective precipitation of zinc sulfide was possible by operating the bioreactor at pH 4.0 and supplementing the synthetic mine water with 4 mM glycerol. Analysis of the microbial populations in the bioreactors showed that they changed with varying operational parameters, and novel acidophilic bacteria (including one sulfidogen) were isolated from the bioreactors. The acidophilic sulfidogenic bioreactors provided "proof of principle" that segregation of metals present in mine waters is possible using simple on-line systems within which controlled pH conditions are maintained. The modular units are versatile and robust, and involve minimum engineering complexity.

5.1 Introduction

Waters draining abandoned metal mines and mine waste repositories are characteristically acidic (sometimes extremely so) and enriched with dissolved transition metals and aluminium (Nordstrom, 2000). The physico-chemical characteristics of mine impacted waters (MIWs) vary from location to location, as these are dictated by a number of geochemical, climatic, hydrological and other factors. Microbially-enhanced oxidative dissolution of sulfide minerals is a prime cause of water pollution associated with metal mines (Johnson and Hallberg, 2003). Bacteria such as *Acidithiobacillus* spp. and *Leptospirillum* spp. are well known for their abilities to use reduced chemicals (ferrous iron and/or reduced sulfur) as sources of energy, and to use the energy released from these reactions to fix carbon dioxide and thereby produce new biomass. These autotrophic bacteria have minimal nutritional requirements, and their abilities to tolerate elevated concentrations of dissolved metals in acidic solutions enables them to exploit the seemingly hostile environments that characterise mine spoils, mineral tailings and MIWs. Acidity derives from the oxidation of the reduced sulfur moiety in sulfide minerals, and also the hydrolysis of ferric iron in the case of iron-containing minerals, such as the most ubiquitous of all sulfides, pyrite (FeS_2). Equation [1] depicts the complete oxidation of the most abundant copper sulfide mineral in the lithosphere, chalcopyrite:

$$CuFeS_2 + 4.25\ O_2 + 2.5\ H_2O \rightarrow 2\ H^+ + 2\ SO_4^{2-} + Fe(OH)_3 + Cu^{2+} \qquad [1]$$

The low pH of the leach liquors produced allows metals, such as copper and zinc, which are released from the oxidative dissolution process to remain in solution. Aluminium does not occur as a sulfide mineral, but many aluminosilicates are susceptible to acid dissolution and, as a results concentrations of this metal are also usually much higher in MIWs than in non-impacted (circum neutral-pH) streams.

The severe impact that MIWs can have on the local and wider environment means that control of their formation or, if this is not pragmatic, remediation of waters draining metal mines is generally regarded as a priority issue for regulatory authorities. The most widely used approach for remediating MIWs is to aerate (to oxidize ferrous iron to ferric) and add an alkalizing chemical (such as CaO) in order to raise water pH and to precipitate metals as hydroxides and carbonates. Aggregation and thickening of the metal hydroxide flocs produces a sludge which typically contains ~ 30% solids in the "high density sludge" application. However, this

active chemical process has numerous drawbacks, including operating and reagent costs, and the need to dispose of the polymetallic sludge generated in designated landfill sites. In addition, potentially useful and valuable metal resources are not recovered in chemical remediation of MIWs. Compost bioreactors (sometimes called "constructed anaerobic wetlands") that use microbial reductive processes to immobilise metals in MIWs also suffer from a number of drawbacks (Johnson and Hallberg, 2002) These reactors are fuelled by bulky organic materials (usually a mixture of straw or sawdust, and animal manure) which require periodic replacement, and again metals are not recovered but are "locked up" within the spent compost, which is therefore usually categorized as a toxic waste.

An alternative approach for remediating MIWs which, like compost bioreactors, harnesses the abilities of microorganisms to generate alkalinity and to immobilise metals, is referred to generically as "active biological treatment". In such systems, microorganisms that catalyse redox transformations of iron or sulfur are maintained in reactors where conditions can be optimised for their activities and, like active chemical treatment, this approach requires continuous inputs of reagents and more intensive management. Two distinct variants have been demonstrated as pilot-scale or full-scale systems. One uses acidophilic bacteria to oxidize ferrous iron and thereby facilitate iron removal from MIWs by hydrolysis and precipitation of the ferric iron produced. A pilot-scale operation of this kind has been operating at Nochten, in east Germany, for over 3 years, removing iron from contaminated groundwater and producing schwertmannite ($Fe_8O_8(OH)_6SO_4$) as a by-product (Heinzel *et al.*, 2009). Other metals often present in MIWs are, however, more effectively removed as sulfides than as hydroxide or carbonate phases. Since metal sulfides have different solubilities, they can be selectively precipitated by controlling solution pH, which will determine the concentration of the reactant, S^{2-} (Steudel, 2000). Sulfidogenic bacteria generate hydrogen sulfide primarily by using either sulfate or elemental sulfur as an electron acceptor, and an organic (e.g. ethanol) or inorganic (e.g. hydrogen) electron donor. Since MIWs are usually contain elevated concentrations of sulfate (which, like many of the metals present, derives from the oxidation of sulfide minerals; equation [1]) using sulfate-reducing bacteria (SRB) for mine water amelioration is particularly pertinent. A drawback to this strategy has been, however, that characterised species of SRB are highly sensitive to even mild acidity, and do not grow at pH <5.5 (Koschorreck, 2008). This characteristic has required SRB used for remediation of MIWs to be maintained in reactors where direct contact with the

acidic wastewaters is avoided, which has implications for the design and operating costs of engineered systems.

There have been some reports of novel species of SRB that are acid-tolerant or acidophilic. Kimura *et al.* (2006) reported that a mixed culture of a SRB isolated from a geothermal site on the Caribbean island of Montserrat ("*Desulfosporosinus acidophilus*" M1) and an acidophilic heterotroph (*Acidocella* sp. PFBC) grew in a synthetic liquid media maintained at pH 3.8 – 4.2, where it promoted the selective precipitation of zinc sulfide. Senko *et al.* (2009) also isolated an acid-tolerant *Desulfosporosinus* sp. (from sediment of MIW from a coal mine) that reduced sulfate, iron (III), manganese (IV) and uranium (VI) at pH 4.2. Rowe *et al.* (2007) reported that SRB other than *Desulfosporosinus* spp. were responsible for precipitating copper (as CuS) in a microbial "mat" found in a stream draining an abandoned copper mine in south-west Spain.

Here we describe the commissioning and performances of continuous flow bioreactors containing mixed communities of acidophilic SRB and other bacteria, designed to remove selectively copper and zinc (as sulfides) from MIWs.

5.2 Experimental procedures

5.2.1 ***Design and commissioning of the sulfidogenic bioreactor.*** Two sulfidogenic upflow biofilm reactors, based on a system described previously by Jameson *et al.* (2010), were commissioned and assessed as separate units, each for a period of over 300 days. A pre-trial bioreactor was set up initially, as shown in Fig. 5.1, in which bacteria were immobilised on a bed (12.5 cm deep) of 1 - 2 mm diameter porous glass beads (Poraver Dennert GmbH, Germany). The sterile beads were inoculated with active pure cultures of *Desulfosporosinus* M1 (Kimura *et al.*, 2006) and "*Db. acidavidus*" strain CL4 (Jameson *et al.*, 2010), and an enrichment culture of an anaerobic streamer mat from an acidic (pH 2.5) stream draining of an abandoned copper mine in south-west Spain (Rowe *et al.*, 2007). The bioreactor vessel had a working volume of 2.3 L and was coupled to a FerMac 310/60 unit (Electrolab., U.K.) which controlled pH, temperature and agitation. Two pH electrodes (Broadley James, U.K.) were inserted into the bioreactor vessel: one extended into the porous glass bead bed (and was used only to monitor the pH in this part of the reactor) while the other (shorter) electrode extended only in the liquid phase above the biofilm (bead) bed, and was coupled to the acid-input pump of the control unit.

This pump controlled the flow of feed (influent) liquor into the reactor, which was added at a variable rate, required to maintain the pH of the bioreactor liquor at any set value. Since microbial sulfidogenesis is a proton-consuming reaction, it was essential that the feed liquor was always at a lower pH than that of the

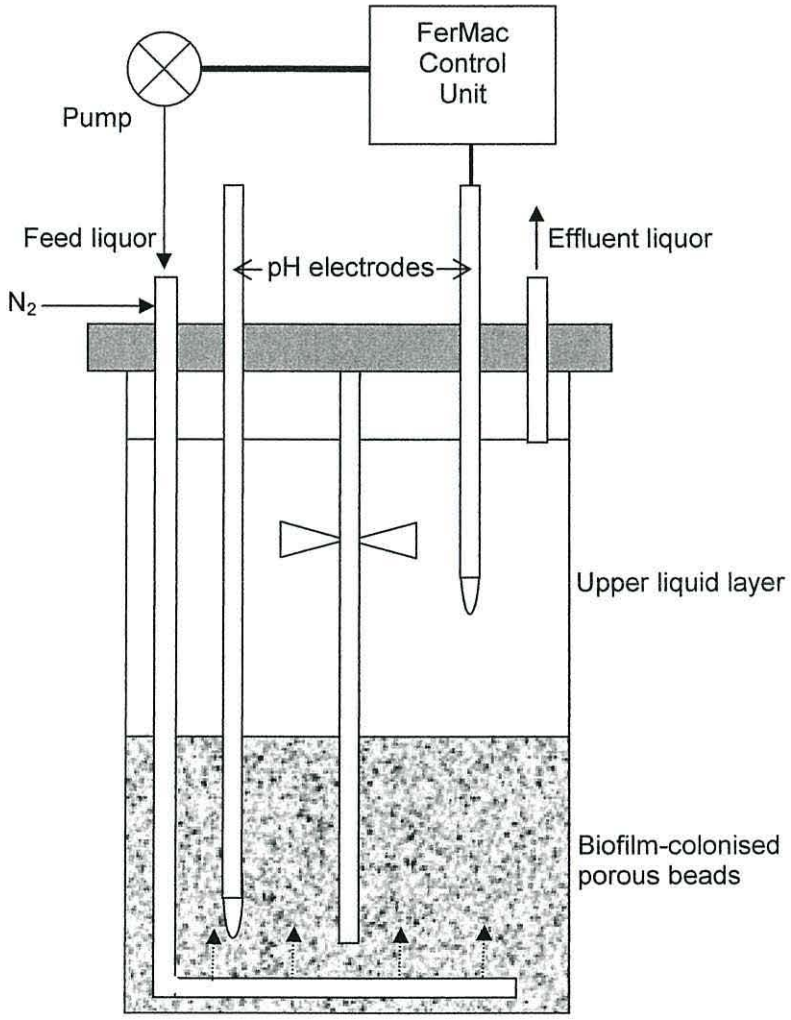

Fig. 5.1. Schematic of the bioreactor vessels used in the present study. The arrows indicated the direction of liquid/gas flow.

bioreactor liquor in order to maintain pH homeostasis. As indicated in Fig. 5.1, the feed liquor was delivered via an L-shaped tube which had perforations in its lower length, causing it to migrate through the biofilm bed before it entered the liquid phase above the beads. A drain tube placed above the liquid surface coupled to a second pump on the control unit that was synchronized with the pH pump, ensured that the liquid volume within the bioreactor remained constant. The temperature of the bioreactor was set at 30°C, and the vessel contents were stirred gently (at 50 rpm)

using a single impellor blade located within the top liquid phase of the bioreactor, in order not to disrupt the bead bed. A continuous stream of nitrogen (~ 200 ml min^{-1}) was used to off-set any oxygen ingress into the bioreactor.

The pre-trial bioreactor was operated at pH varying between 3.5 and 4.5, and was supplied with an influent liquor (pH 2.5) containing 2 mM glycerol (as electron donor), 0.01% (w/v) yeast extract, 2 mM zinc, 1 mM ferrous iron, and basal salts (Wakeman *et al.*, 2008). After 5 months of operation as a continuous flow system, the bioreactor was decommissioned, and the biofilm-containing beads removed and mixed with fresh sterile beads. The bead mixture was split into two equal parts, each of which was placed into a fresh bioreactor vessel, and the two vessels were coupled to two FerMac 310/60 control units (Supplementary Fig. S5 1). The two new systems were operated as described for the pre-trial bioreactor for 1 - 2 months to allow pre-conditioning of the units, and then at different operating parameters (described below).

5.2.2 ***Operation of the sulfidogenic bioreactors.*** The feed liquor for bioreactor I (Table 5.1) contained copper, zinc and iron (all at 1 mM), varying concentrations of glycerol (initially 5 mM, decreasing to 0.7 mM), yeast extractT (0.01%, w/v) and basal salts. The pH of the feed liquor was set at 2.5 for the day 0 to 21, and at 2.1 for the next 290 days. In contrast, the bioreactor liquor pH was lowered progressively throughout the experimental period, from a starting value of 3.6 to a final value of 2.2 (Fig. 5.2). After day 311, the feed liquor for this bioreactor was changed to a synthetic mine water based on the chemistry of a stream draining the abandoned Mynydd Parys copper mine in north Wales (Table 5.1; Coupland and Johnson, 2004). The main differences between the Mynydd Parys mine water and its synthetic equivalent were the lower pH (2.1, compared with ~2.5) and more variable ferrous iron concentration (5 to 10 mM) of the latter. The performance of bioreactor I with a feed liquor of synthetic Mynydd Parys mine water (supplemented with glycerol and yeast extract; Table 5.1) was tested for 56 days.

The composition of the feed liquor for bioreactor II was based on mine water draining an abandoned zinc mine (Cwm Rheidol) in mid-Wales (Table 5.1; Edwards and Potter, 2007) which contained zinc, iron and aluminium as major soluble metals. During the course of the experiment, aluminium concentrations in the feed liquor increased from 0.5 to 30 mM, while concentrations of other components of the influent solution remained unchanged. The glycerol and yeast extract concentrations

Table 5. 1 Compositions of feed liquors supplied to bioreactors I and II

	Bioreactor I feed liquor	Bioreactor II feed liquor*	Synthetic Mynydd Parys mine water** (Bioreactor I)
pH	2.5 →2.1	2.5	2.1
$^{a}Fe^{2+}$	1.0	3.0	5.0→10
SO_4^{2-}	3.9→6.4	12.1→56.4	11.7
Cl^-	0.27	trace	2.1
$H_2PO_4^-$	0.15	trace	0.02
Cu^{2+}	1.0	trace	0.71
Zn^{2+}	1.0	3.0	1.07
Al^{3+}	Trace	0.5→30	3.33
Mn^{2+}	Trace	0	0.22
Na^+	0.38	0.5	2.12
NH_4^+	2.72	trace	0.02
K^+	0.42	trace	0.04
Mg^{2+}	0.81	2.0	0.66
Ca^{2+}	0.05	0.5	1.05
Glycerol	5.0→0.7	4.0	3.0→0.7
Yeast extract	0.01	0.01	0.01→0.005

*based on AMD draining the abandoned Cwm Rheidol zinc mine, mid-Wales; *, ** data kindly supplied by Dr. Hugh Potter, Environment Agency, U.K.; [a] at point of discharge. All concentrations shown are mM, except yeast extract (w/v), and arrows indicate changes in concentrations during the course of the experiment.

in the feed liquor for bioreactor II were maintained at 4 mM and 0.01% respectively, and the pH at 2.5. The bioreactor pH (upper liquid phase) was set at 4.9 initially and was lowered, in stages, to 4.0 during the course of the 335 day experiment.

5.2.3 ***Physico-chemical analysis.*** Concentrations of transition metals, acetate and glycerol were determined by ion chromatography (Wakeman *et al.,* 2008; Ňancucheo and Johnson, 2010). Soluble transition metal were determined using a Dionex-320 ion chromatograph fitted with an IonPAC® CS5A column and an AD absorbance detector. Acetate concentrations were measured using a Dionex IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity detector. Glycerol was determined using a Dionex ICS 3000 ion chromatography system fitted with a Carbo Pac MA1 column and ED amperometric detector. Aluminium was determined by atomic absorption spectrophotometry using a Varian SpectrAA 220 FS. Metal sulfide precipitates taken from both bioreactors at the end of the experiment were characterized by X -ray diffraction (XRD) analysis. Solid materials were collected by centrifugation, dried in a vacuum desiccator for 48 h and then ground to a powder. Samples were analyzed using a Philips PW3040/60 X'Pert PRO and the data analyzed using the PANalytical search-match program "Highscore".

5.2.4 ***Microbiological and molecular analysis of bioreactor microbial populations.*** DNA was routinely extracted from liquid samples taken from both bioreactors using Ultraclean Soil DNA extraction kits (MoBio, CA, U.S.A.) The extracted DNA (typically 1 µl) served as template for the amplification of the 16S rRNA genes by PCR as described previously (Okibe *et al.*, 2003). The primers used for amplification for bacterial 16S rRNA were 27F (Lane, 1991) and 1387R (Marchesi *et al.*, 1998). Archaeal 16S rRNA genes were amplified using the primers 20F (Orphan *et al.,* 2000) and 1392R (Lane, 1991), with DNA from the euryarcheote *Ferroplasma acidiphilum* acting as a positive control. Bacterial communities were analyzed by T-RFLP as described previously by Hallberg *et al.* (2006). The labelled DNA that was amplified by PCR in three independent reactions was digested with the restriction enzymes MspI, CfoI and HaeIII. Assignment of T-RFs to known acidophilic was accomplished by comparison with a database of previously identified microbes detected in acidic environments (Rowe *et al.*, 2007). T-RFs of bacteria isolated from the bioreactors were determined using the same protocols. Bacteria in liquid samples were enumerated using a Helber counting chamber marked with Thoma ruling

(Hawksley, U.K.), and viewed with a Leitz Labolux phase contrast microscope at x400 magnification.

5.2.5 ***Isolation and identification of bacteria from the sulfidogenic bioreactors.*** Bacteria were isolated from the bioreactors at different stages of operation, by streaking liquid samples onto an overlay medium (Johnson and Hallberg, 2007) supplemented with 4 mM glycerol, 0.02% (w/v) yeast extract, 7 mM zinc and 0.5 mM ferrous iron (pH ~3.7). Plates were incubated in an anaerobic atmosphere (using the AnaeroGen™ system; Oxoid, U.K.) at 30°C and removed periodically for visual examination. Representative colonies were sub-cultured on the same solid medium incubated both anaerobically and aerobically (to test for aerobic growth) and also in a liquid medium equivalent of this medium. Phylogenetic analysis of three isolates obtained was carried out using the method described by Rowe *et al.* (2007).

5.3 Results

The long-term performances of the two 2.3 L working volume sulfidogenic bioreactors were assessed by operating them in parallel as anaerobic continuous flow systems for over 300 days. During this time, flow rates of both systems fluctuated depending on several factors, including the pH differential between the influent liquor and that set for the bioreactor, and rates of bacterial sulfidogenesis which were also affected by pH. In general, pH values measured in the lower biofilm layers in the reactors were similar to those of the liquid phases above the bead beds, though they tended to be lower (~0.2 to 1.0 pH unit) when flow rates were relatively fast and when the pH of the influent liquors were lowered.

5.3.1 ***Bioreactor I.*** The major objective with bioreactor I was to determine conditions which allowed the selective precipitation of copper sulfide from feed liquors that also contained soluble zinc and ferrous iron. The mean flow rate with this bioreactor was 49 (+/- 27, standard deviation) ml h^{-1}, corresponding to a mean dilution rate of 0.021 h^{-1}, with maximum and minimum rates of 135 and 20 ml h^{-1} (Fig. 5.2). All of the copper present in the influent liquor was precipitated in the bioreactor for the greater part of the experiment (Fig. 5.3).

