DOCTOR OF PHILOSOPHY

A study of novel acidophilic Firmicutes and their potential applications in biohydrometallurgy

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Award date:
2018

Awarding institution:
Bangor University

A study of novel acidophilic *Firmicutes* and their potential applications in biohydrometallurgy

A thesis submitted to the Bangor University in candidature for the degree of Philosophiae Doctor

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U.K.

2018

Abstract

The application of biotechnologies in the mining sector has intensified over the last 30 years, driven by the increasing demand for metals associated with the rise in energy costs and the awareness for environmentally responsible mining practices. Acidophilic prokaryotes play an important role in biohydrometallurgy, facilitating the solubilisation and recovery of base metals from ores and waste materials. The potential of novel acidophiles of the phylum *Firmicutes* for applications in biohydrometallurgical processes is examined in this thesis. Eight strains of extremely acidophilic bacteria were studied and shown to belong to the proposed novel genus *"Acidibacillus"*. These had been isolated previously from several distinct global locations and were shown to be obligately heterotrophic bacteria with potential to carry out tasks critical to biomining such as regenerating ferric iron (by catalysing the dissimilatory oxidation of ferrous iron), generating sulfuric acid (by the oxidation of zero-valent sulfur and tetrathionate; two strains only), and removing potentially inhibitory dissolved organic carbon. These isolates also demonstrated the ability to catalyse the dissimilatory reduction of ferric iron in anaerobic conditions. Results obtained during this study provide the basis for future research to assess their potential roles in microbial consortia applied in the bio-processing of metal ores. A novel obligately anaerobic acidophilic *Firmicute* (strain I2511) isolated from sediment obtained from an abandoned copper mine, was characterised in terms of its phylogeny and physiology. This isolate formed a separated clade within the *Firmicutes,* and was considered to represent a novel candidate genus. It also displayed a unique set of physiological traits, distinct from currently validated species of acidophilic *Firmicutes*. The isolate was an obligate anaerobe that grew *via* zero-valent sulfur (ZVS) respiration, generating H_2S over a wide pH range (1.8 - 5.0), and also catalysed the dissimilatory reduction of ferric iron. Strains of acidophilic sulfate-reducing bacteria (aSRB), also *Firmicutes,* were shown to reduce ZVS at pH as low as 3. These aSRB, together with isolate I2511, populated a novel variant of a low pH sulfidogenic bioreactor. The "hybrid sulfidogenic bioreactor" (HSB) operated using both sulfate and ZVS as electron acceptors, and glycerol as electron donor. The bioreactor successfully remediated and recovered zinc from circum-neutral pH mine-impacted waters with distinct chemical composition collected from two abandoned lead/zinc mines in the U.K. The microbial consortium used in this system proved to be robust, in which the HSB generated H_2S consistently under a wide pH range (2 – 7). Experiments demonstrated that H_2S could also be generated abiotically in a non-inoculated low pH reactor, by the chemical reaction of ZVS and zero-valent iron to form iron sulfide, and the consequent acid dissolution of the latter. Operational costs and the advantages of biogenic and abiotic generation of H_2S for recovery of transition metals from mine waters are discussed.

Acknowledgements

I would like to dedicate this Ph.D. thesis to my wonderful family and friends for their unconditional love and encouragement. In special, to my father and my sister Juliana, that enthusiastically have supported me in every decision I have made in life, including the one to come to Bangor to live this incredible adventure, and to my mother (*in memoriam*), the greatest love I have ever experienced. I am immensely grateful for my uncle Lauro and my cousin Germana that have always guided, encouraged and inspired me to pursue a successful career. I am also deeply grateful for Donato, which his love and care motivated me daily to never give up despite all challenges. You will always be in my heart.

I would like to express my sincere and deep gratitude to my supervisor Prof. D. Barrie Johnson, for allowing me to be part of his group, his patience and contribution towards my development, for sharing his knowledge and providing me with the best training so, I could have the necessary skills to embrace future professional endeavours with competence and confidence.

I want to thank Dr. Barry M. Grail with all my heart and gratitude for his sincere friendship, encouragement, technical support and professional guidance throughout my Ph.D. He has stood by my side at every moment I needed, with endless patience, tranquillity and wisdom, in which I cannot put words to describe how important he has been to my life.

I am very grateful for all the wonderful people I have met at Bangor University, in special BART members (Ana, Sarah, Laura Kelly, Eva, Rose Jones and Carmen), Wendy and Shila. I have learnt so much from all of you and I will keep in my heart every moment we spent together.