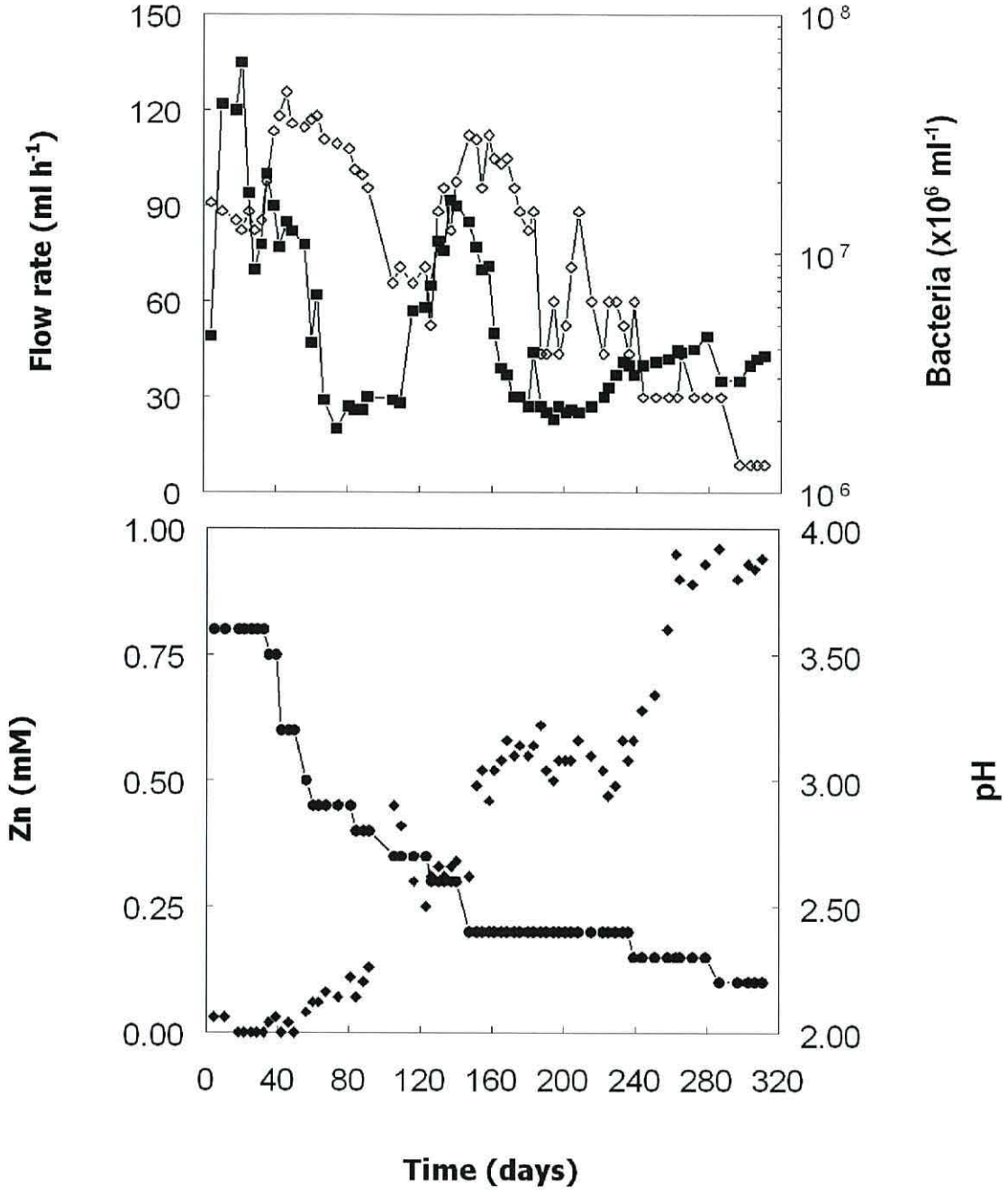

Fig. 5.2. Changes in flow rates (■), pH (●), concentrations of soluble zinc (♦) and numbers of planktonic-phase bacteria (◊) in bioreactor I during the first phase of operation. The feed liquor contained 1 mM of copper, zinc and ferrous iron and its pH was progressively lowered from 2.5 to 2.1 on day 21.

At times when soluble copper was detected in the bioreactor liquor, this was due to short-term perturbations in the system (reflux of copper sulfate from the gas trap into the reactor, due to low nitrogen pressure) and, on each occasion, the bioreactor recovered rapidly. In contrast, none of iron in the influent liquor was retained in the bioreactor. Concentrations of iron were invariably greater than the 1 mM present in the feed water, due to: (i) dissolution of small amounts of FeS deposited during the initial commissioning of the bioreactor, and (ii) microbially- and acid-enhanced corrosion of some of the stainless steel components of the bioreactor (Dinh *et al.*, 2004). Since separation of copper and iron as solid and liquid phases was readily achieved in bioreactor I, the main challenge was to establish conditions where copper would precipitate (as a sulfide) but zinc would be retained in solution. When the bioreactor pH was set at 3.6, >99% of the zinc in the influent liquor was precipitated, along with the copper, within the bioreactor (Fig. 5.2). By progressively lowering both the bioreactor pH and the concentration of the electron donor (glycerol) in the influent liquor, it was possible to retain increasing amounts of zinc in solution. Between days 147 and 236, the bioreactor was maintained at pH 2.4, and the amount of zinc precipitated stayed reasonably stable at 47% +/- 1 6% (23 sampling time points) even though influent glycerol concentrations was lowered from 4 to 1.5 mM during this time. However, by decreasing the bioreactor pH still further (ultimately to pH 2.2) and the influent glycerol concentration to 0.7 mM, it was possible precipitate only 8 +/- 2% (9 samples) of zinc within the bioreactor, while maintaining >99% removal of copper from solution. Analysis of the solid residue that accumulated in bioreactor I confirmed that it was predominantly copper sulfide.

Bacterial numbers in the upper liquid phase in bioreactor I varied from between 1.3×10^6 and 4.75×10^7 ml^{-1} (Fig. 5.2) and were more strongly correlated with influent glycerol concentrations (r =0.77) than with bioreactor pH (r = 0.56; Supplementary Fig. S5.2). Most of the glycerol added to the bioreactor was oxidized completely to carbon dioxide; although acetic acid was detected in all samples analyzed, it was always present in low concentrations, and corresponded to a mean of ~26% of the glycerol that was oxidized throughout the 311 trial period.

Bioreactor I was subsequently operated (for 56 days) with a feed liquor similar in composition to water draining the abandoned Mynydd Parys copper mine, with the bioreactor pH maintained at 2.6. All of the copper, but none of the ferrous iron, aluminium or manganese, were precipitated within the bioreactor under these conditions (Fig. 5.4). As before, some precipitation of zinc (sulfide) occurred (~75% of

that in the influent) at pH 2.6 when the glycerol concentration in the feed liquor was 3 mM. Decreasing the glycerol concentration to 0.7 mM lowered the amount of zinc

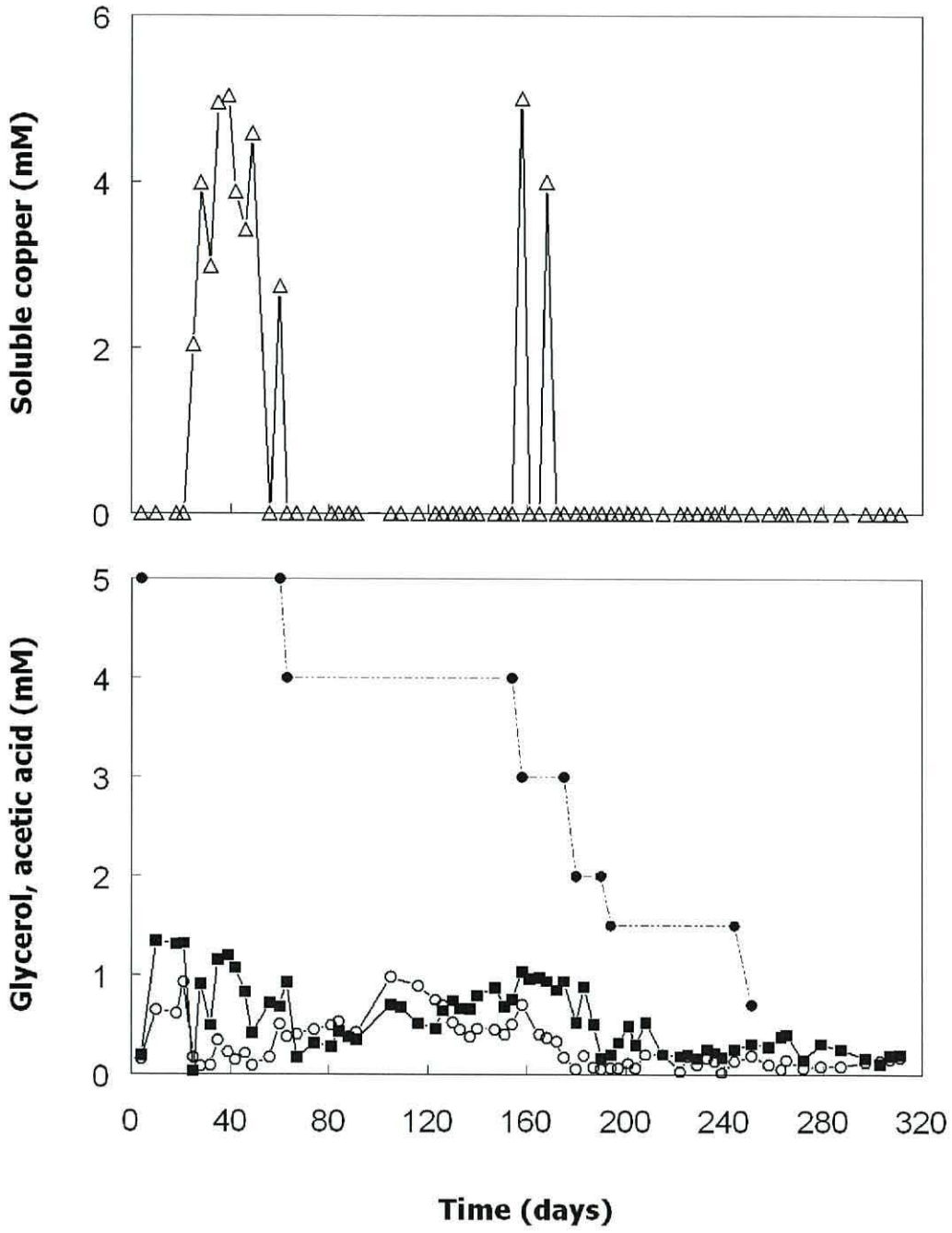

Fig. 5.3. Changes in concentrations of soluble copper (Δ), glycerol (o) and acetic acid (■) in bioreactor I during the first phase of operation. Concentrations of glycerol in the feed liquor are also shown (●). The feed liquor contained 1 mM of copper, zinc and ferrous iron and its pH was progressively lowered from 2.5 to 2.1 on day 21.

precipitated to about 30% of that in the synthetic mine water, and this was depressed further (to ~25%) when the yeast extract content of the influent was lowered from 0.01% to 0.005%. Numbers of bacteria in the upper liquid phase of the bioreactor decreased significantly as a result of lowering the concentrations of both glycerol and yeast extract (Fig. 5.4). Concentrations of acetic acid corresponded to a mean of ~9.5% of the glycerol that was oxidized during this phase of operation.

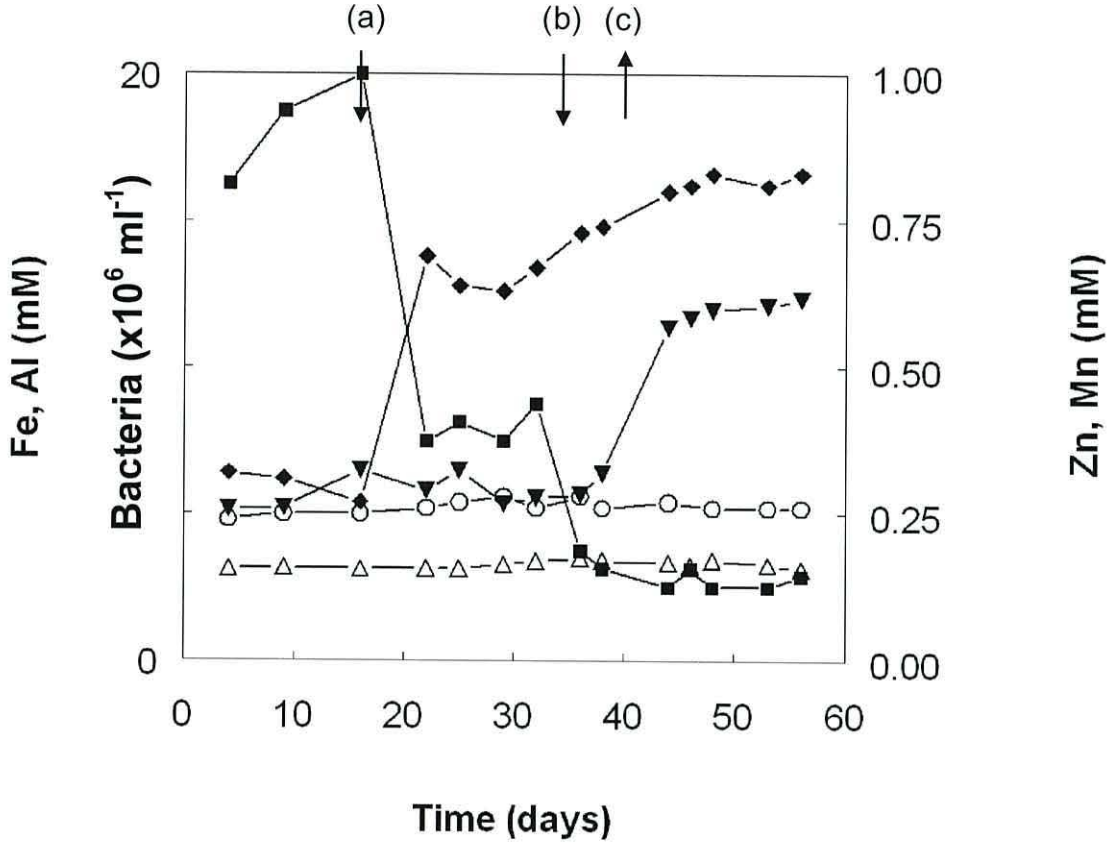

Fig. 5.4. Changes in concentrations of soluble zinc (♦), iron (▼), aluminium (Δ) and manganese (o), and numbers of planktonic-phase bacteria (■) in bioreactor I during the second phase of operation. The feed liquor was a synthetic mine water based on the chemical composition of a stream draining the abandoned Mynydd Parys copper mine in north Wales. The arrows indicate the points at which: (a) the glycerol concentration in the feed liquor was lowered from 3 mM to 0.7 mM; (b) the yeast extract concentration was lowered from 0.01% to 0.005% (w/v); (c) the ferrous iron was increased from 5 mM to 10 mM.

5.3.2 *Bioreactor II.* Flow rates into and out of bioreactor II were comparable with those of bioreactor I, with a mean value and standard deviation of 57 +/- 18 ml h^{-1} (corresponding to a dilution rate of 0.025 h^{-1}) and maximum and minimum values of 105 and 17 ml h^{-1} respectively (Fig. 5.5) from day 0 to day 335. For most of this time, ferrous iron concentrations in the bioreactor were slightly greater than the 3 mM present in the influent liquor (3.2 +/- 0.23 mM between days 88 and 335; n = 49 sampling points; Fig. 5.5), though between days 0 and 84, the mean ferrous iron concentration in the effluent liquor was slightly less than that in the influent (2.8 +/- 0.41; n = 18 sampling points), possibly due to the higher pH (4.9 – 4.8) at which the bioreactor was maintained up to day 84 than afterwards (pH 4.5 to 4.0). Concentrations of zinc were below levels of detection in most of the samples analyzed (Fig. 5.5), though significant concentrations of soluble zinc (27% of that in the influent) were measured in the bioreactor shortly after the aluminium content of the feed liquor was increased from 16 to 30 mM. On the four occasions when aluminium concentrations in the bioreactor liquor were determined, these were found to be very similar to those present in the feed used at those times (Fig. 5.6a) confirming that very little of the aluminium was precipitated within the bioreactor. Analysis of the solid residue that accumulated in bioreactor II confirmed that it was predominantly zinc sulfide.

Bacterial numbers in bioreactor II declined from a mean of 8.1+/-2.6 x 10^6 ml^{-1} from days 0 to 259, when the maximum aluminium concentration in the feed liquor was 1.5 mM, to 4.7 +/- 1.2 x 10^6 ml^{-1} from days 259 to 335, when aluminium concentrations were increased to up to 30 mM (Fig. 5.5). Analysis of glycerol in the bioreactor indicated that effective oxidation (98%, mean figure; n = 64 sampling points) of glycerol was occurring from day 0 to day 300 but that this declined to a mean of 92% (n = 8 sampling points) when the aluminium concentration was increased from 6 to 30 mM (Fig. 5.6b). Acetic acid was detected in the bioreactor throughout the experiment (Fig. 5.6b), but generally in small concentrations (0.04 to 1.27 mM; n = 73 sampling points), corresponded to a mean of ~13% of the glycerol that was oxidized throughout the 335 day experiment.

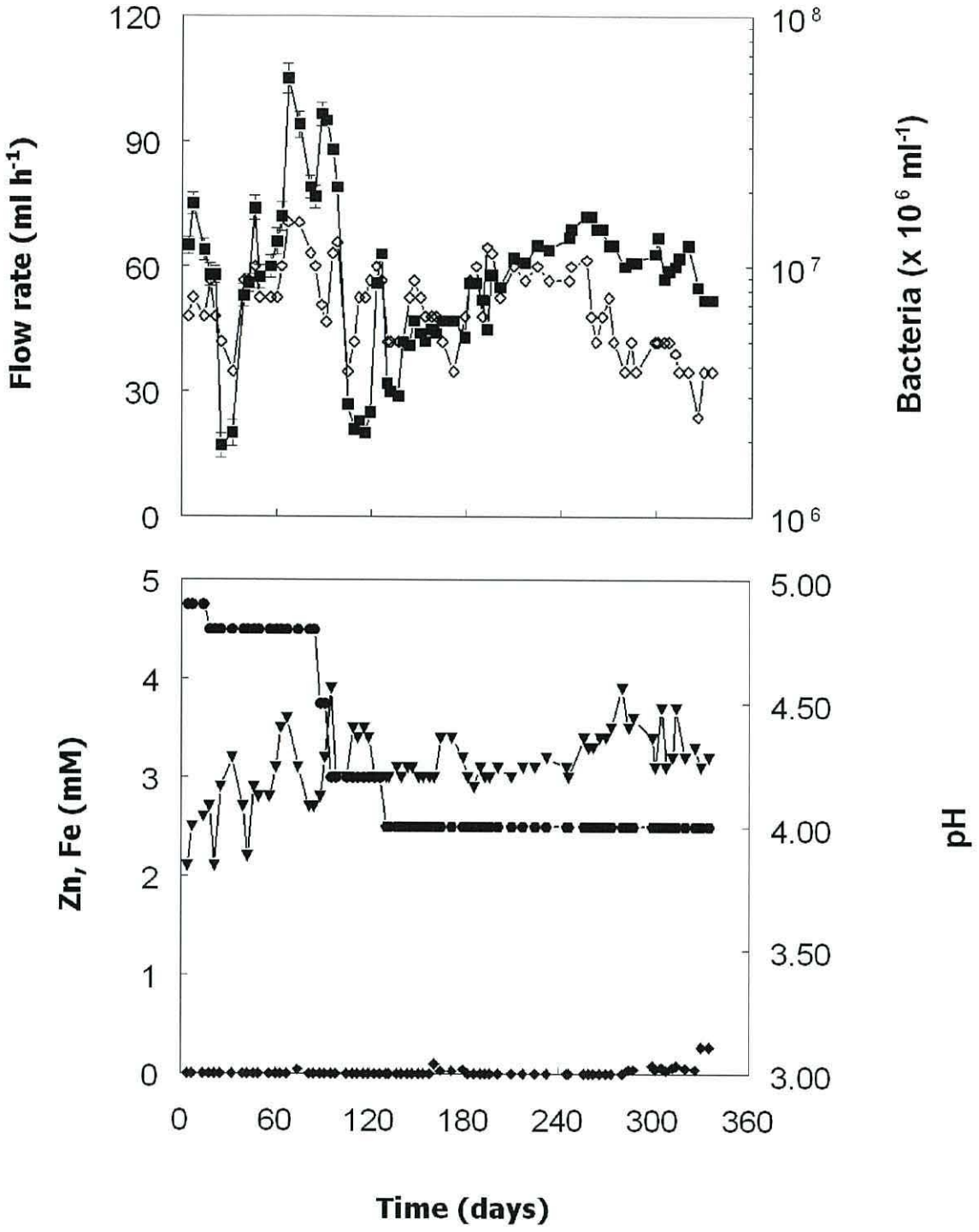

Fig. 5.5. Changes in flow rates (■), pH (●), concentrations of soluble zinc (♦) and soluble iron (▼), and numbers of planktonic-phase bacteria (◊) in bioreactor II. The composition of the feed liquor was based on that of an acidic (pH 2.5) stream draining the abandoned Cwm Rheidol mine in mid-Wales, and contained 3 mM of ferrous iron and 3 mM of zinc and varying concentrations of aluminium, as major soluble metals.