A special thanks to Ivan Ñancucheo and Julio Espirito Santo that were responsible for connecting me with Prof. D. Barrie Johnson and therefore to this Ph.D. I am deeply grateful for the life changing opportunity you offered me and for believing in my potential.

I am deeply grateful for having in my life Rita, Marcelo, Lynne, Ian, Verity and all members of Soka Gakkai International (UK) that wholehearted encouragement me during the most challenging times, in particular towards the completion of this thesis. My Buddhist practice has been fundamental to my life, giving me the strength, wisdom, courage and compassion to overcome all hardships, transforming the perception of the dignity and preciousness of my own life and all living beings, and therefore allowing me to create value from each moment I live.

Finally, I would like to express my gratitude to the National Council of Technological and Scientific Development (CNPq) in Brazil for providing the scholarship for this Ph.D research.

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List of Abbreviations

ABS	acidophile basal salts medium
AMD	acid mine drainage
AAI	amino acid identity
aSRB	acidophilic sulfate reducing bacteria
BART	Bangor Acidophile Research Team
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
CBS	chloride basal salts
CO_2	carbon dioxide
ºC	degrees Celsius
CTAB	cetyl trimethylammonium bromide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
EDTA	ethylene diamine-tetra-acetic acid
E_H/ E^o	redox potential
EQS	Environmental Quality Standards
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fig.	figure
g	gram
G+C mol%	guanine-cytosine content
h	hours
H_3O^+	hydronium ions (or protons)
H_2S	hydrogen sulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRT	hydraulic residence (or retention) time
HS^-	sulfide
HSB	“hybrid” low pH sulfidogenic bioreactor

L	liter
m	meter
M	molar
min	minute
mM	millimolar
mL	millilitre
mm	millimetre
mmoles	millimoles
mV	millivolts
nm	nanometres
ND	not determined
nt	nucleotide
OD_x	optical density at x nanometres
OFN	oxygen free nitrogen
pK_a	dissociation constant of an acid
pK_{sp}	solubility product of a soluble salt
pH	-log $[H^+]$
PCR	polymerase chain reaction
POCP	percentage of conserved proteins
ppm	parts per million
r	regression coefficient
RISC	reduced inorganic sulfur compound
RNA	ribonucleic acid
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
rpm	revolutions per minute
TE	trace elements solution
Tris	tris(hydroxymethyl)aminomethane
TSB	tryptone soya broth
USA	United States of America
UK	United Kingdom
UV	ultraviolet light

v	volume
w	weight
x*g*	gravitational force
ZVS	zero-valent sulfur
ZVI	zero-valent iron
μL	microliter
μm	micromolar
μmoles	micromoles
w/v	weight per volume
<	less than
>	greater than
~	approximately
ΔG	Gibbs free energy

Genus abbreviations

Acidianus	*Ad.*
Acidibacillus	*Ab.*
Acidicaldus	*Acd.*
Acidiferrobacter	*Af.*
Acidimicrobium	*Am.*
Acidisphaera	*As.*
Acidiphilium	*A.*
Aciditerrimonas	*Adm.*
Acidithrix	*Atx.*
Acidithiobacillus	*At.*
Acidobacterium	*Abm*
Acidocella	*Ac.*
Alicyclobacillus	*Alb.*
Bacillus	*B.*
Desulfobacillus	*Ds.*
Desulfosporosinus	*D.*
Desulfovibrio	*Dv.*
Desulfurella	*Dsf.*
Desulfuromonas	*Dm.*
Ferrithrix	*Fx.*
Leptospirillum	*L.*
Stygiolobus	*Sg.*
Sulfobacillus	*Sb.*
Sulfurisphaera	*Ss.*
Sulfurospirillum	*Sp.*
Wolinella	*W.*

Chapter 1. Introduction

1.1 Diversity of acidophilic prokaryotes in low pH environments.

1.1.1 Acidic ecosystems on planet Earth

Many environments on Earth (including freshwater and soils) have pH below neutrality (pH 7), and those that have pH values < 5 are considered to be acidic. They can be divided into moderately acidic (pH 3 – 5) or extremely acidic (pH < 3) (Johnson and Aguilera, 2016). These environments are distributed worldwide and can be of natural or anthropogenic origin. Extremely acidic environments are far less common, formed in most cases due to biogenic generation (and accumulation) of sulfuric acid, and can exhibit features such as high temperatures, elevated concentrations of inorganic substances, transition metals and metalloids.