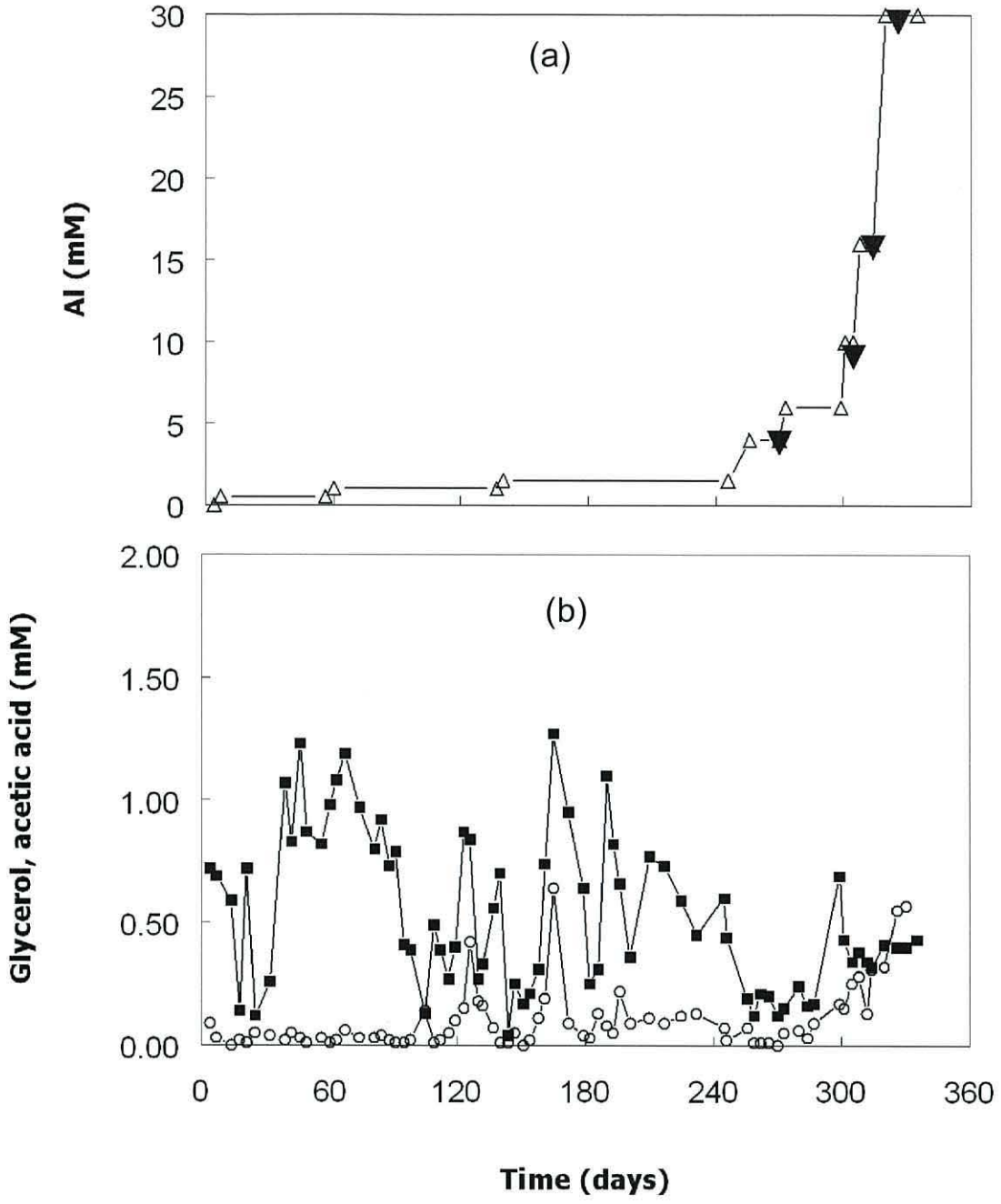

Fig. 5.6. (a) Concentrations of aluminium of the feed liquor (Δ) and those determined in bioreactor liquor on four sampling occasions (▲); (b) concentrations of glycerol (o) and acetic acid (■) in the bioreactor. Concentrations of glycerol in the feed liquor were maintained at 4 mM throughout the experiment.

5.3.3 ***Molecular analysis of bioreactor communities.*** Bacterial 16S rRNA genes were routinely amplified from both bioreactors, and subjected to semi-quantitative T-RFLP analysis to assess changes in microbial community structures with varying operating conditions. All attempts at amplifying archaeal 16S rRNA genes were unsuccessful (in contrast to positive controls), indicating that methanogenic prokaryotes were absent in both bioreactors. Results from T-RFLP analysis (Fig. 5.7) show that bacterial populations in both bioreactors changed in response to varying operational parameters. In the case of bioreactor I, the dominant terminal restriction fragment (T-RF) when the reactor was maintained at pH 3.6 corresponded to a novel *Firmicute* (coded IR2) isolated from this bioreactor (see below). The same T-RF was present when the pH of bioreactor I was lowered to pH 2.4 and then to 2.2, but in smaller relative abundance (Fig. 5.7a). T-RFs corresponding to sulfidogenic bacteria were 138 nt (which accounted for 54% of the total peak area at pH 2.4, but which was detected in a small proportion at pH 3.6 and not detected at lower pH values than 2.4) and 214 nt (which accounted for, at most, 6% of total peak area) in length (HaeIII digests). The 138 nt corresponded to a novel acidophilic SRB (CEB3) which was also isolated from bioreactor I (see below) while the 214 nt restriction fragment is common to both *Desulfosporosinus* M1 and "*Desulfobacillus* (*Db.) acidavidus*". Other T-RFs identified were 253 nt (corresponding to *Acidithiobacillus ferrooxidans*), and 231 nt, which was only founded when the reactor I was maintained at pH 3.6, and corresponded to a Gram-positive *Actinobacterium* isolate (IR1) isolated from bioreactor II maintained at pH 4.0 (described below). The relative abundance of the T-RF corresponding to *At. ferrooxidans* increased as the pH of bioreactor I was lowered, from 6% of total peak area at pH 3.6 to 20% at pH 2.2 (Fig. 5.7a).

In the case of bioreactor II, T-RFs corresponding to confirmed sulfidogenic bacteria accounted for more of the summated T-RF peak areas of all four samples analyzed than was the case with bioreactor I. The T-RF corresponding to the novel SRB isolate CEB3 (138 nt) increased progressively in relative peak area from <1 to 26% as the aluminium concentration in the feed liquor was increased, while that corresponding to *Desulfosporosinus* M1/ "*Db. acidavidus*" (214 nt) was the dominant T-RF in three of the four samples analyzed (Fig. 5.7b). T-RFs corresponding to *At. ferrooxidans*, and to the novel isolates IR1 and IR2, were also found in T-RFLP profiles of samples from bioreactor II but all three were less abundant than in bioreactor I.

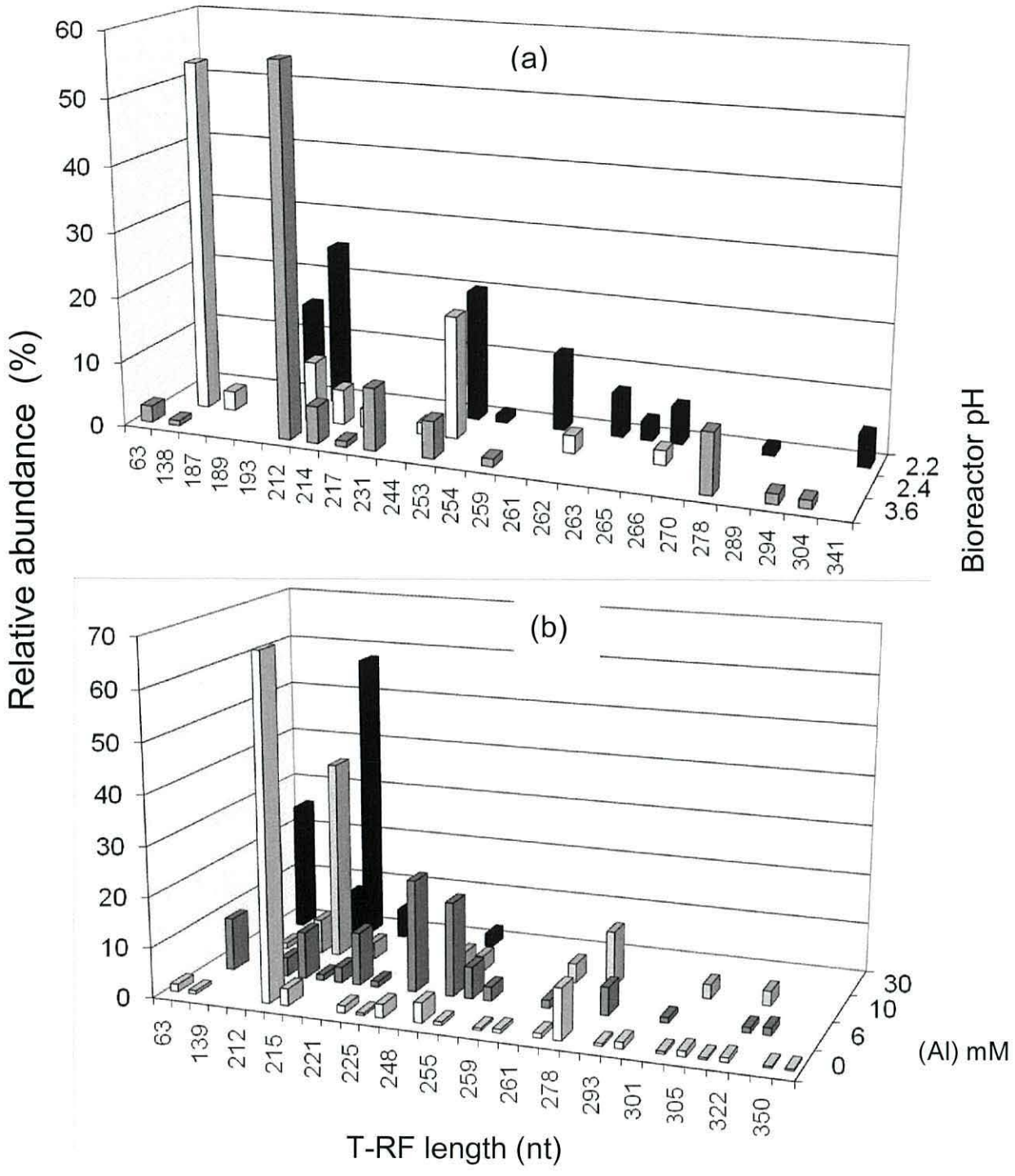

Fig. 5.7 Terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis of planktonic phase bacterial communities: (a) bioreactor I, sampled at different pH values; (b) bioreactor II, sampled at different aluminium concentrations in the feed liquor. Terminal restriction fragments (T-RFs) are of HaeIII-digests of 16S rRNA genes.

5.3.4 ***Isolation and phylogenetic analysis of bacteria.*** The appearance of T-RFs of sizes that were not registered in the acidophile databank held in the authors' laboratory prompted attempts to isolate the "unknown" bacteria that corresponded to these T-RFs. Three such isolates were obtained (Table 5.2). One of these (isolate CEB3) was confirmed to be an acidophilic SRB (sulfide was produced in pH 3.7 growth medium) while sulfidogenesis was not observed with isolates IR1 or IR2. The presence of *At. ferrooxidans* in the bioreactors inferred from T-RFLP profiles (T-RF of 253 nt. with HaeIII digests) was confirmed by isolating this facultative anaerobe from the bioreactors on ferrous iron-containing overlay media (Johnson and Hallberg, 2007).

5.4 Discussion

Microbiological sulfate reduction is of fundamental importance in engineered systems, such as compost bioreactors and permeable reactive barriers, which are used to remediate acidic, metal-rich streams and ground-waters (Johnson and Hallberg, 2005). Biological sulfidogenesis has three features that contribute towards mitigation of MIWs: (i) it is a proton-consuming reaction, (ii) many transition metals react with the end product (sulfide) to form highly insoluble mineral phases, and (iii) it lowers the sulfate concentrations of MIWs. Active mine water bioremediation systems that use SRB technology (e.g. Boonstra *et al.*, 1999) can be highly effective, but their widespread application has been limited by construction and operational costs. The prototype system described in the present work minimises the latter as: (i) it utilises a single reactor within which both sulfidogenesis and selective metal precipitation can occur; (ii) control of the system is possible by monitoring and maintaining the pH within the reactor, using low-cost equipment and empirical technology (a pH electrode and meter, and coupled pump); (iii) selective precipitation facilitates recovery of metals (e.g. copper and zinc) that have commercial value, and recycling these can off-set net costs (Pott and Mattiasson, 2004); (iv) the electron donor used to fuel the process (glycerol) is relatively inexpensive; (v) the electron acceptor used (sulfate) is present in all MIWs, and therefore no extraneous electron acceptor is required, as in the "BioSulphide" process where elemental sulfur is used as an electron acceptor (Bratty *et al.*, 2006).

Table 5.2. Some physiological and phylogenetic characteristics of the novel bacteria isolated from bioreactor I and II

Isolate	Bioreactor pH*	Major Physiological Characteristics	Amplification Product Length (nt) (16S rRNA Gene)	GenBank Accession No.	T-RF Length** (nt)	Nearest Relative	Identity (%)
CEB3	2.4	Spore-forming motile rods; obligately anaerobic sulfidogen	1314	JF346160	138	Uncultured *Desulfitobacterium* sp. clone E41 (Winch *et al.*, 2009)	95.0
IR1	4.0	Rod-shaped motile cells; spores not observed; facultative anaerobe; non-sulfidogen	1303	JF346161	231	Uncultured *Actinobacterium* clone (Akob *et al.*, 2008)	98.0
IR2	2.2	Spore-forming motile rods; facultative anaerobe; non-sulfidogen	1302	JF346159	212	Uncultured *Alicyclobacillus* sp. clone (G13_bac) (Winch *et al.*, 2009)	99.0

*at the time of isolation; ** digested with the restriction enzyme HaeIII

The two bioreactors which ran in parallel in the present study, processing different feed liquors, both achieved their key targets of demonstrating selective precipitation of transition metals. In the early stages of operation, with an influent glycerol concentration of 4-5 mM, both zinc and copper were precipitated (as sulfides) within bioreactor I while ferrous iron remained in solution (equation [2]):

$$4\ C_3H_8O_3 + 10\ H^+ + 7\ SO_4^{2-} + Cu^{2+} + Zn^{2+} + Fe^{2+} \rightarrow 12\ CO_2 + 5\ H_2S + CuS + ZnS + Fe^{2+} + 16\ H_2O \quad [2]$$

The amount of free hydrogen sulfide produced under such conditions involved a significant consumption of protons, and therefore a relatively large pH increase. To minimise this, and therefore preclude mineralisation of ZnS, smaller concentrations of glycerol were required. With a concentration of glycerol in the influent liquor of 0.7 mM, and a bioreactor pH maintained at 2.2, the net reactions that occurred were as depicted in equation [3], with only ~8% of the zinc, >99.9% of the copper and < 0.1% of the iron in the feed liquor being precipitated:

$$0.7\ C_3H_8O_3 + 0.45\ H^+ + 1.225\ SO_4^{2-} + Cu^{2+} + Zn^{2+} + Fe^{2+} \rightarrow 2.1\ CO_2 + 0.225\ H_2S + CuS + Zn^{2+} + Fe^{2+} + 2.8\ H_2O \quad [3]$$

In the case of bioreactor II, the feed liquor chemistry was based on water draining an abandoned zinc mine where concentrations of soluble copper were negligible, and the objective here was to produce a "clean" ZnS precipitate. Again, by maintaining an acidic bioreactor pH (though not as low as that of bioreactor I), precipitation of FeS was readily avoided. However, like many other MIWs, that draining the Cwm Rheidol mine contained significant amounts of soluble aluminium. Although it does not form a sulfide mineral, aluminium can precipitate as an hydroxide phase (gibbsite) in moderately acidic (pH >5: Bache, 1986) solutions. Gibbsite forms bulky, gelatinous deposits in such circumstances, and these could cause severe blockage problems in continuous flow bioreactors. In the event, segregation of zinc and aluminium was readily achieved by maintaining bioreactor II at pH 4.0 - 4.5, and supplementing the feed liquor with 4 mM glycerol (equation [4]):

$$4\ C_3H_8O_3 + 8\ H^+ + 7\ SO_4^{2-} + 3\ Zn^{2+} + Fe^{2+} + Al^{3+} \rightarrow 12\ CO_2 + 4\ H_2S + 3\ ZnS + Fe^{2+} + Al^{3+} + 16\ H_2O \quad [4]$$

One of the risks with maintaining potentially toxic ions, such as aluminium, in solution is that they may have a negative impact on the SRB that are critical to the remediation process. However, even when the concentration of aluminium in the feed liquor was as high as 30 mM (810 mg l^{-1}; a concentration far greater than that of most MIWs) sulfidogenic activity was maintained in bioreactor II, though increasing concentrations of aluminium caused changes in the bioreactor microbial community, and also resulted in smaller amounts of glycerol being oxidized. Further evidence of the potential of the acidophilic sulfidogenic bioreactors to remediate complex MIWs came from tests with bioreactor I where synthetic Mynydd Parys mine water was used as feed liquor. No precipitation of iron, aluminium or manganese occurred within the reactor, though >99% of the copper and smaller amounts of zinc were removed.

The key component of the system described is the acidophilic sulfate-reducing bacterial community. Acidophilic SRB are not well known, though some acid-tolerant species have been reported (e.g. Johnson, 1995a; Hard *et al.*, 1997; Alazard *et al.*, 2010). We included two pure cultures of SRB isolated during earlier studies on the microbial ecology of mine-impacted environments, in the bioreactor consortium. One of these (*Desulfosporosinus* M1) was the first acid-tolerant SRB to be described (Sen and Johnson, 1999) though the proposed species designation ("*Desulfosporosinus acidophilus*") was also later used for another isolate (Alazard *et al.*, 2010). "*Db. acidavidus*" is a more acidophilic isolate (capable of growth in pure culture in pH 3 liquid media) which was isolated from an anaerobic "mat" community at the Cantareras mine, an enrichment culture of which was also included in the original inoculum used in the pre-trial bioreactor, and from which the other bacteria detected (and, in some cases, isolated) in the bioreactor liquors would have arisen. A T-RF restriction fragment that corresponded to both *Desulfosporosinus* M1 and "*Db. acidavidus*" dominated the DNA amplified from bioreactor II, but was less abundant in bioreactor I, probably due to the lower pH at which the latter was operated. One of the "unknown" T-RFs was found to correspond to a novel bacterium (strain CEB3) isolated from bioreactor I, but also detected in bioreactor II. Interestingly, strain CEB3 appeared to be the dominant bacterium present when bioreactor I was operated at pH 2.4, though it was not detected at pH 2.2. It also appeared to became increasingly abundant in bioreactor II when the aluminium concentration in the feed liquor was increased. Preliminary work with a pure culture of isolate CEB3 have confirmed that it is acidophilic, and further characterization of is continuing. The other bacteria detected in bioreactor I, however, are more enigmatic. Sulfidogenesis was confirmed

to be a major metabolic process at all pH values at which it was operated, and the stoichiometry of copper and zinc precipitated as sulfides when the feed liquor contained only 0.7 mM glycerol indicated that most of the glycerol oxidized was being coupled to the reduction of sulfate to hydrogen sulfide (equation [3]). However, on most sampling occasions the dominant T-RFs obtained of amplified DNA from bioreactor I corresponded to either of *At, ferrooxidans* or a *Firmicute* related to bacteria of the genus *Alicyclobacillus* (isolate IR2). *At. ferrooxidans* is best known as an iron/sulfur-oxidizing aerobe, though it can grow anaerobically using ferric iron as electron acceptor, or (in some strains) by sulfur reduction (Pronk *et al.*, 1991; Ohmura *et al.*, 2002). It does not, however, reduce sulfate to hydrogen sulfide, and its presence in both of the sulfidogenic bioreactors was thought due to the fact that the feed liquors were not de-oxygenated, so *At. ferrooxidans* was probably using the small amounts of dissolved oxygen present to oxidize ferrous iron to ferric. Oxidized iron would have been rapidly reduced back to ferrous in the prevailing conditions (presence of sulfide etc.) within the bioreactor. Isolate IR2 was also confirmed to be a facultative anaerobe, though it displayed no propensity to use sulfate as a terminal electron acceptor. This was also the case for other bioreactor isolate (IR1, an *Actinobacterium*). The roles and significance of isolates IR1 and IR2 in the sulfidogenic bioreactors cannot, at this stage, be ascertained, though they may be involved in syntrophic interactions with the SRB. Kimura *et al.* (2006) reported that *Desulfosporosinus* M1 and a strain of *"Acidocella aromatica"* (a non-sulfidogenic, obligately heterotrophic acidophile) formed a stable syntrophic partnership wherein PFBC metabolised the acetic acid produced by the SRB by its partial oxidation of glycerol, generating molecular hydrogen which was used as a secondary electron donor, by *Desulfosporosinus* M1.

The sulfidogenic bioreactors described here provide "proof of principle" that segregation of metals present in MIWs is possible in on-line systems by controlling the pH of the reactors. The modular units themselves are versatile and robust, and can be configured to fit into a variety of design options. For example, a single acidic sulfidogenic bioreactor could be used to recover both copper and zinc, selectively, from mine water, by adding sufficient electron donor (glycerol) to cause hydrogen sulfide to be over-produced in a pH 4, ZnS-precipitating bioreactor, the excess being delivered in a gas-stream to a vessel containing the "raw" mine water, where pH control could be used to facilitate selective mineralization of copper sulfide.

Supplementary Material: Chapter 5

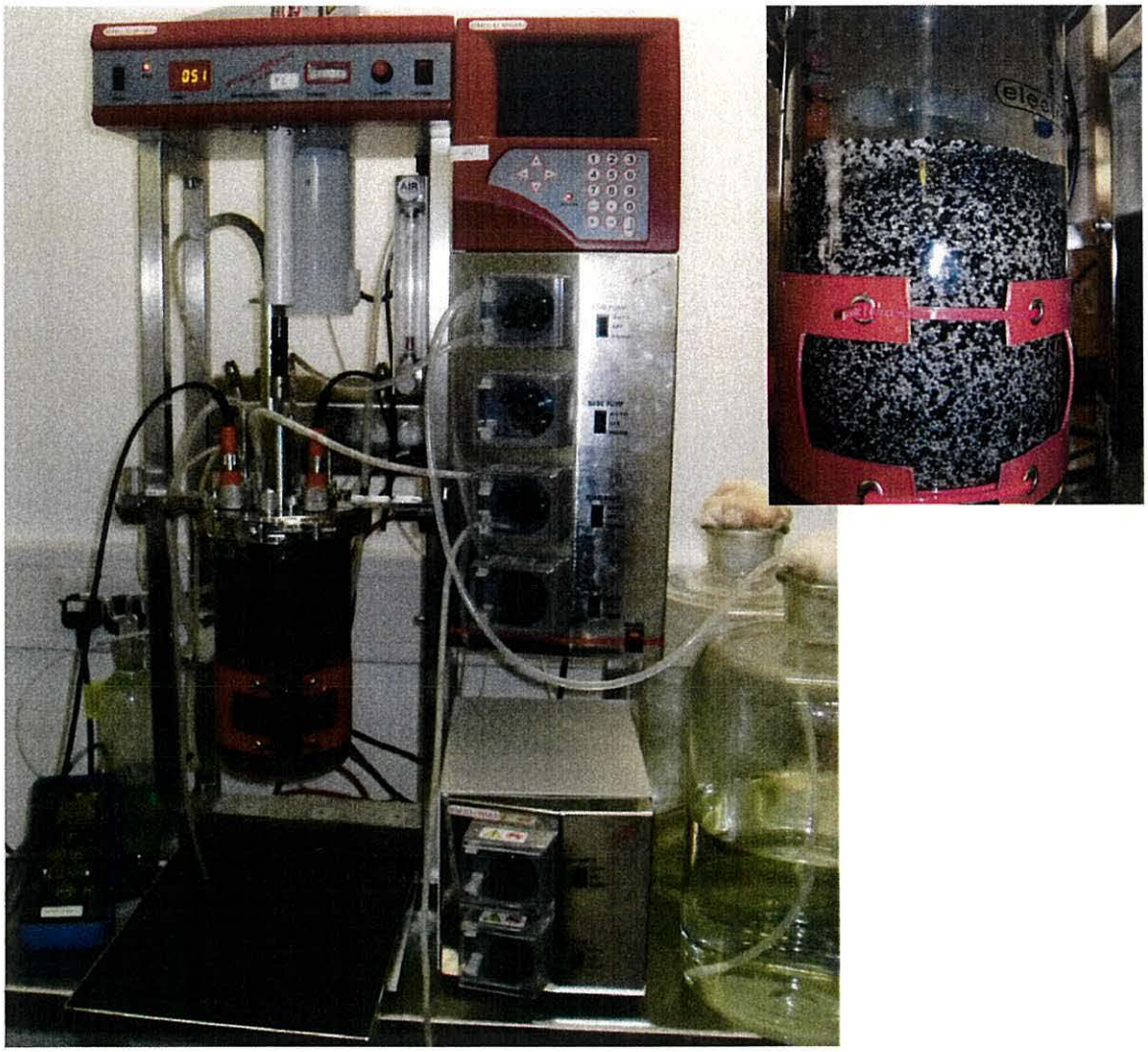

Fig. S5.1. Bioreactor II culture vessel and FerMac control unit. Inset: image of Poraver beads at the outset of the experiment, showing dark-coloured colonized beads mixed with new (white-coloured) non-colonized beads.

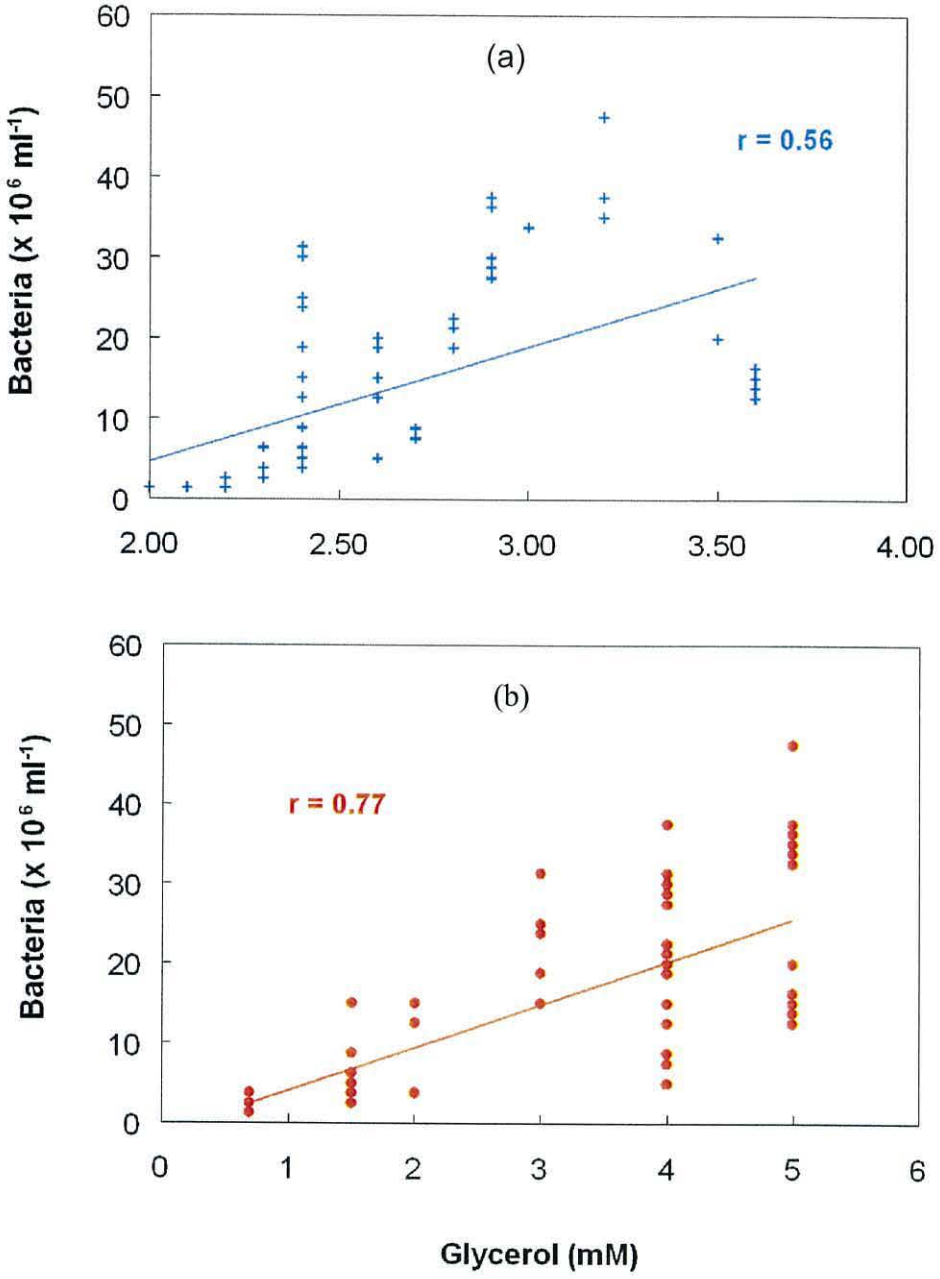

Fig. S5.2. Comparison of correlations between numbers of planktonic phase bacteria in bioreactor I during the first phase of operation with, (a) bioreactor pH, and (b) glycerol concentration in the feed liquor.

Chapter 6

Safeguarding reactive mine tailings by ecological engineering: the significance of microbial communities and interactions

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Abstract

Pyritic mine tailings, common waste products of metal mining, pose significant environmental hazard since they are major sites of production of acidic, metal-rich effluents (acid mine drainage; AMD). Accelerated oxidative dissolution of pyrite and other sulfide minerals in tailings by acidophilic chemolithotrophic prokaryotes has been widely reported. However, other acidophiles (heterotrophic bacteria that catalyze the dissimilatory reduction of iron and sulfur) can reverse the reactions involved in AMD genesis, and have been implicated in "natural attenuation" of some AMD streams. We have investigated whether by inoculating tailings with mixed populations of iron/sulfur reducing acidophiles and sustaining their growth and activities with organic carbon derived from phototrophic acidophiles, it is possible to mitigate the impact of the acid-generating and metal-mobilizing chemolithotrophs that are indigenous to tailings deposits. For this, sixty tailings mesocosms were set up using five different inoculation variants and analyzed at intervals for physico-chemical and microbiological properties for up to 12 months. Significant differences between treatment protocols were most apparent with tailings that were inoculated with acidophilic algae, as well as aerobic and anaerobic heterotrophic bacteria. These included greater pH and redox potentials and much lower concentrations of soluble copper and zinc than in tailings that were inoculated with only pyrite-oxidizing chemolithotrophs. The results suggest that empirical ecological engineering of tailings lagoons to promote the growth and activities of iron- and sulfur-reducing bacteria could minimize the risk of AMD production, and that by promoting surface growth of suitable algae these ecosystems could become self-sustaining.

6.1 Introduction

Mining of metals has been integral to the development of human civilization. As higher-grade ores become depleted, the primary ores that are processed by mining companies are increasingly of lower grade (metal content) and the amount of waste material produced by mining operations is consequently greater. In many cases, metal ores are crushed and ground, and the target metal minerals concentrated by a process known as froth flotation (Dold, 2010). This involves the addition of chemicals ("collectors") to ground ore suspensions which attach to the target minerals causing their surfaces to become hydrophobic. Controlled aeration of the treated suspension allows air bubbles to attach to the modified minerals, causing them to float and facilitating their separation from hydrophilic minerals which settle or remain in suspension. Other chemicals, such as lime, can enhance the separation of target and non-target minerals. The fine-grain mineral wastes which results from froth flotation are referred to as "tailings" and, in copper mining, these can account for 95-99% of the crushed and ground ores (Dold, 2010).

Many base metal ores are sulfidic, and frequently contain large concentrations of the most abundant of all metal sulfides, pyrite (FeS_2) which, with other non- target minerals, mostly ends up in the tailings wastes. Pyrite and other sulfide minerals are potentially highly reactive minerals. In the presence of both air (oxygen) and water, pyrite oxidizes ultimately to sulfate and ferric iron with the concomitant production of proton acidity, as illustrated in equation [1] where the oxidized iron mineral phase generated is shown as schwertmannite (a commonly encountered ferric iron mineral in ferruginous waters with pH values of ~ 3 to 5):

$$8\ FeS_2 + 30\ O_2 + 18\ H_2O \rightarrow Fe_8O_8(SO_4)(OH)_6 + 15\ SO_4^{2-} + 30\ H^+ \qquad [1].$$

Pyritic mine tailings therefore have the potential to become extremely acidic and enriched with soluble sulfate and, because of their greater solubilities in acidic liquors, transition metals and aluminum. Surface waters percolating through tailings and other mine wastes (low-grade waste rocks etc.) can transfer these potentially noxious solutes to the wider environment where they can have severe detrimental impact on impacted stream and river ecosystems, as "acid mine drainage" (AMD; Nordstrom, 2000).

The mechanisms by which the oxidation pyrite and other sulfide minerals occurs has been the subject of a considerable body of research (reviewed in

Rohwerder *et al.*, 2003). In acidic liquors, the primary oxidant is ferric iron rather than molecular oxygen, and this is reduced to ferrous iron when it reacts with metal sulfides. For the reaction to continue, ferric iron needs to be regenerated, which requires oxygen. This can occur by spontaneous chemical oxidation at circum-neutral pH values but abiotic iron oxidation becomes increasingly slow with decreasing pH, even in oxygen-saturated waters (Stumm *et al.*, 1981). However, some species of acidophilic bacteria and archaea exploit this situation by using ferrous iron as an energy source (sometimes as the sole electron donor) to support their growth. By so doing, acidophilic iron-oxidizing prokaryotes can accelerate the rate of pyrite oxidation in acidic liquors by several orders of magnitude compared to the abiotic process, and therefore these microorganisms are perceived to have a crucial role in generating AMD (Johnson, 2003).

To reduce the risk of potentially reactive mine tailings generating acidic, metal-rich effluents, these are generally stored under water to minimize their exposure to oxygen. However, this is only a partial solution to the problem, as oxygen diffusion will facilitate ferrous iron oxidation in the tailings/water surface zones, and migration of the ferric iron produced to lower zones can cause oxidation of pyrite and other sulfide minerals even in anoxic zones. In situations where tailings impoundments are allowed to drain, distinct zonation has been reported, with a surface "oxidation zone" overlying (in sequence) a "neutralization zone" and an unaltered "primary zone" (Diaby *et al.*, 2007). Bacteria were been detected in largest numbers in the "oxidation front" (the junction between the oxidation and neutralization zones) in stratified tailings, with *Leptospirillum ferrooxidans* and *Sulfobacillus* spp. (and smaller numbers of *Acidithiobacillus (At.). ferrooxidans*) identified as the mineral-oxidizing bacteria present;heterotrophic *Acidiphilium* and *Acidobacterium* spp. were detected in the oxidation zone (Diaby *et al.,* 2007). Kock and Schippers (2008) found that greatest numbers of bacteria (which were more numerous than both archaea and eukaryotes) were also present in the oxidation zones of acidic mine tailings sampled in Botswana, Sweden and Germany, and that *Acidithiobacillus* spp. were more abundant than *Leptospirillum* spp. in two of the three sites. In contrast, the indigenous microfloral community in a highly oxidized and extremely acidic (pH 2.2) tailings at an abandoned copper mine (São Domingos) in Portugal was dominated by *Firmicutes* (*Alicyclobacillus*- and *Sulfobacillus*-like) and sulfur-oxidizing acidithiobacilli (Bryan *et al.*, 2006).

Besides their importance in AMD genesis, microorganisms can also have a role in mitigating the environmental effects of mine water pollution. There have been several reports of natural attenuation of AMD in which microbially-catalyzed changes in water chemistry has caused streams to become less bio-toxic as they flow from their points of discharge. For example, oxidation of ferrous iron and spontaneous hydrolysis and precipitation of the ferric iron produced has been reported to significantly lower concentrations of soluble iron in streams in the Iberian pyrite belt (IPB; Sanchez Espaňa *et al.*, 2005), and bacterial oxidation of soluble arsenic (III) to arsenic (V), which readily co-precipitates with ferric iron minerals such as schwertmannite, or as scorodite ($FeAsO_4$), has been reported in Carnoulès, southern France (Bruneel *et al.*, 2011) and in Yellowstone National Park, Wyoming (Donahoe-Christiansen *et al.*, 2004). Elsewhere within the IPB, Rowe *et al.* (2007) described a complex natural attenuation of AMD draining an abandoned copper mine that involved iron-reducing bacteria, sulfate-reducing bacteria (SRB) and several species of acidophilic algae. Acidic (pH 2.65) water inside the main adit at this mine (Cantareras) contained elevated concentrations of soluble ferrous iron (1,100 mg/l) and copper (140 mg/l), as well as other metals and sulfate. While there were no obvious microbial growths within the adit itself, on exiting the mine the drainage channel was ramified with thick (10 - 20 cm) gelatinous streamer/mat growths. The surfaces of these were green colored and dominated by acidophilic algae (unicellular *Euglena, Chlamydomonas*, and filamentous *Zygnema*) while the stratified under-layers were dominated by iron-oxidizing and iron-reducing bacteria (*Acidithiobacillus* spp., *Ferrimicrobium, "Ferrovum"*, *Acidiphilium* and *Acidobacterium*) and the lowest (black-colored) layers by uncharacterized species of SRB, some of which were isolated and shown to be acidophilic. Water analysis showed that, while microbial ferrous iron oxidation was occurring in the surface waters, iron reduction was more prevalent streamer/mat growth and sulfate reduction at the lowest depths. Sulfidogenesis resulted in the formation of CuS (though not FeS, as the mine water at depth was also of pH <3), and resulted in some of the copper that had been liberated from the residual copper minerals within the mine being immobilized with the streamer/mat growths. The role of the acidophilic algae was shown to be critical to this natural attenuation process as these phototrophs provided the organic carbon that supported the growth of the heterotrophic iron- reducing bacteria and SRB housed in the streamer/mat growths.

Given the conflicting roles of acidophilic microorganisms in generating or mitigating AMD pollution, we have sought to test the hypothesis that, by promoting the growth and activities of acidophilic communities that can counter the processes of acid generation and metal mobilization, it would be possible to mitigate the potential environmental impact of reactive mine tailings.

6.2 Materials and Methods

6.2.1 *Mine tailings.* Pyritic tailings were obtained from the Aguablanca nickel-copper mine, located in south-east Spain. This mining operation produces a nickel-copper concentrate from a sulfidic ore body by conventional crushing, grinding and froth flotation. The waste ground ore is stored underwater at the mine site in a tailings lagoon. Tailings obtained from the mine were ground and washed with 3 M sulfuric acid to remove residual alkalinity, rinsed repeatedly with reverse osmosis (RO)-grade water and dried at 50°C. The pH of the tailings slurry (1:2.5 water suspension) was 2.5 following acid treatment, and the grain size was between 61 and 200 µm. The pyrite content of the tailings was determined using the method described by Dacey and Colbourn (1979). Mineralogical analysis was carried by X- ray diffraction (XRD) using a Philips PW3040/60 X' Pert PRO and data analyzed using the PANalytical search-match program "Highscore".

6.2.2 *Microorganisms.* Several cultures of acidophilic bacteria and algae, sourced from the *Acidophile Culture Collection* maintained at Bangor University, were used to inoculate the tailings mesocosms, as described below. These were: (i) iron- and pyrite-oxidizing autotrophic bacteria, *Acidithiobacillus ferrooxidans*^{T} (ATCC 23270), *Acidithiobacillus ferrivorans*^{T} (strain NO37 (Hallberg *et al.*, 2010)) and *Leptospirillum ferrooxidans* strain CF12 (Johnson, 1995b). In addition to being able to couple the oxidation of ferrous iron and reduced sulfur to the reduction of molecular oxygen, both *Acidithiobacillus* spp. are able to catalyze the dissimilatory reduction of ferric iron in anaerobic environments.; (ii) iron-reducing heterotrophic bacteria (*Acidiphilium* strain SJH, *Acidocella* strain PFBC and *Acidobacterium* strain Thars1 (Bridge and Johnson, 2000; Coupland and Johnson, 2008); (iii) an acidophilic SRB consortium taken from two laboratory bioreactors used to selectively remove transition metals from mine waters at low pH (2-4; Ňancucheo and Johnson, 2011a). Acidophilic SRB identified in this consortium included *Desulfosporosinus* strain M1, "*Desulfobacillus*"

strain CL4 and *Desulfitobacterium* strain CEB3; (iv) two pure cultures of unicellular algae (*Chlorella prothecoides* var. *acidicola* and *Euglena mutabilis* (Ňancucheo and Johnson, 2011b). The acidophilic microorganisms were all grown in appropriate liquid media (Johnson and Hallberg, 2007; Wakeman *et al.*, 2008). These were (i) 1% (w/v) of pyrite /basal salts (pH 2.0) for the iron-oxidizers; (ii) 5 mM fructose /0.02% (w/v) yeast extract (pH 3.5) for the iron-reducing heterotrophs; (iii) 3 mM glycerol, 0.01% (w/v) yeast extract (pH 3.0) for the bioreactor SRB consortium (iv) Basal salts/trace elements supplemented with 100 µM of ferrous sulfate (pH 2.5) for the acidophilic algae. Each microorganism (except the anaerobic consortium) was grown as a separate pure culture and combined inocula of each physiological group (iron-oxidizing autotrophs, iron-reducing heterotrophs, sulfate reducers and algae) used to inoculate the mesocosms, as described below.