Natural acidic environments include terrestrial geothermal sites (e.g. solfatara, geothermal springs), deep-sea hydrothermal systems, cave systems, acid sulfate soils and sulfide-rich rock strata exposed to air and water. Since indigenous microorganisms that inhabit acidic environments are adapted not only to acidity but also to the other extreme conditions that may occur in those ecosystems (e.g. high metal and salt concentrations), they have potential to be applied in biomining operations where conditions can be similar or more extreme (Hedrich and Schippers, 2016).

Acidic environments associated with the oxidation of sulfide minerals are very often due to anthropogenic activity, in contrast with environments impacted by oxidation of sulfur compounds originated from volcanic and geothermal areas (Johnson and Aguilera, 2019). Man-made low pH environments are mostly associated with mining of both metals and coals and comprise the most widely studied acidic ecosystems (Hedrich and Schippers, 2016).

1.1.1.1 Natural acidic environments

Mineral soils and peatlands can become acidic in situations where precipitation exceeds evapo-transpiration potentials and where non-basic bedrocks are prevalent. Acid sulfate soils can develop very low pH values (pH< 4) due to the presence of sulfide minerals that undergo oxidation processes generating sulfuric acid. Other types of mineral soils rarely reach pH values below 4.2, while in the case of organic peatlands pH values tend to be above 3. This is mostly due to aluminium buffering in most mineral soils while aliphatic and aromatic organic acids that have pK_a values between 3 and 5 being more abundant in organic peatlands (Johnson and Aguilera, 2019).

Natural low pH saline lakes are much less abundant than alkaline saline lakes, and can be found for instance in the Salar de Gorbea, Northern Chile. These water bodies are characterised by low pH varying from 1 to < 5 and high salinity (100-300% total dissolved solids) with temperatures ranging from 8 – 20 °C. Studies of the microbial population of these water bodies indicated the most abundant bacterial species were from the phyla *Firmicutes* (e.g. *Sulfobacillus* spp. and *Alicyclobacillus* spp.)*, Proteobacteria, Cyanobacteria and Actinobacteria*. The most abundant archaeal species were members of *Euryarchaeota* (e.g. *Thermoplastales* and *Halobacteriales*) (Escudero *et al*., 2013; Davis-Belmar *et al*., 2013).

Acidic cave systems are formed when underground environments containing sulfide-rich ground water bodies are subjected to microbial oxidation of sulfide generating sulfuric acid, and thus causing dissolution of carbonate rock strata. A well-studied acidic ecosystem is the Frasassi cave, in Italy, where microbial community analysis of biofilms ("snottites" of pH ~0) revealed low diversity with bacteria related to the sulfur-oxidiser *Acidithiobacillus thiooxidans* as the most abundant member, while other species such as *Sulfobacillus* spp., *Acidimicrobium ferrooxidans* and uncultivated archaea were much less abundant (Macalady *et al.*, 2007).

Terrestrial geothermal areas are distributed widely on planet Earth, located mainly around the borders of tectonic plates. These sites often encompass thermal springs, fumaroles, geysers and solfatara fields. Solfatara fields occur where the earth's crust is relatively thin, and near to active volcanoes, and are characterised by the presence of acidic hot springs, mud pots and soils, comprising the most pervasive natural sulfur-rich environments on Earth (Dopson and Johnson, 2012). These areas contain elevated concentrations of sulfur compounds in different oxidation states, e.g. zero-valent sulfur (ZVS), SO_4^{2-}, and a variety of gases such as H_2O (steam), H_2, CO_2, CO, O_2, CH_4 and H_2S (Golyshina *et al.,* 2016a). Elevated temperatures and high acidity of these areas accelerate the dissolution of minerals generating high concentration of soluble metals and silica. The great temperature range found in these environments (from ambient to ~100 °C), together with the variety of substrates and oxygen concentrations, allows for a vast diversity of indigenous acidophilic prokaryotes, which has been confirmed by the isolation of several novel species of archaea and bacteria and by studies using cultivation-independent approaches (Inskeep *et al.,* 2010, Johnson *et al.,* 2003; 2001). Examples of solfataric areas where acidophiles have been isolated include Yellowstone National Park (USA), Montserrat (Caribbean), Pozzuoli and Vulcano Island (Italy), Hokkaido (Japan), Java (Indonesia) and São Miguel (Azores) (Johnson, 2016; Golyshina *et al.,* 2016a).