6.2.3 *Mesocosm set up.* Stainless steel cylinders (5 cm high x 5 cm diameter; 100 cm^3 capacity) designed for sampling soil cores (Eikelkamp Agrisearch Equipment bv, the Netherlands) were filled with 100 g of dry, acid-washed tailings, Plastic covers were pushed on to the base of each cylinder, and silicon sealant used to prevent water seepage. The amount of water required to produce 100% saturation of the tailings mesocosms was evaluated. Subsequently, sterile RO-grade water (with or without a bacterial/algal inoculum) was added initially to produce 75% saturation of each mesocosm. Five weeks into the experiment it was noted that this amount of water was insufficient to allow good growth of the acidophilic algae on the tailings surface, and therefore additional water was added to bring the tailings mesocosms to 95% saturation.

Five tailings mesocosm variants were set up. These were: (i) treatment I (TI) control mesocosms, to which only water was added; (ii) treatment II mescosms, which were inoculated with iron-oxidizing autotrophs (~ 7.4 x 10^8 cells/mesocosm); treatment III mescosms: as TII but also inoculated with iron-reducing heterotrophs (~ 1.6 x10^9 cells/mesocosm); treatment TIV mesocosms: as TIII, but also inoculated with the SRB bioreactor liquor (~ 3.3 x 10^8 cells/mesocosm); treatment V mesocosms: as TIV, but also inoculated with acidophilic algae (~ 4.1 x10^8 cells/mesocosm). All bacteria inocula were included with the bulk water added to the tailings, but the algae were added subsequent to this, in an attempt to concentrate the phototrophs at the surface of the mesocosms.

A total of 60 tailings mesocosms were prepared (12 for each treatment). These were weighed individually, covered loosely with sterile plastic petri plate tops and placed in a plant growth room, maintained at 22°C and illuminated with 70 µmol of photons $m^{-2} s^{-1}$. Each mesocosm was reweighed on a weekly basis and water loss corrected for by adding the equivalent amount of RO water.

6.2.4 ***Sampling of mesocosms and analysis of tailings.*** The experiment was set up during March 2010 and samples were removed for analysis at 3 month intervals up to April 2011. On each sampling occasion, three mesocosms for each treatment variant were removed and sampled destructively. The tailings cores were removed carefully from their stainless steel containers, sectioned with a knife and inspected visually for signs of mineral oxidation (presence of rust-colored ferric iron precipitates) and sulfate reduction (evolution of hydrogen sulfide following addition of hydrochloric acid to tailings samples). Vertical sections (~10 g) from each core were removed and mixed to give a homogeneous representative tailings sample, and sub-samples of this analyzed for physico-chemical and microbiological parameters, as described below. Samples removed from the surfaces of algal-inoculated mesocosms were examined with a phase contrast microscope (Leitz Labolux, at x400 magnification).

6.2.5 ***Physico-chemical analysis.*** One gram (dry weight equivalent) of homogenized tailings sample was mixed with 2.5 ml of RO water, mixed by vortexing and left standing at room temperature for 30 minutes. The pH and redox potential (E_h values, relative to a standard hydrogen reference cell) of the suspension were measured using a pHase combination glass electrode and a platinum combination redox electrode (VWR, UK), both coupled to a Accumet pH/redox meter. Concentrations of total soluble iron, ferrous iron, copper, zinc, nickel and sulfate were also determined in 2.5:1 water extracts of homogenized tailings, filtered through 0.2 µm pore size nitrocellulose membranes. Acid (5 M HCl)-extractable iron in tailings samples were determined using the method described by Dopson and Lindstrom (Dopson and Lindstrom, 1999). Metal concentrations in water- and acid-extracts were determined using a Dionex-320 ion chromatograph fitted with an IonPAC® CS5A column and an AD absorbance detector. Sulfate concentrations in water extracts were measured using a Dionex IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity detector. Concentrations of ferrous iron were determined using the ferrozine assay (Lovley and Phillips, 1987).

6.2.6 ***Microbiological and molecular analysis.*** To enumerate aerobic iron-oxidizing and heterotrophic bacteria, 1 g of a homogenized tailings sample from each mesocosm was added to 2.5 ml of basal salts solution (pH 3.5) and mixed by vortexing. The samples were serially diluted and spread onto ferrous iron overlay solid medium (Johnson and Hallberg, 2007) to enumerate iron-oxidizing autotrophic bacteria, and 5 mM fructose/0.02% (w/v) yeast extract solid medium (pH 3.5) to enumerate heterotrophic iron-reducing heterotrophic bacteria. These plates were incubated, aerobically, at 30°C for 20 days, and colony forming units (CFUs) counted. To enumerate anaerobic acidophiles, tailings suspensions (prepared as above) were serially diluted and spread onto an overlay medium (pH ~3.7) containing 4 mM glycerol, 0.02% (w/v) yeast extract, 7 mM zinc and 0.5 mM ferrous iron (24). Plates were incubated anaerobically (using the AnaeroGen™ system; Oxoid, U.K.) at 30°C for 30 days and numbers of CFUs recorded.

Bacterial colonies were enumerated and physiological groups identified from colony morphologies, as described elsewhere (Johnson and Hallberg, 2007). In order to confirm the identities of isolates (and to identify colonies corresponding to bacteria that were not present in the inocula) colonies were picked off plates and resuspended in basal salts, DNA extracted and 16S rRNA genes amplified, sequenced and compared to those in public databases, as described previously (Rowe *et al.*, 2007).

6.2.7 ***Dissimilatory ferric iron reduction.*** Dissimilatory reduction of ferric iron by a *Curtobacterium* sp. isolated from the tailings was tested in anaerobic liquid media. Duplicate universal bottles containing 100 mg of amorphous ferric hydroxide (Bridge and Johnson, 2000) were filled with a liquid medium containing 5 mM glucose and 0.02% (wt/vol) yeast extract (pH 4.0), and inoculated with an aerobically-grown culture of the isolate. The bottles, together with a non-inoculated control, were incubated at 30°C and sampled at regular intervals to determine concentrations of ferrous iron (ferrozine assay) and cell numbers (using a Thoma counting chamber). The pH values of inoculated and non-inoculated media were recorded at the end of the experiment.

6.2.8 ***Statistical analysis.*** Data were analyzed using SPSS version 17.0 (SPSS, Chicago, IL). One way Anova was carried out to compare means of different treatments and pairwise comparisons were done using Tukey test at 0.05 significance level

6.3 Results

6.3.1 ***Chemistry and mineralogy of the tailings material.*** Chemical analysis of the tailings from the Aguablanca mine showed that it had an equivalent pyrite (FeS_2) content of 6.5% (by wt.). XRD analysis confirmed that silicates were the dominant minerals in the tailings. Peaks corresponding to pyrite and arsenopyrite, but not to any other sulfide minerals, were apparent. Acid-washing was required to remove residual alkalinity in the fresh tailings. Lime is often added to suppress pyrite flotation during the production of metal concentrates, but tends to be readily washed out of tailings impoundments and has only a short-term effect on pH-buffering of pyritic wastes (Dold, 2010). Analysis of the acid-washed tailings showed that they contained very small concentrations of soluble iron (~ 1 µg/g dry tailings) and that concentrations of soluble copper, zinc and nickel were below detectable levels.

6.3.2 ***Physico-chemical changes in incubated mesocosms.*** Time course changes in both physico-chemical and microbiological properties of the tailings mesocosms, on a treatment by treatment basis, are shown in Figs. 6.1-6 Supplementary Tables S6.1-11 show data obtained on each sampling occasion and indicate where these displayed significant differences.

Three months after the start of the experiment, the pH values of all of the inoculated mesocosms were significantly lower than those of the non-inoculated control. During the course of the experiment, the pH of the non-inoculated tailings declined a little, though tended to remain slightly higher than those of the inoculated mesocosms. The exception to this were the tailings that had been inoculated with the acidophilic algae, where mean pH values increased progressively during the course of the experiment (Fig. 6.1) and were significantly higher by month 12 than in all other mesocosms (Supplementary Table S6.1). Redox potential were more positive in most of

the inoculated mesocosms than in the TI controls after three months and tended to remain as such, though there was a notable increase in the non-inoculated tailings by month 12 (Fig. 6.1). Again, the algal-inoculated tailings did not follow this trend. E_h values tended to remain at around +400 mV throughout (Fig. 6.1) and were significantly less positive on all sampling occasions (except on month 6) than in all other inoculated mesocosms (Supplementary Table S6.2).

Concentrations of soluble ferrous tended to decline with time in both control and inoculated mesocosms, with the exception of those that were inoculated with acidophilic algae where they increased significantly from month 3 to month 6 (Fig. 6.2). Comparison of different treatments on each sampling time showed that, while there were no significant differences at month 3, ferrous iron contents were significantly greater in algal-inoculated tailings on all subsequent sampling occasions (Supplementary Table S6.3). In contrast, total soluble iron tended to be greatest in tailings mesocosms that had been inoculated only with pyrite-oxidizing bacteria (Fig. 6.2), though differences were not always significant (Supplementary Table S6.4).

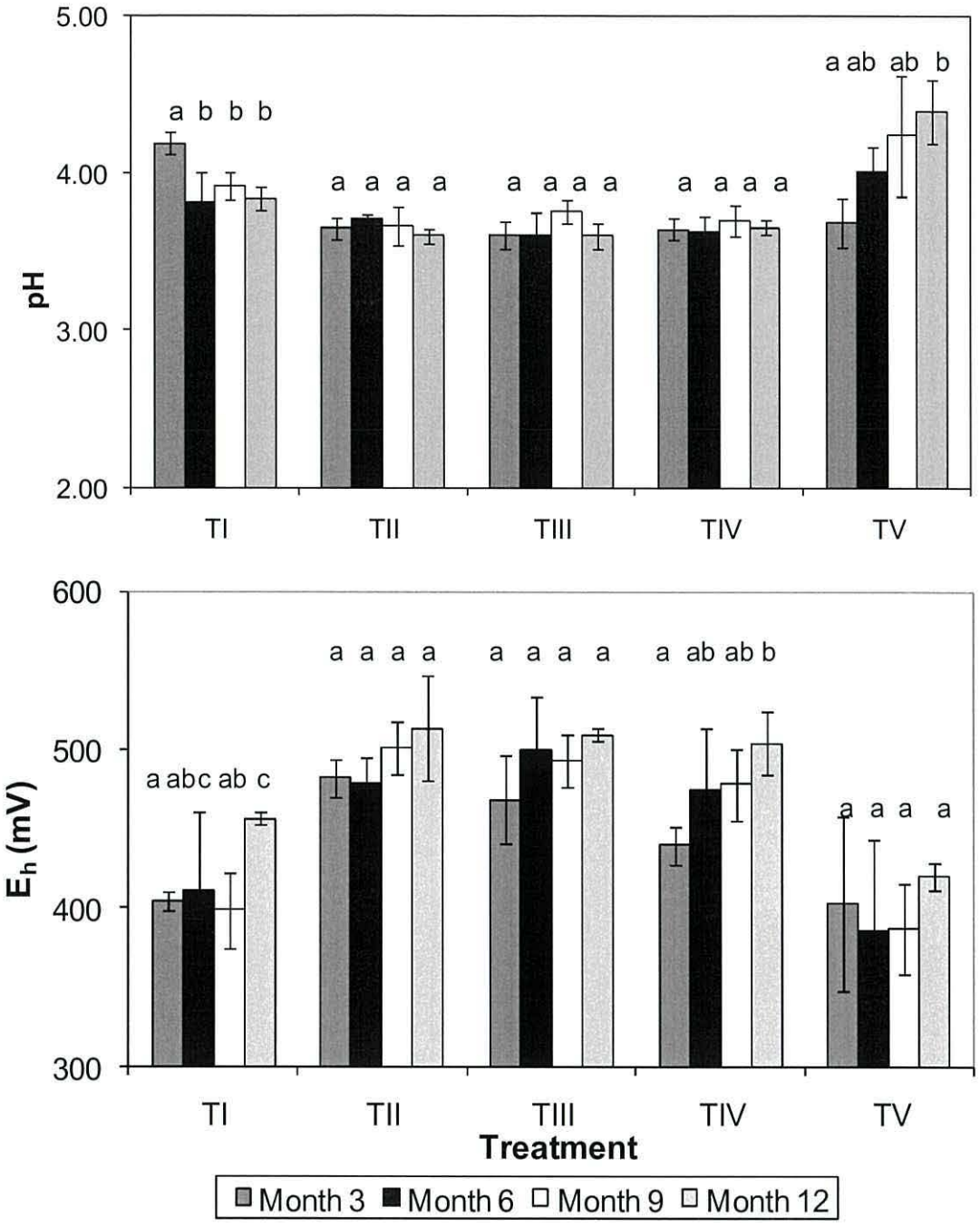

Fig. 6.1. Time-course changes in pH values (top) and redox potentials (bottom) in mine tailings mesocosms inoculated with different populations of acidophilic microorganisms (treatments II-V) and non-inoculated controls (treatment I). For each treatment data set, bars not annotated with the same letter are significantly different (95% confidence limit). Error bars represent standard deviations.

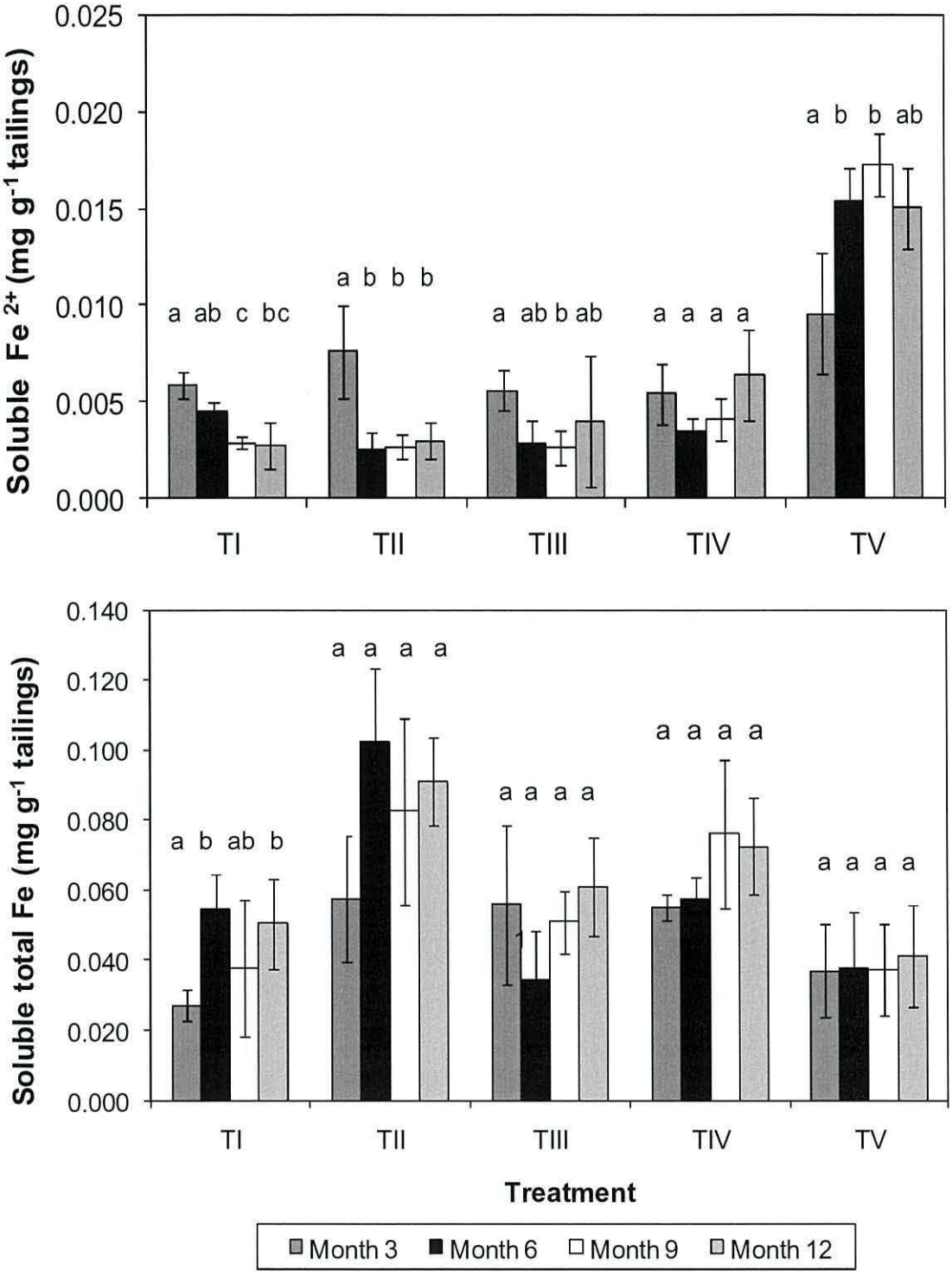

Fig. 6.2. Time-course changes in ferrous iron (top) and total iron (bottom) in water extracts of mine tailings inoculated with different populations of acidophilic microorganisms (treatments II-V) and non-inoculated controls (treatment I). For each treatment data set, bars not annotated with the same letter are significantly different (95% confidence limit). Error bars represent standard deviations.

Acid-extractable iron, which would have included secondary ferric iron precipitates as well as soluble iron (Dopson and Lindstrom, 1999) showed a general increase with time in all tailings mesocosms, but differences were often not significant (Fig. 6.3 and Supplementary Table S6.5). Visual inspection of sectioned cores showed that secondary ferric iron precipitates tended to accumulate in a distinct layer below the surface algal growths in treatment V mesocosms but were more randomly distributed in other mesocosms (Supplementary Fig. S6.1). It was also noted that hydrogen sulfide was released from acidified treatment V tailings mesocosms on month 12 though no odor was detected for other treatments on any sampling occasion. Concentrations of soluble sulfate decreased from month 6 in tailings mesocosms that had been inoculated with the mixed culture of SRB but only significantly so in those tailings that had also been inoculated with algae (Fig. 6.3). The greater sulfate concentration in inoculated compared to control mesocosms at month 3 was probably due to sulfate added with the inoculating liquors.

Several transition metals, in addition to iron, were detected in water-extracts of the tailings mesocosms. Copper concentrations increased from month 3 to month 6 in control mesocosms and remained relatively stable thereafter, and all time-related changes in treatment II and III mesocosms were not significant. This contrasted with mesocosms that had been inoculated with sulfate-reducing acidophiles (treatments IV and V) where copper concentrations declined with time and were significantly less in both cases at month 12 than at months 3-6 (Fig. 6.4). Changes in soluble copper concentrations with incubation time were more accentuated in treatment V (algal-inoculated tailings) and decreased significantly throughout the greater part of the experimental period. Zinc concentrations in the tailings mesocosms showed similar trends to those of copper (Fig. 6.4 and Supplementary Table S6.8) though the lowest concentrations measured were much greater than those of copper (mean values of 17 and 0.05 µg/g tailings, respectively, recorded in treatment V mesocosms at month 12). Concentrations of soluble nickel in the tailings, which varied from *ca.* 20 to 40 µg/g tailings, did not display any obvious time-related or treatment-related effects (data not shown).

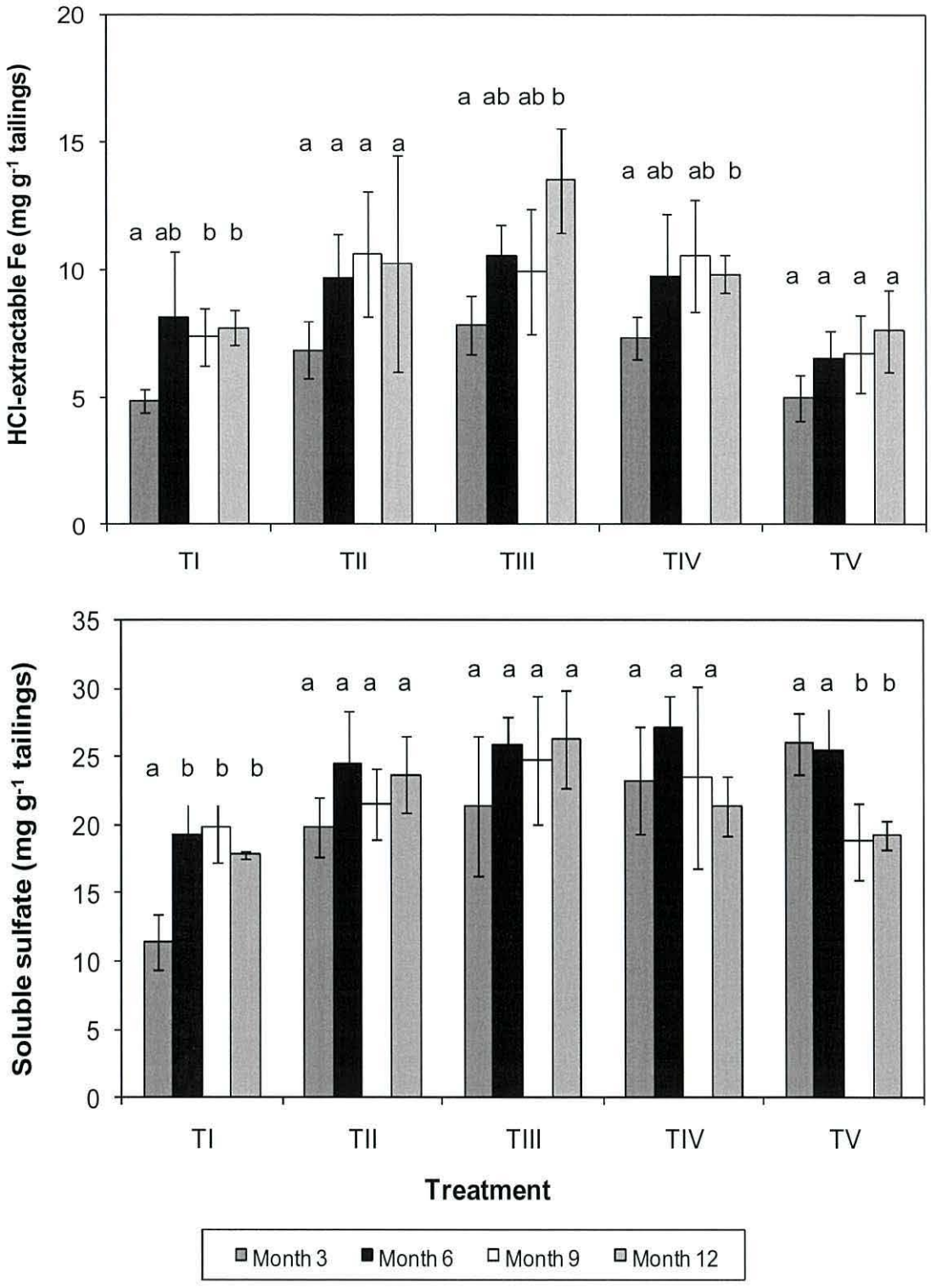

Fig. 6.3. Time-course changes in concentrations of HCl-extractable iron (top) and soluble sulfate (bottom) in mine tailings inoculated with different populations of acidophilic microorganisms (treatments II-V) and non-inoculated controls (treatment I). For each treatment data set, bars not annotated with the same letter are significantly different (95% confidence limit). Error bars represent standard deviations

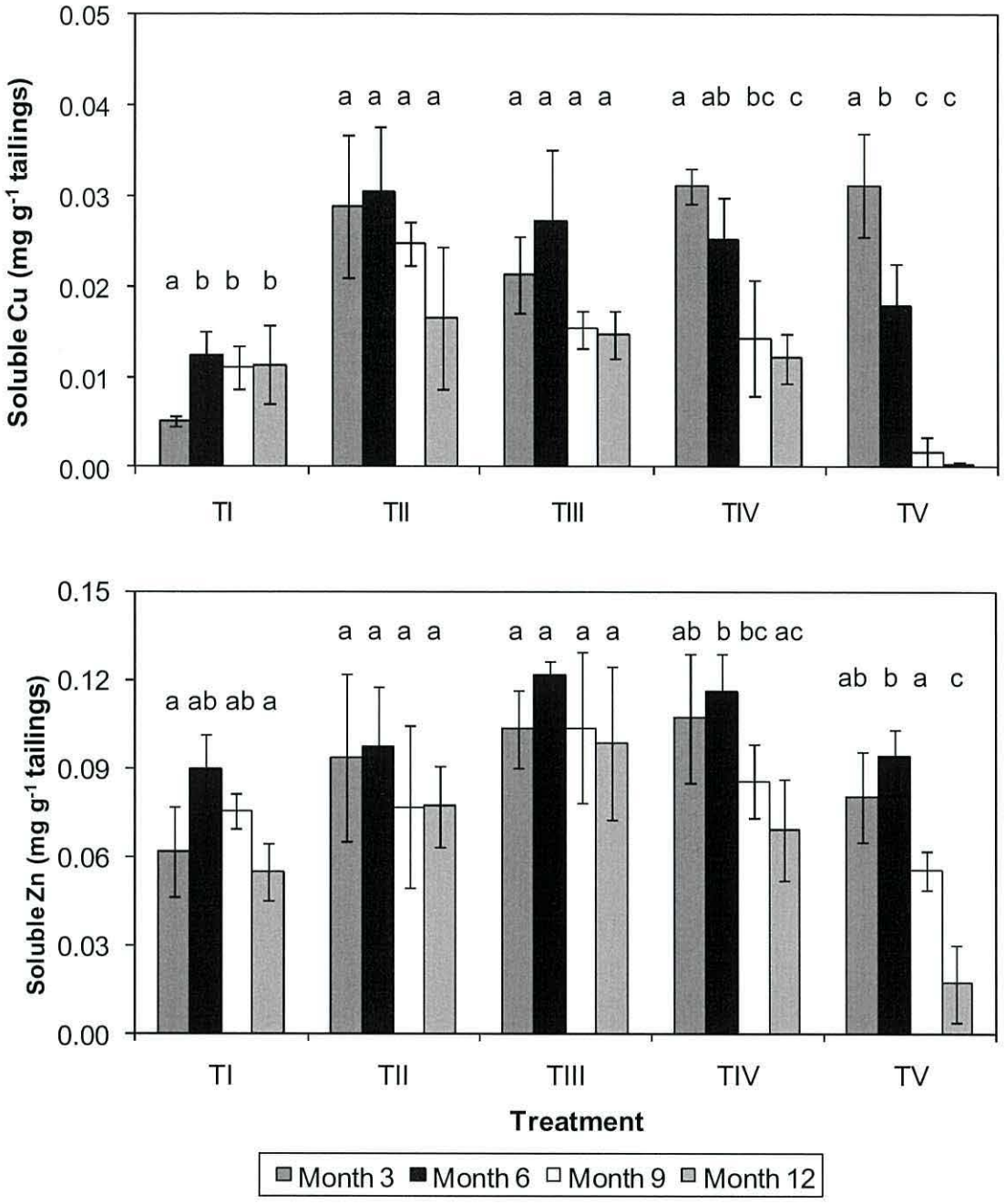

Fig. 6.4. Time-course changes in concentrations of copper (top) and zinc (bottom) in water extracts of mine tailings inoculated with different populations of acidophilic microorganisms (treatments II-V) and non-inoculated controls (treatment I). For each treatment data set, bars not annotated with the same letter are significantly different (95% confidence limit). Error bars represent standard deviations.

6.3.3 ***Microbiological changes in incubated mesocosms.*** Although no iron-oxidizing autotophs were detected in control (non-inoculated) tailings mesocosms on month 3 of the experiment, CFUs of these bacteria, equivalent to *ca.* 0.1 – 10% of those in inoculated mesocosms, were found on subsequent sampling occasions (Fig. 6.5). All tailings mesocosms displayed significant increased numbers of CFUs of iron-oxidizing acidophiles from months 3 to 6, though these tended not to change greatlybeyond month 6, except in treatment III mesocosms. However, when compared on different sampling occasions, the different inoculation regimes used at the outset of the experiment appeared to have little impact on numbers of iron/pyrite-oxidizing bacteria that were detected in the incubated tailings (Supplementary Table S6.9).

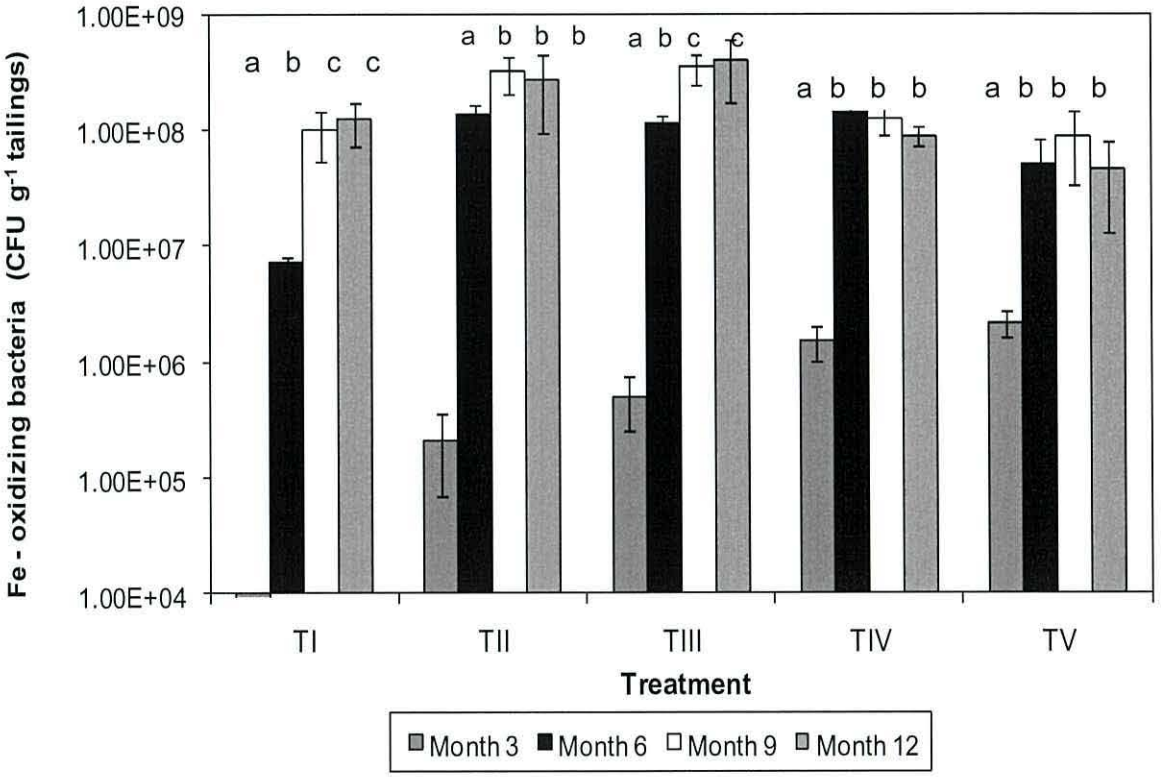

Fig. 6.5. Time-course changes in CFUs of acidophilic iron- and pyrite-oxidizing bacteria in mine tailings inoculated with different populations of acidophilic microorganisms (treatments II-V) and non-inoculated controls (treatment I). For each treatment data set, bars not annotated with the same letter are significantly different (95% confidence limit). Error bars represent standard deviations.

Numbers of aerobic heterotrophic bacteria increased in those inoculated (TIII, IV ans V) mesocosms from months 3 to 6, and followed the same trend as iron-oxidizing acidophiles (Fig. 6.6). After month 6, changes in numbers of aerobic heterotrophs isolated did not appear to be time-related. No aerobic heterotrophs were detected in treatments I and II mesocosms on months 3, 6, and 9, which was not surprising as these tailings had not been inoculated with these bacteria. However, as with the iron-oxidizers, aerobic heterotrophs were detected in all tailings samples at month 12. The dominant CFUs (small crescent-shaped yellow-colored colonies) in TI and TII mesocosms (76% and 63% of total CFUs) was distinct to those of the three aerobic heterotrophs used as the original inocula. The same colony form corresponded to only 14% of total CFUs in TIII mesocosms, and ~1% in TIV and TV mesocosms at the same time. Total numbers of aerobic heterotrophs were significantly greater on all sampling occasions (except month 3; TIV and TV mesocosms) in tailings that had been inoculated with algae (Supplementary Table S6.10).

Anaerobic heterotrophs followed similar trends to those of aerobic heterotrophs, in that their numbers increased significantly from months 3 to 6 in those mesocosms where they had been inoculated, but were not apparent in tailings that had not been inoculated until month 12 (Fig. 6.6). On three of the four sampling occasions, numbers of anaerobic heterotrophs were found to be significantly greater in tailings that had been inoculated with acidophilic algae (treatment V) than in those that has been inoculated with the same suite of bacteria, but no algae (treatment IV; Supplementary Table S6.11).

Microscopic examination of algal growths on the tailings surface (Supplementary Fig. 1) found that *Euglena*-like cells but no *Chlorella* were present on all sampling occasions.

6.3.4 ***Identification of bacterial isolates.*** Several of the bacteria isolated from the tailings mesocosms on solid media were identified from sequence analysis of their 16S rRNA genes. A representative of the single morphological type of ferric iron-

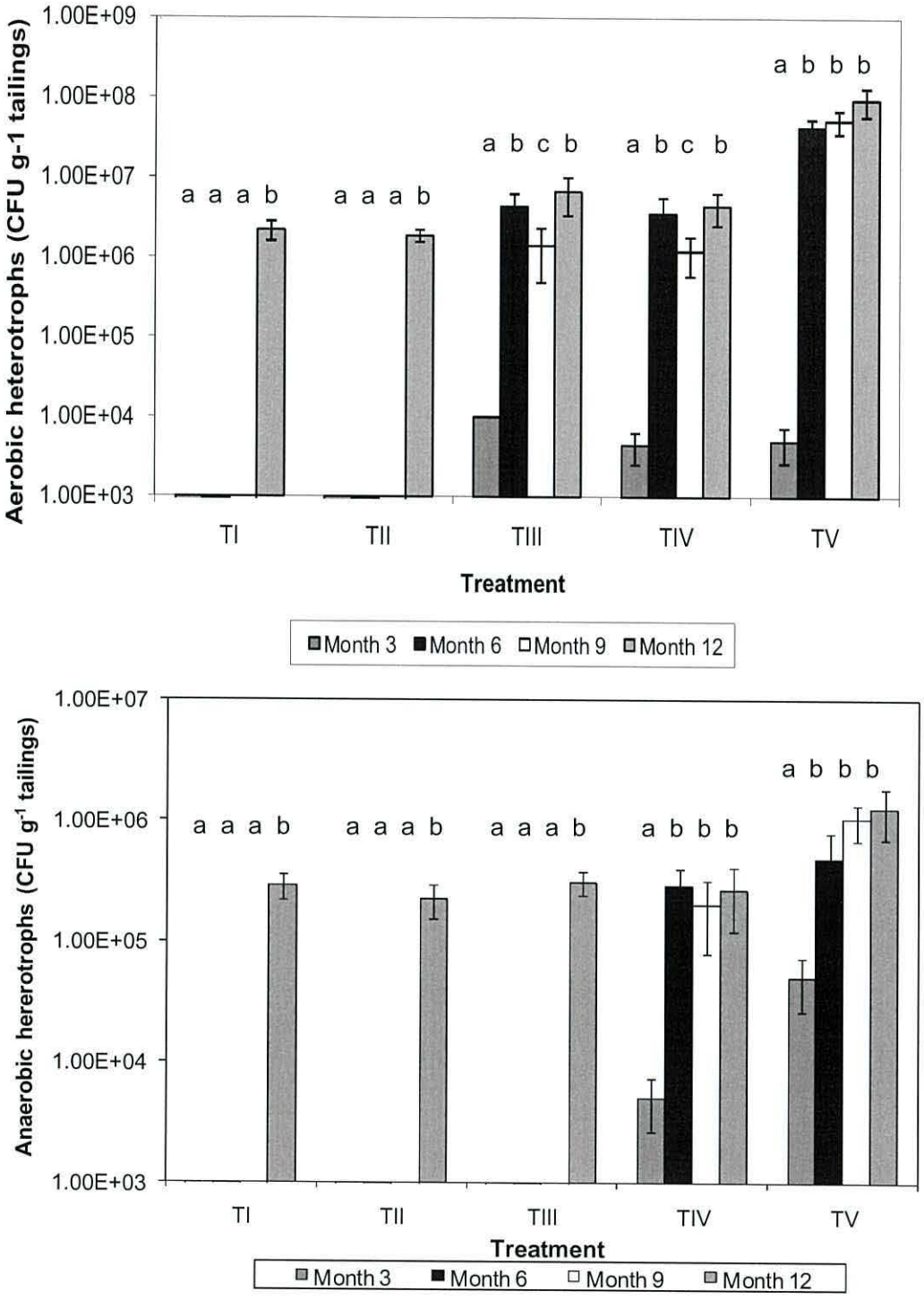

Fig. 6.6 Time-course changes in CFUs of aerobic (top) and anaerobic (bottom) heterotrophic acidophiles in mine tailings inoculated with different populations of acidophilic microorganisms (treatments II-V) and non-inoculated controls (treatment I). For each treatment data set, bars not annotated with the same letter are significantly different (95% confidence limit). Error bars represent standard deviations.

stained colony obtained from the non-inoculated (TI) mesocosms was identified as *At. ferrooxidans* (100% 16S rRNA gene similarity to the type strain; 1305 nt PCR product length). The dominant iron-oxidizers isolated from inoculated tailings were also *Acidithiobacillus*-like bacteria, though whether these were *At. ferrooxidans* or *At. ferrivorans* was not ascertained. The closest cultivated relative of the dominant and distinct aerobic heterotrophic colony variant isolated from control and TII mesocosms at month 12 mesocosms was *Curtobacterium ammoniigenes*, with which it shared 99.0% 16S rRNA gene similarity (1296 nt PCR product length). In contrast, the most numerous heterotrophic bacterium in inoculated tailings was *Acidiphilium* SJH (100% gene similarity to the bacterium included in the inoculum). The most abundant colony morphology on anaerobic plates inoculated with treatment III and V tailings, and also with the non-inoculated controls, was related (98.0% gene similarity; 1291 nt PCR product length) to *Alicyclobacillus* sp. AGC-2. The gene sequences of the *At. ferrooxidans*, *C. ammoniagenes* and *Alicyclobacillus* isolates have been deposited in GenBank, and allocated the accession numbers xxxxx, yyyyy and zzzz, respectively. The *Curtobacterium* isolate was shown to cause the reductive dissolution of amorphous ferric hydroxide, as evidenced by greater concentrations of ferrous iron concentrations (Fig. 6.7) and higher pH values (4.26 +/- 0.3) in inoculated anaerobic cultures than in a sterile control (final pH 4.07). Concomitant increases in cell numbers implied that this isolate could also grow *via* ferric iron respiration (Fig. 6.7).