The low pH of solfatara areas is a consequence of the biogenic generation of sulfuric acid by microbial oxidation of sulfur compounds, e.g. ZVS (Eq.1.1) and hydrogen sulfide (Eq.1.2). Zero-valent sulfur can be formed by the reaction between sulfur dioxide and hydrogen sulfide

(Eq.1.3). These gases are partly soluble in H_2O, and therefore can also be subjected to microbial oxidation generating sulfuric acid (Dopson and Johnson, 2012; Johnson and Aguilera, 2019).

$$S^0 + 1.5\ O_2 + 3\ H_2O \rightarrow 2\ H_3O^+ + SO_4^{2-} \qquad \text{Eq.1.1}$$

$$H_2S + 2\ O_2 + 2\ H_2O \rightarrow 2\ H_3O^+ + SO_4^{2-} \qquad \text{Eq.1.2}$$

$$SO_2 + 2\ H_2S \rightarrow 3\ S^0 + 2\ H_2O \qquad \text{Eq.1.3}$$

The most diverse and abundant geothermal terrestrial system on Earth, Yellowstone National Park, is also the most studied natural extremely acidic hydrothermal area. Cultivation-based studies have identified several novel acidophilic bacteria (e.g. moderate thermophilic iron- and sulfur-metabolising *Acidicaldus organivorans*; Johnson *et al.,* 2006) and archaea (e.g. thermophilic sulfur-metabolising *Acidianus brierleyi*; Brierley, 1973). Metagenomic studies of thermophilic populations at acidic iron- and sulfur-rich sediments and hot springs has identified novel deeply rooted representatives of the archaea domain (e.g. "*Geoarchaeota"* and *Thaumarchaeota*; Beam *et al.,* 2014; Kozubal *et al*., 2013).

Submarine hydrothermal systems are characterised by gradients of temperature, pH (extreme acidity areas may be formed close to venting ducts) and electron donors/acceptors, and are affected by the high buffering capacity of seawater. These characteristics generate chemoclines and thermoclines with varying pH and temperatures and limited niches for acidophilic microorganisms to populate (Johnson, 2016; Golyshina *et al*., 2016a).

1.1.1.2 Extremely acidic mine-impacted environments

The formation of extremely acidic (pH < 3) environments is often due to the (microbiologically-accelerated) oxidative dissolution of sulfide-minerals, where sulfur exhibits oxidation states of either -1 or -2. Sulfidic ores are the main sources of several base and semi-precious transition metals (e.g. copper, cobalt, nickel, zinc and silver) and often are associated with pyrite (FeS_2) in ore bodies. The latter is the most abundant of all sulfide minerals and is often present in much greater quantities in the ore bodies than the sulfidic mineral bearing the target metal(s). The mining of ore bodies involves excavation, comminution and disposal of reactive mineral waste such as fine-grain tailings, which often contain large amounts of pyrite and pyrrhotite ($Fe_{(1-x)}S$, where x= 0 - 2) due to their relatively low commercial value. These minerals are susceptible to oxidation when exposed to water and oxidising agent (O_2 or Fe^{3+}) as a purely abiotic process. This occurs relatively slowly, but can be greatly accelerated by microbial activity. Abiotic oxidation of pyrite generates (initially) ferrous iron and thiosulfate (Eq.1.4) (Vera *et al.,* 2013). Acidophilic prokaryotes oxidise ferrous iron when oxygen is available regenerating ferric iron that will oxidise pyrite producing more ferrous iron and thiosulfate (Eq.

1.5). The regeneration of ferric iron is a necessary step in allowing the oxidation process to continue and therefore acidophilic prokaryotes perform a critical task and are considered the primary agents of accelerating the oxidative dissolution of sulfidic minerals in acidic environments. The thiosulfate generated is unstable at low pH, especially in the presence of ferric iron, and decomposes into zero-valent sulfur and sulfur oxy-anions, which are in turn also oxidised by indigenous prokaryotes generating sulfuric acid (e.g. Eq.1.2) (Schippers *et al.,* 1996).The oxidative dissolution of pyrite at low pH, in which all products are in their most oxidised form, is summarised in equation 1.6.

$$FeS_2 + 6\ Fe^{3+} + 9\ H_2O \rightarrow 7\ Fe^{2+} + S_2O_3^{2-} + 6\ H_3O^+ \qquad \text{Eq.1.4}$$

$$Fe^{2+} + 2\ H_3O^+ + 0.5\ O_2 \rightarrow Fe^{3+} + 3\ H_2O \qquad \text{Eq.1.5}$$

$$FeS_2 + 3.75\ O_2 + 1.5\ H_2O \rightarrow Fe^{3+} + 2\ SO_4^{2-} + H_3O^+ \qquad \text{Eq.1.6}$$

Mining activity can negatively impact adjacent water bodies, generating acidity and greatly increasing concentrations of dissolved transition metals, aluminium, and metalloids such as arsenic. Such water bodies include subterranean lakes formed within abandoned deep mines, pit lakes formed in the opencast voids and acidic streams draining mines and mine spoils (Johnson, 2016).

When opencast mining of coal and metals terminates, water drainage pumps are switched off and the abandoned voids become increasingly filled with groundwater and rainfall, forming pit lakes. This process can take between months and many years, depending on the hydrology of the local area. These lakes can become acidic if the exposed geological strata adjacent to the voids is composed by reactive minerals such as iron sulfides, and the potential acidity generated by the metal oxidative dissolution is not counterbalanced by the dissolution of basic minerals (e.g. carbonates). The water column of acidic pit lakes are often stratified in terms of pH, redox potentials and oxygen concentrations, where the upper zones usually are oxic and can develop higher acidity and redox potentials, while the deeper zones are anoxic with higher pH and lower redox potentials (Sanchez-España *et al.,* 2008). Metal mining activity carried out at the Iberian Pyrite Belt (IPB) has generated more than 25 pit lakes with complex geochemistry in terms of the variety of transition metals and metalloids that they may contain (Santofimia *et al.,* 2013). The oxidative dissolution of pyrite and consequent acidification of water in these lakes caused dissolution of other sulfides (e.g., chalcopyrite, sphalerite, arsenopyrite, galena), sulfosalts (e.g., tetrahedrite–tennantite) and gangue aluminosilicates (e.g., feldspars, chlorite, sericite) from the rock strata. Consequently, most pit lakes in the IPB have high concentrations of dissolved metals and acidity (Sanchez-España *et al.,* 2008).

Mynydd Parys ('Parys mountain'), located in North Wales, is an abandoned copper mine where first mining activity dates back to the Bronze Age. The mining site consisted of underground works and two opencast voids; pyrite, chalcopyrite ($CuFeS_2$), sphalerite (ZnS), and galena (PbS) were the major sulfide minerals present. Mynydd Parys was the world's largest copper-producing mine in the 18th century with extractive mining activities terminated at around 1880. However copper was extracted by "*in situ"* bioleaching until 1950 when the site was abandoned and controlled flooding and draining the mine (to produce "cement copper") ended, resulting in an underground lake developing within impounded shafts and adits. This lake remained undisturbed for over 50 years, but the risk of a concrete dam failure resulted in dewatering of the mine. Acidic metal-rich water, *ca*. 274,000 m^3, was pumped out of the underground mine flowing, untreated, into the Irish Sea. Microbial community composition analysis of the subterranean water indicated that the dominant bacteria present belonged to *Acidithiobacillus* spp. Other iron-oxidisers such as *Leptospirillum* spp., *Ferrimicrobium acidiphilum* and acidophilic bacteria related to *Gallionella*, *Acidiphilium, Acidobacterium* and *Acidisphaera* were also detected (Coupland and Johnson, 2004). The limited oxygen ingress in the underground lake at Parys mine favoured the dissimilatory reduction of ferric iron by autotrophic and heterotrophic bacteria, generating high concentrations of ferrous iron, ~ 600 mg L^{-1}, which caused ferrous iron to be the primary electron donor in the aerated drainage stream. Cultivation-independent analysis of archaeal members (clone libraries) revealed that methanogens were dominant while *Thermoplasma/Ferroplasma*-like represented a minor fraction of the clones detected. Large 'drapes' (two dimensional gelatinous materials) were found in the freshly-drained underground chamber at Mynydd Parys. These had distinct bacterial compositions from those of streamers in the drainage stream outside of the mine (described below) and the drainage water itself. Heterotrophic rather than autotrophic acidophilic bacteria were identified in the cultivation-independent analysis, e.g. *Ferrimicrobium* and *Acidimicrobium,* species of which oxidise ferrous iron and use organic compounds. In contrast to the bacterial populations, cultivation-independent analysis of archaeal community showed that the composition in the 'drapes' and streamers within Mynydd Parys were very similar, though the former exhibited higher diversity. (Kay *et al.*, 2013).