6.4 Discussion

Ecological engineering in the context of mine waste management and restoration, has been defined as "the application of knowledge of natural biological systems known to be present on rock or minerals or in sediments, to practically and beneficially achieve human and industrial objectives in a natural self-sustaining way" (Kalin, 2011). In the current work, we have sought to demonstrate whether bacteria that catalyze the dissimilatory reduction of iron or sulfur, and sustained by organic carbon derived from acidophilic algae, could minimize or reverse the impact of acid-generating, metal-mobilizing acidophiles in reactive mine tailings. Prior removal of residual alkali materials and inoculation of all mesocosms except the controls with active populations of iron/pyrite-oxidizing *Acidithiobacillus* spp. and *L. ferrooxidans* ensured that the tailings were chemically and biologically primed for oxidizing pyrite and other sulfides present, and presented a "worst case" scenario so far as their potential for mineral dissolution and metal release was concerned. The use of small-

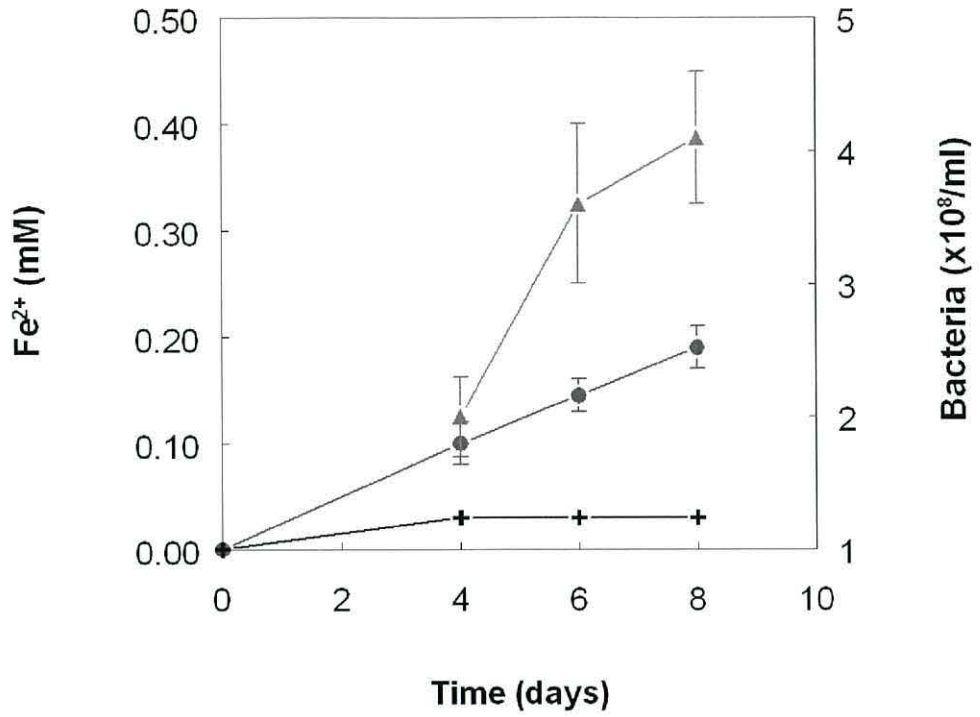

Fig. 6.7. Reductive dissolution of amorphous ferric hydroxide by the *Curtobacterium* sp. isolated from mine tailings, and changes in cell numbers during anaerobic growth by ferric iron respiration. Key: ●, ferrous iron (inoculated cultures); +, ferrous iron (control cultures); ▲, bacterial numbers. Data points show the mean values of duplicate cultures and error bars indicate the range of values.

scale mesocosms allowed several synchronized microbial permutations to be set up and evaluated. Evidence for both oxidative (e.g. dissolution of pyrite) and reductive (formation of sulfides) processes, as well as the isolation of aerobes and anaerobes from the same mescosms, pointed to the existence of microsites with contrasting oxygen contents, redox potentials and possible pH within the tailings.

One of the more surprising observations was that washing the tailings with strong mineral acid did not appear to eliminate indigenous iron-oxidizing or heterotrophic bacteria. The origin of these bacteria is uncertain, but the identification of bacteria related to *C. ammoniigenes* as the dominant aerobic heterotroph and to *Alicyclobacillus* sp. AGC-2 as the dominant anaerobic heterotroph (neither of which were present in the inocula used) strongly suggests that these bacteria (and, by inference, the *At .ferrooxidans* isolated from non-inoculated mesocosms) were

indigenous to the tailings. This is the first report of a *Curtobacterium* sp. proliferating in an acidic, metal-rich minerals environment. *C. ammoniigenes* is an ammonium-oxidizing species of the class *Actinobacteria* that was isolated from acidic (pH 2 – 4) swamps in Vietnam (Aizawa *et al.*, 2007). The current isolate was partially characterized as a moderate acidophile that, like *Acidiphilium, Acidocella* and *Acidobacterium* spp., catalyzed the dissimilatory reduction of ferric iron, which is another trait previously not identified for this genus. There are no published data on *Alicyclobacillus* sp. AGC-2, though its GenBank entry (accession number AF450135) states that it was isolated from a hot spring in Alaska. Some *Alicyclobacillus* spp. are facultative anaerobes that can grow by ferric iron respiration though none are known to catalyze the dissimilatory reduction of sulfate to sulfide (Johnson and Hallberg, 2009). While none of the SRB strains included in the inocula were subsequently isolated from the tailings, there was evidence for sulfidogenesis within the mesocosms, as discussed below.

Iron-reducing bacteria and SRB were inoculated in order to promote processes that are, essentially, the reverse of those carried out by pyrite-oxidizing autotrophic bacteria. Dissimilatory reduction of ferric iron can be either a proton-generating reaction, in the case of soluble ferric iron, or a proton-consuming reaction, in the case of amorphous and crystalline ferric minerals as illustrated in equation [2] where schwertmannite is shown as the mineral phase subjected to bacterial reductive dissolution, and "CH_2O" represents a generic organic electron donor):

$$Fe_8O_8(SO_4)(OH)_6 + 2\ CH_2O + 2\ H_2O \rightarrow 8\ Fe^{2+} + 14\ OH^- + SO_4^{2-} + 2\ CO_2 \qquad [2].$$

The ferrous iron solubilized will, however, re-oxidize either *in situ* or downstream unless it is otherwise immobilized (e.g. as a sulfide mineral) and the ferric iron produced will hydrolyze with concomitant net production of protons. Bacterial iron reduction does, however, have a role in mitigating AMD production at its source since it removes, at least in part, the chemical (ferric iron) which is the prime oxidant of sulfide minerals at low pH.

Dissimilatory sulfate reduction at low pH is a proton-coming process, as illustrated in equation [3]:

$$SO_4^{2-} + 2\ CH_2O + 2\ H^+ \rightarrow H_2S + 2\ CO_2 + 2\ H_2O \qquad [3].$$

In addition, the hydrogen sulfide produced reacts with a variety of metals generating highly insoluble metal sulfides, Selective immobilization of different transition metals by SRB can be achieved by controlling liquor pH (Ňancucheo and Johnson, 2011a).

While some species of acidophilic iron-reducing bacteria are autotrophs, most appear to be heterotrophs (Coupland and Johnson, 2008). In contrast, few acid-tolerant and acidophilic SRB have been isolated and studied *in vitro*, but those that have are also heterotrophic, though some species appear also to use hydrogen as electron donor (Kimura *et al.*, 2006; Ňancucheo and Johnson, 2011a). Therefore, in order to stimulate the growth and activities of both iron- and sulfate-reducing acidophilic, a supply of organic carbon is often essential. While adding suitable organic carbon from an extraneous source is one possible scenario, this is not desirable (or economical) in a mine waste context, and continuous provision of small concentrations of organic carbon from a sustainable source is preferable for mine waste management. Autotrophic microorganisms that inhabit acidic, mineral-rich environments include bacteria and archaea that use iron and/or sulfur as energy sources to fuel the fixation of carbon dioxide (chemolithotrophs) and some species of micro-algae that use solar energy for this purpose (phototrophs). Small molecular weight organic compounds can originate from both of these groups as exudates and lysates, and some of these are utilized by heterotrophic acidophiles that live in association with the primary producers. Ňancucheo and Johnson (2010) showed that glycolic acid was a major exudate produced by acidophilic iron-oxidizing chemolithotrophs but that relatively few species of acidophilic heterotrophs were able to metabolize this organic acid. In contrast, monosaccharides identified in cell-free cultures of the acidophilic algae used in the current work (*Euglena* and *Chlorella*) were much more widely used as carbon and energy sources by heterotrophic acidophiles. Using organic carbon derived exclusively from chemolithotrophs to stimulate iron- and sulfate-reducing acidophiles also has the major drawback that the energy that fuels CO_2 fixation by the former is ultimately the oxidation of pyrite (or other sulfides), which is the process that gives rise to the problem of acidification and metal-mobilization. Acidophilic algae have been relatively little studied, partly because they are not perceived to have any role in the major biotechnology ("biomining") which involves the use of prokaryotic consortia. Das *et al.* (2009) described the significance of eukaryotic microorganisms in the context of mine water and alluded to the possible role of algae in sustaining populations of SRB. More recently, Senko *et al.* (2011) have provided evidence for the role of algae in inhibiting

or reversing iron oxidation in AMD environments. Oxygen evolution by acidophilic algae can also influence iron and sulfur cycling, and was probably the reason why ferric iron precipitates accumulated immediately below the algal surface layer in TV mesocosms (Supplementary Fig. S6.1d).

The current mesocosm experiments have illustrated the how ecological engineering could be used to improve long-term management of reactive mine tailings by minimizing their generation of acidic, metal-rich effluents. Although none of the inoculation regimes completely retarded the oxidative dissolution of the reactive sulfidic minerals present in the tailings (as illustrated by the larger concentrations of soluble iron present on any sampling occasions compared to those present in the acid-washed material), there was clear evidence of mitigation of this process in those mescosms that had been inoculated with heterotrophic iron-reducing bacteria and SRB, more particularly when acidophilic algae were also inoculated. The current data suggest that these bacteria influence tailings geochemistry directly by redox transformations of iron and sulfur, rather than indirectly by inhibiting the growth of pyrite-oxidizing bacteria, numbers of which were similar in mesocosms that were inoculated or not with acidophilic heterotrophs. The net positive effects highlighted with the heterotrophic bacteria/acidophilic algae consortium were: (i) evidence for production of alkalinity, as evidenced by higher pH values; (ii) lower redox potentials, which correlated with higher concentrations of ferrous iron; (iii) immobilization of copper and zinc released *via* dissolution of residual sulfide minerals in the tailings. Lower redox potentials and greater concentrations of soluble ferrous iron provided evidence of the activities of iron-reducing heterotrophs within the algal-inoculated tailings, while the presence of nascent sulfide minerals (H_2S evolution when hydrochloric acid was added) indicated that sulfidogens were also metabolically active in the same mesocosms. Additional evidence for the latter came from the decreasing concentration of sulfate with incubation time), and also from the relative degrees of immobilization of copper, zinc and nickel within treatment V tailings. These three metals have very different solubility products (the log K_{sp} values for CuS, ZnS and NiS are -35.9, -24.5 and -21.0, respectively (Diaz *et al.*,1997) and the fact that soluble copper declined by 97%, zinc by 83% (figures calculated by comparing concentrations of soluble metals at month 12 to the maximum determined during the experiment) while nickel concentrations remained relatively unchanged, provided convincing evidence that these metals were precipitated as sulfides rather than being immobilized by adsorption etc. In an earlier report, Fortin and co-workers (2002) also found that cycling of iron and sulfur occurred within mine tailings in field samples, and

proposed that removal of sulfate in the tailings was mediated by SRB rather than by chemical precipitation.

A model of the biogeochemical transformations implied to have occurred within the mesocosms that were inoculated with chemolithotrophic and heterotrophic bacteria, and phototrophic algae, is shown in Fig. 6.8.

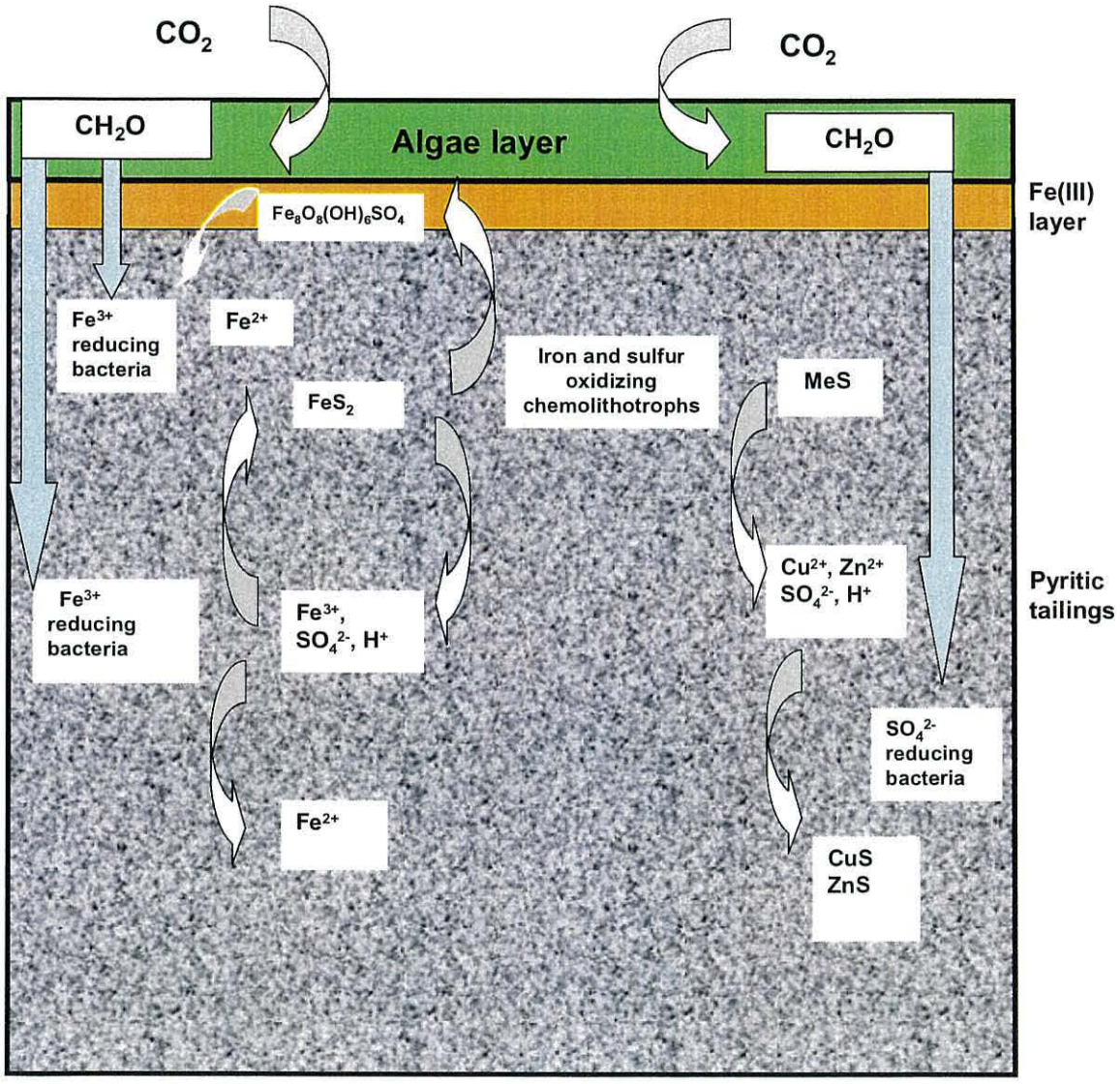

Fig. 6.8. Proposed biogeochemical transformations of carbon, iron and sulfur in incubated tailings mesocosms inoculated with iron-oxidizing bacteria, aerobic and anaerobic heterotrophic bacteria and acidophilic algae.

In the context of mine tailings management, the results of the current work suggest that by ensuring that acidophilic/acid-tolerant species of iron-reducing bacteria, SRB and algae are present in tailings lagoons (e.g. by inoculating with suitable strains of these microorganisms, as the tailings are being deposited) the environmental impact of these potentially hazardous mine wastes could be much reduced. Surface growths of algae is aided by the widespread current practice of storing tailings under a water cover. However, growth of the algae would probably be enhanced by adding small amounts of inorganic nitrogen and phosphorus to nutrient-poor mine tailings.

Supplementary Material: Chapter 6

Supplementary TABLE S6.1. pH values of water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	4.19+/-0.07 a	3.82+/-0.19 ab	3.92+/-0.09 a	3.84+/-0.08 a
II	3.65+/-0.07 b	3.72+/-0.03 b	3.67+/-0.12 a	3.61+/-0.05 b
III	3.61+/-0.09 b	3.61+/-0.14 b	3.76+/-0.07 a	3.60+/-0.08 b
IV	3.65+/-0.07 b	3.63+/-0.10 b	3.70+/-0.10 a	3.66+/-0.05 b
V	3.69+/-0.16 b	4.02+/- 0.16 a	4.24+/-0.39 a	4.40+/-0.20 c

Supplementary TABLE S6.2. Redox potential (E_h values, as mV) of water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	404+/-6 a	411+/-50 a	399+/-23 a	457+/-4 a
II	482+/-12 b	479+/-17 ab	502+/-17 b	514+/-33 b
III	469+/-28 bc	500+/-34 ab	494+/-17 b	510+/-4 b
IV	440+/-12 bc	475+/-39 abc	479+/-23 b	505+/-20 b
V	404+/-55 ac	386+/-58 ac	387+/-29 a	421+/-9 a

Supplementary TABLE S6.3. Ferrous iron concentrations (µg/g dry tailings) in water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	6+/-0.7 a	4+/-0.5 a	3+/-0.3 a	3+/-1.2 a
II	8+/-2.4 a	3+/-0.9 a	3 +/- 0.6 a	3+/-0.9 a
III	6+/-1 a	3+/-1.2 a	3+/-0.9 a	4+/-3.4 a
IV	5+/-1.5 a	3+/-0.7 a	4+/-1.1 a	6+/-2.4 a
V	10+/-3 a	15 +/-1.7 b	17+/-1.6 b	15+/- 2.1 b

Supplementary TABLE S6.4. Total soluble iron concentrations (µg/g dry tailings) in water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	27+/-4.4 a	55+/-10 a	38+/-19 ab	51+/-13 a
II	57+/-18 b	102+/-21 b	83+/-27 b	91+/-13 b
III	56+/-23 ab	34+/-14 a	51+/-8.9 ab	61+/-14 ab
IV	55+/-38 b	58+/-6.2 a	76+/-21 b	73+/-14 ab
V	37+/-13 ab	38+/-16 a	37+/-13 a	41+/-15 ab

Supplementary TABLE S6.5. Total iron concentrations (mg/g dry tailings) in 5M HCl extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	4.87+/-0.46 a	8.17+/-2.54 ab	7.39+/-1.12 a	7.74+/-0.69 a
II	6.86+/-1.11 ab	9.72+/-1.67 ab	10.63+/-2.45 a	10.24+/-4.24 abc
III	7.83+/-1.17 b	10.54+/-1.24 b	9.93+/-2.43 a	13.52+/-2.04 c
IV	7.34+/-0.83 b	9.73+/-2.46 ab	10.57+/-2.21 a	9.84+/-0.74 b
V	4.99+/-0.91 a	6.53+/-1.09 a	6.71+/-1.52 a	7.63+/-1.60 ab

Supplementary TABLE S6.6. Sulfate concentrations (mg/g dry tailings) in water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	11.42+/-2.02 a	19.26+/-3.11 a	19.84+/-2.56 a	17.85+/-0.26 a
II	19.82+/-2.16 b	24.54+/-3.78 ab	21.58+/-2.59 a	23.67+/-2.80 b
III	21.38+/-5.14 bc	25.83+/-2.13 b	24.77+/-4.68 a	26.30+/-3.63 b
IV	23.29+/-3.89 bc	27.18+/-2.35 b	23.52+/-6.72 a	21.39+/-2.23 bc
V	25.98+/-2.22 c	25.54+/-3.18 c	18.82+/-2.86 a	19.34+/-1.06 ac

Supplementary TABLE S6.7. Copper concentrations (µg/g dry tailings) in water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	5.0+/-0.6 a	12+/-2.5 a	11+/-2.4 a	11+/-4.3 a
II	29+/-7.8 b	30+/-7.2 b	25+/-2.4 b	17+/-7.9 a
III	21+/-4.2 bc	27+/-7.7 b	15+/-2.0 a	15+/-2.6 a
IV	31+/-2.0 b	25+/-4.7 b	14+/-6.4 a	12+/- 2.8 a
V	31+/-5.7 bc	18+/-4.5 ab	2.0+/-1.8 c	0.05+/-0.4 b

Supplementary TABLE S6.8. Zinc concentrations (µg/g dry tailings) in water extracts of tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	62+/-15 a	90+/-12 a	75+/-5.8 a	55+/-9.7 a
II	93+/-28 ab	98+/-20 ab	77+/-27 abc	77+/-14 ab
III	103+/-13 b	122+/-4.6 b	104+/-26 ab	99+/-26 b
IV	107+/-22 b	116+/-13 ab	86+/-13 ab	69+/-17 ab
V	81+/-15 ab	94+/-9.3 ab	56+/-6.5 c	17+/-13 c

Supplementary TABLE S6.9. Numbers of CFUs of iron/pyrite-oxidising acidophilic bacteria (/g dry tailings) in tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	$<10^2$ a	$7.1+/-1.0x10^6$ a	$9.9+/-45x10^7$ a	$1.2+/-0.5x10^7$ ab
II	$2.1+/-0.014x10^5$ b	$1.4+/-0.3x10^8$ b	$3.1+/-1.1x10^8$ b	$2.7+/-1.7x10^8$ ab
III	$5.0+/-2.5\ x10^5$ bc	$1.1+/-0.2x10^8$ bc	$3.5+/-1.0x10^8$ b	$3.9+/-2.2x10^8$ b
IV	$1.5+/-0.5x10^6$ cd	$1.4+/-0.4x10^8$ b	$1.3+/-0.4x10^8$ a	$8.9+/-1.7x10^7$ ab
V	$2.2+/-0.5x10^6$ d	$5.0+/-0.3x10^7$ c	$8.8+/-0.5x10^7$ a	$4.6+/-3.3x10^7$ a

Supplementary TABLE S6.10. Numbers of CFUs of aerobic heterotrophic acidophilic bacteria (/g dry tailings) in tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	$<10^2$ a	$<10^2$ a	$<10^2$ a	$2.2+/-0.6x10^6$ a
II	$<10^2$ a	$<10^2$ a	$<10^2$ a	$1.9+/-0.3x10^6$ a
III	$1.0+/-0.0x10^4$ b	$4.3+/-1.9x10^6$ b	$1.4+/-0.9x10^6$ b	$6.7+/-0.3x10^6$ a
IV	$4.4+/-1.9x10^3$ c	$3.6+/-2.0x10^6$ b	$1.2+/-0.6x10^6$ b	$4.4+/-1.9x10^6$ a
V	$5.0+/-2.4x10^3$ c	$4.3+/-1.1x10^7$ c	$5.2+/-1.7x10^7$ c	$9.6+/-3.7x10^7$ b

Supplementary TABLE S6.11. Numbers of CFUs of anaerobic heterotrophic acidophilic bacteria (/g dry tailings) in tailings mesocosms. Data are shown as mean values and standard deviations (n = 3 samples for each data set). For each sampling time, data not annotated with the same letter are significantly different (95% confidence limit)

	Time (month)			
Treatment	3	6	9	12
I	$<10^2$ a	$<10^2$ a	$<10^2$ a	$2.9+/-0.7x10^5$ a
II	$<10^2$ a	$<10^2$ a	$<10^2$ a	$2.2+/-0.7x10^5$ a
III	$<10^2$ a	$<10^2$ a	$<10^2$ a	$3.1+/-0.7x10^5$ a
IV	$5.0+/-2.4x10^3$ b	$2.9+/-1.2x10^5$ b	$2.0+/-1.2x10^5$ b	$2.7+/-1.5x10^5$ a
V	$5.0+/-2.4x10^4$ c	$4.8+/-3.1x10^5$ b	$1.0+/-0.3x10^6$ c	$1.3+/-0.6x10^6$ b

(a) (b)

(c)

(d)

Fig. S6.1. Tailings mesocosms: (a) and (c), treatment III; (b) and (d), treatment V. All images are of mesocosms sampled at month 12.

Chapter 7

General Conclusions

This thesis has sought to obtain a better understanding of the various interactions that occur within consortia of acidophilic microorganisms that are frequently encountered in extremely acidic, metal-rich environments, and to use this knowledge to propose and to develop novel approaches for securing mine wastes and remediating mine waters. Particular emphasis was placed on those acidophilic bacteria that catalyse the dissimilatory reduction of iron and of sulfate, and of their relationships with autotrophic acidophiles. The main conclusions of the experimental work carried out were:

- Ecological engineering-based solutions offer new, sustainable and environmentally-benign solutions to the problem of mine water pollution. New approaches may be based on exploiting the potentials of well-known species of acidophiles or novel microorganisms.
- The primary producers in mine-impacted environments include both chemolithotrophic bacteria and archaea, and phototrophic eukaryotes. Organic carbon in extremely acidic environments can derive from both groups of microorganisms, as well as from extraneous sources.
- Glycolic acid is a significant exudate produced by acidophilic chemolithotrophs (*Acidithiobacillus* spp. and *Leptospirillum*). However, relatively few species of organotrophic acidophiles appear to use this organic acid as sole carbon and energy source and, like many other small molecular weight aliphatic acids, it is toxic to many acidophiles in relatively low concentrations. However, many acidophilic *Firmicutes* can metabolise glycolic acid, and this may be the major reason why these bacteria rather than others are the major organotrophic acidophiles in stirred tank biomining operations, where the primary producers are exclusively chemolithotrophs.
- Exudates produced by acidophilic micro-algae include monosaccharides (such as glucose, mannitol and fructose) which have more widespread utilisation by heterotrophic acidophiles, including species (*Acidiphilium, Acidocella* and *Acidobacterium*) that are known to catalyse the dissimilatory reduction of ferric iron minerals, and to "bioshroud" pyrite.

- The biodiversity of acidophilic sulfate reducing bacteria is far greater than previously recognised, and consortia of these have considerable potential for metal recovery from AMD streams and mine process waters where they can be used to facilitate the selective recovery of target metals. Acidophilic SRB can be used for this purpose in on-line bioreactors, which have minimal engineering complexity and relatively low operational costs.
- Reactive mine tailings can be "ecologically engineered" to reduce their risk of environmental pollution by acting as source points of AMD discharge, by promoting the growth of microbial populations that counteract the effects of acid-generating and metal mobilising acidophiles. Dissimilatory iron- and sulfate-reducing bacteria, in tandem with phototrophic micro-algae, could be used to promote a self-sustaining, reduced risk solution to the environmental hazard posed by tailings lagoons.

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Appendix

16S rRNA gene sequences of bacteria and 18S RNA gene sequences of algae isolated as part of this study.

(i) Isolate IR1: 16S rRNA gene sequence
(an *Actinobacterium* isolated from an acidophilic SRB bioreactor consortium)

GTGCTTAACACATGCAAGTCGAACGGTGATGGCAAGCTTGCTTGTCTGATCAGT
GGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCTCCTCTCTGGGATAAGCC
TTGGAAACGGGGTCTAATACCGGATATTCACTTCTGCCTGCATGGGTGGTGGTG
GAAAGCTTTTGCGGTGGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGG
TGATGGCCTACCAAGGCGATGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACG
GCCTTCGGGTTGTAAACCGCTTTCGGCAGGGAAGAAGCCGTAAGGTGACGGTA
CCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTCGT
CGCGTCTGCTGTGAAATCCTCAGGCTCAACCTGGGGCGTGCAGTGGGTACGGG
CGGACTAGAGTGCGGTAGGGGTGACTGGAATTCCTGGTGTAGCGGTGGAATGC
GCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCACTGGGCCGCAACT
GACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGT
CCATGCCGTAAACGTTGGGCACTAGGTGTGGGGTCCATTCCACGGATTCCGTG
CCGCAGCAAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAA
ACTCAAAGAAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATT
CGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGCACTCGTAACACCCAGA
GATGGGTGCCCCGTAAGGCGGGTGCACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCCAT
GTTGCCAGCGGGTTATGCCGGGGACTCATGGGAGACTGCCGGGGTCAACTCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCAC
GCATGCTACAATGGCCGGTACAGAGGGCTGCGATACCGTAAGGTGGAGCGAAT
CCCTAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAG
TCGGAGTCGCTAGTAATCGCAGATCAGCAACGC

(ii) Isolate IR2: 16S rRNA gene sequence
(*Alicyclobacillus* sp. isolated from an acidophilic SRB bioreactor consortium)

AGCGGATGTTTCCGGTGCTTGCACCGGAAAGATGAGCGGCGGACGGGTGAGT
AACACGTGGGTAATCTACCTTTCAGACCGGAATAACGCCTGGAAACGGGTGCTA
ATGCCGGATAGGGTGGTGGGGAGGCATCTTCCTGCCAGGAAAGGGGCAAAGG
CCCCACTGAAAGAGGAGCCCGCGGCGCATTAGCTGGTTGGCGGGGTAACGGC
CCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGGACGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGGCGCAAGCCTGACGGAGCAACGCCGCGTGAGCGAGGAAGGCCTTCGG
GTCGTAAAGCTCAGTCACCTGGGAAGAGCGGTCATGGGAGTGGAAAATCCATG
ACGAGACGGTACCGGGAGAGGAAGCCCCGGCAAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCACTGGGCGTAAAGGGTGCG
TAGGCGGTTTGGCAAGTCCGGGGTGAAAGGCTCCAGCTCAACTGGGGTAATGC
CTTGGAAACGGCTGAACTTGAGTACTGGAGAGGCAAGGGGAATTCCACGTGTA
GCGGTGAAATGCGTAGATATGTGGAGGAATACCAGTGGCGAAGGCGCCTTGCT
GGACAGTGACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGGTCACT
CCTCAGTGCCGAAGGAAACCCAATAAGCACTCCGCCTGGGGAGTACGGTCGCA
AGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCTCTGACG
TCTCTAGAGATAGGGATTCCCTTCGGGGCAGAGGAGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGATCTGTGTTACCAGCGCGTGAAGGCGGGGACTCACAGGTGACTGCCGGC
GTAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTGATGTCCT
GGGCGACACACGTGCTACAATGGGCGGAACAACGGGAAGCGAGACCGCGAGG
TGGAGCGAACCCCTGAAAACCGTTCGTAGTTCGGATTGCAGGCTGCAACCCGC
CTGCATGAAGCCGGAATTGCTAGTAATCGCGGATT

(iii) Isolate CEB3: 16S rRNA gene sequence
(*Desulfitobacterium* sp. isolated from an acidophilic SRB bioreactor consortium)

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAACACATGCAA
GTCGAACGGAGATATTTTGAAGTTTACTTTAGGATATCTTAGTGGCGGACGGGT
GAGTAACGCGTGGGTAACCTACCCATAAGGCCGGGACAACTCCTGGAAACGGG
GGCTAATACCGGATAATCTTTCCGAGGGACATCTCTTGGGAAGGAAAGGCGGC
CTCTGAAGATGCTGCCGTTTATGGATGGACCCGCGTCTGATTAGCTAGTTGGTG
GGGTAATGGCCTACCAAGGCGACGATCAGTAGCCGGCCTGAGAGGGTGAACG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGTATGACGA
AGGCCTTCGGGTTGTAAAGTACTGTCTTTGGGGACGAACGGCGTCTATGTAAAT
AATGTAGGCGAGTGACGGTACCCAAGGAGGAAGCCCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAA
AGGGCGCGTAGGCGGATGCTTAAGTCCGGTGTGAAAGACTGGGGCTCAACCC
CAGGGTTGCATCGGAAACTGGGTGTCTTGAGGACAGGAGAGGAAAGCGGAATT
CCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGG
CGGCTTTCTGGACTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTAGAG
GGTATCGACCCCCTCTGTGCCGCAGTTAACACAATAAGCACTCCGCCTGGGGA
GTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGAC
ATCCTGCGAACCCTTAGGAAACTAAGGGGTGCCCTTCGGGGAGCGTAGAGACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCCTATCTTTAGTTGCTAACAGGTAAGCTTGAGCACTCTAG
AGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGTCTTGGGCTACACACGTGCTACAATGGCCGGTACAGACGGAAG
CGAAGCCGCGAGGTGAAGCGAATCCGAGAAAGCCGGTCTCA

(iv) *Curtobacterium ammoniigenes* –like isolate: 16S rRNA gene sequence (aerobic heterotrophic bacterium isolated from tailings mesocosm)

AACGGGTGAGTAACACGTGAGTAACCTGCCCCTGACTCTGGGATAAGCGTTGG
AAACGACGTCTAATACTGGATATGACAACTGATCGCATGGTCTGGTTGTGGAAA
GATTTTTTGGTTGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATG
GCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTT
CGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGA
AAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGT
CTGCTGTGAAATCCCGAGGCTCAACCTCGGGCTTGCAGTGGGTACGGGCAGAC
TAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGA
TATCAGGAGGAACACCGATGGCGAAGGCANATCTCTGGGCCGTAACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGTTGGGCGCTAGATGTAGGGACCTTTCCACGGTTTCTGTGTCGTAG
CTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATG
CAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGAT
GGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTG
CCAGCGGTTCGGCCGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATG
CTACAATGGCCGGTACAAAGGGCTGCGATACCGTAAGGTGGAGCGAATCCCAA
AAAGCCGGTCTCAGTTCGGA

(iv) *Alicyclobacillus* sp. isolate: 16S rRNA gene sequence
(anaerobic heterotrophic bacterium isolated from tailings mesocosm)

GTCGAGCGGACCTTAGGGTCAGCGGCGGACGGGTGAGTAACACGTGGGCAAT
CTGCCGAGCAGACCGGAATAACGCCTGGAAACGGGTGCTAATGCCGGATAGGC
AGCGAGGAGGCATCTTCTTGCTGGGAAAGGTGCAACTGCGCCCCTGTTCGAGG
AGCCCGCGGCGCATTATCTAGTTGGTAAGGTGAAGGCTTACCAAGGCGACAAT
GCGTAGCCCACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCC
ACACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTG
ACGGAGCAACGCCCCGTGAGCGAAAAAAGCCTTCGGGTTGTAAAGCTCACTCA
CTCGGGAAGAGCGACCTAAGGAGTGGAAAGCCTTATGGGAGACGGTACCGAG
GGAGGAAGCCCCGGCAAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGG
CAAGCGTTGTCCGGAATCACTGGGCGTAAAGCGTGCGTAGGCGGTTTTTTGGG
TCTGGGGTGAAAGTTCAGGGCTCAACCTTGAGAATGCCTTGGAAACTATAAGAC
TTGAGTGCTGGAGAGGCAAGGGGAATTCCACGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAATACCAGTGGCGAAAGCGCCTTGCTGGACAGTGACTGACGCT
GAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAGGTGTTGGGGGGTCATACTCTCAGTGCCGAAGGAA
ACCCAATAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCAGGGCTTGACATCCCTCTGACCGGTACAGAGATGGAC
CTTCCCTTCGGGGCAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTGTGTTACC
AGCACGTGATGGTGGGGACTCACAGGTGACTGCCGGCGTAAGTCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCTTTATGTCCTGGGCGACACACGTGCT
ACAATGGGCGGTACAACGGGAAGCGAGACCGCGAGGTGGAGCGAAACCCTAA
AAGCCGTTCGTAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAA
TTGCTAGTAATCGCGGATCAGCA

(v) *Chlorella protothecoides* var. *acidicola* isolate: 18S rRNA gene sequence (acidophilic micro-algae isolated from the abandoned Cantareras mine)

TGCATGTCTAAGTATAAACTGCTTTATACTGTGAAACTGCGAATGGCTCATTAAA
TCAGTTATAGTTTATTTGATGGTACCTACTTACTCGGATAACCGTAGTAAATCTA
GAGCTAATACGTGCGCACATCCCGACTTCTGGAAGGGACGTATTTATTAGATA
AAAGGCCGACCGGGCTCTGCCCGCCTCGCGGTGACTCATGATAACTTCACGAA
TCGCACGGCCTCGTGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTT
GATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGAATTAGGGT
TCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAA
TACCGGGCCTTTGGTCTGGTAATTGGAATGAGTACAATCTAAACCCCTTAACGA
GGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGGTGG
GGCCTGTCGGTCCGCCGTTTCGGTGTGCACTGACCGGGCCCACCTTGGTGCC
GGGGACGGGCTCCTGGGCTTCACTGTCCGGGACCCGGAGTCGGCGAGGTTAC
TTTGAGTAAATTAGAGTGTTCAAAGCAGGCCTACGCTCTGAATACATTACCATGG
AATAACACGATAGGACTCTGGCCTATCTTGTTGGTCTGTANGAACGGANTAATG
ATTAAGAGGGACAGTCGGGGGCATTCGTATTTCATTGTCACAGGTGAAATTCTT
GGATTTANGAAAGACGAANTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAA
TCAAGAACGAAACTTGGGGGCTCGAAGACGATTAGATACCGTCATAGTCTCAAC
CATAAACGATGCCGACTAGGGATCGGCGGGTGTTTTTTCGATGACCCCGCCGG
CACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGG
CTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGC
TTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGACATAGTGAGGATTGA
CAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAG
TTGGTGGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTG
CTAAATAGTCACGGCCGCTCCGGCGGTCGGCGGACTTCTTAGAGGGACTATTG
GCGATTAGCCAATGGAAGCATGAGGCAATAACAGGTCTGTGATGCCCTTAGATG
TTCTGGGCCGCACGCGCGCTACACTGATGCAGGCAACGAGCCTATCCTTGGCC
GAGAGGTCCGGGTAATCTTTGAATCTGCATCGTGATGGGGATAGATTATTGCAA
TTATTAATCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGA
TTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTGC
TGGTGAAGTGTTCGGATTGGCGACCGGTGGCGGTTTCCGCCTTCGGCCGCCGA
GAAGTTCATTAAA

(vi) *Euglena mutabilis* isolate: 18S rRNA gene sequence
(acidophilic micro-algae isolated from the abandoned Parys mine)

AGGACACTGTGAATCGAGAGTTGATCTGTGAATGGCTCCTTAGACCAGTTGCCA
TCGACGTGATAGACCGTGTGTGGCACCAGCCTCTCGCCCAAGGCGCTCAGTTG
GACATCCCTCAAAACCTGGTGGCTAATACACGATCAAGAGGTCAGCCAAAGGAC
CTGTGGTGATGCAACAACCCTCAAAGTCGCTTCGCGTCTCACCCTCAGTGGTAG
ACTGCAACCAGCTGGTGATCAAGACCTGTGCTCCGCAACTCGGAGCAGCGTTG
ACCCCGCGATGAACCAAGCTGGATCTCAATGGCGTATCGAAACGAAAGGCAAG
GGACAGTCAGCTCTGGCGATTTGATCTCGGCTGTGGCTCAGGCCTCAGCTGGA
TCTACACGACATGTGTGCTCGTGGTCTCACCGAGGCCTCGTTCACTCAGCTTGA
TCATGCGAACTTCTGACCTATCAGCTCGATGGTGATGCAATGGATCACACTGGC
CTCGACGGGTAACGGGGAATGAGGGTCTGATTCCGGAGAGGGAGCCTTAGAG
ACGGCTACCACTGCTACGGCGGGCAGCAGGCACGCAAATTGCCCCATGAACGA
ACACCCATTGAGGCAGCAACGAACTGCAGCAACCGTGCATGACCTCATTGGTC
GGTACGGCTTGTAATGGATGCAACCCAAACACAGTTATGAGTAGCCACTGGAGA
GCAAGTCTGGTGCCAGCAGTCGCGGTAATTCCAGCTCCGAGTGCGTATGCTGT
CATTGCTGCTGTTAAAGCGCTCGTAGTCAGACAATGGGTTGCAGGGACCGTGTA
GCCACGCAGGCCCAGTGGGCTCCCAGATGCCCCGTGCAGATGGTCCCATCGG
ACAACCCAGCAGCTGCACGTGACCCACGTCCCTCGGCAACTAGGACGTCTCTC
TGCTTCCCACAGCTCATTGCTGCTGCATCGGCTCACCCGTTGGCCCGGTGCAC
CCAGTCTGGATGGCATGATGTGCTCTTGCTGCAAGCGCCCTTTACTGGACCCTG
AGGCGGTGATGCTCACAACGCTAGTCATTGGCAACAGTCCGTGTGGAACCACG
ACCCCGTGTCTGGAAGCCCAGATCCACACCAGCCAGCCCTCAGAGCCTGCCAT
CATTGTCACTCTGGTGAAAGCAGTGTGCTCCAAGCGTCCAACCGAAGTCCCCC
GCTCGACGGGGGACCCTCAGTCAAGGCTGAATGTGCAACCAGTGAATGCCAAC
CACACGACCGCGTGAGGCAATGCTTGTGCCGGTCTTGAGACCCACGACTTGGG
TAGTGTGGCTGGGGGGAACCGGGGACTGGGTCGCTCCTCGCAGAGGACGAGC
CTGACGCGTGCTTCCCTGTCCCAGCCACTCGATCCCGGATGGGATGCAGCCTC
ACGGGGTGAAGGGAACCTTTGTGTCAGAGGTGAAATTCTTAGACCGCAATGAG
ACCACCTGAGGCGAAGGCTCTTCGACCAACTAGCCTGTCTCTCTCGATCAAGAA
TGGCAGTTTGGGGAGCCAAGATGATCAGACACCGTCGTAGTCCAGCGCCGTAA
ACCGTACCATCCGAGTCCTGCAGCTATGCGTGCTTCCCATTGCCCCAGGACTCA
CACCCCTGACCGAGAGAAATCCCAAGCGGGTCCAGGGGGGAGTACAGTCGCA
AGGCAGAAACTTAAAGGAATTGACGGAGTGGCACCACAAGGCGTGGAGTATGC
GGCTTAATTTGACTCAACGCGGGGAATGTTACTAGGCCAGCACATGGCAAGGAT
GGACAGACTGAGAGCTCTTTCT