A nine year study was carried out in the Afon Goch North, a stream which had not been affected by acid mine drainage, to evaluate the impact of the overflow AMD from Mynydd Parys being diverted into this stream (Kay, *et al.,* 2013). The study focused in the microbiology and geochemistry of the Afon Goch North and showed that autotrophic iron-oxidising acidophiles dominated the acid streamers (macroscopic off-white filamentous microbial growths, Fig. 1.1) during the nine years. Two species*, "Ferrovum myxofaciens"*

and *Acidithiobacillus ferrivorans,* represented > 70% (average) of the bacterial population during the course of the study. The low variation of the physico-chemical properties of the flowing mine water during the study period (Table 1.1), e.g. temperature variation < 1°C regardless the season, explains the stability of the streamer community. An interesting finding in this study was the absence of "*Fv. myxofaciens"* in the subterranean water and "drapes" in the underground mine, possibly explained for the low concentration of oxygen in the impounded lake, since this bacterium is an obligate aerobe.

Figure 1.1. Acid streamer growths within the Afon Goch North: (a) portal at the upper end of the drainage channel; (b) acid streamer growths near to the portal; (c) 15 m downstream of the portal; (d) 90 m downstream of the portal (figure origin: Kay *et al.*, 2013)

Table 1.1. Physico-chemical data (mean values; n=46) of the Afon Goch North adit portal obtained from October 2003 to November 2007.Concentrations shown are mg L^{-1}, except where specified (data from Kay *et al*., 2013).

Analyte	Value (standard error) (mg L^{-1})
pH	2.53 (0.04)
Redox potential Eh (mV)	669 (2.6)
Conductivity (µS cm^{-1})	2506 (66)
Temperature (°C)	11.3 (0.1)
Oxygen (%)	12.7 (0.4)
Sulfate-S	800 (13)
Fe (II)	378 (14)
Fe_{total}	563 (11)
Zn (II)	67 (1.6)
Cu (II)	49 (2.2)
Mn (II)	15.8 (0.7)
Al (III)	2.15 (0.16)
DOC*	4.50 (0.29)

*Dissolved organic carbon

1.1.2 Characteristics of acidophilic prokaryotes

Acidophilic microorganisms are generally described as life forms that are able to tolerate high concentrations of hydronium ions (H_3O^+), and which can grow more favourably in environments where pH is below 7. In the last 30 years a large diversity of acidophilic microorganisms, distributed randomly in the tree domains of life (Bacteria, Archaea and Eukarya) have been described. Tolerance to acidity has therefore been considered to be a characteristic that has developed on several occasions during evolution of life on Earth, rather than representing a singular adaptation episode (Johnson and Aguilera, 2019).

A common characteristic of extremophiles is that they are highly specialised organisms; most extreme acidophiles, for example, are not able to grow in neutral pH conditions (Johnson and Aguilera, 2019). Acidophilic species that also tolerate other extreme conditions (e.g. very high salinity or temperature) tend to be less acidophilic than species that tolerate only extreme acidity (e.g. *Hydrogenobaculum acidophilum*). Nevertheless, the Archaea domain contains several species which are able to grow in more than one physico-chemical extreme, e.g. some species are extreme acidophilic as well as hyper-thermophilic.

In general, acidophilic species maintain intracellular pH values close to neutral, while they inhibit environments containing high concentrations of H_3O^+, and most of their cytoplasmic enzymes that have been characterised, have pH optima ~7 (Johnson and Aguilera, 2016). Acidophiles are also able to tolerate pH gradients across cytoplasmic membranes that are several orders of magnitude greater than neutrophiles. Extracellular enzymes or redox-active proteins (e.g. rusticyanin) and enzymes located in periplasmic region of Gram-negative acidophiles (i.e. the area between the internal and external membranes, and which has a pH value similar to the external environment) are active at low pH (Nunzi *et al.,* 1994; Chi *et al.,* 2007). Studies have suggested that acidophiles use several mechanisms to cope with pH stress: (i) changes to cell membranes (e.g. acidophilic archaea have cytoplasmic membranes composed of lipids containing up to eight cyclopentane moieties which have extremely low proton permeabilities); (ii) use and structure of cellular pumps (e.g. putative cation transporters and proton efflux systems, which are abundant in many acidophilic genomes); (iii) production of cytoplasmic buffers (e.g. increase in the synthesis of alkaline amino acids with decrease in the environmental pH); (iv) DNA and protein repair (by the presence in the acidophile genomes of a large number of genes responsible for the repair of DNA and proteins) and (v) reversed membrane potential ($\Delta\omega$): generation of positive (inside) $\Delta\omega$ formed by the influx of cations (e.g. K^+), that retards proton entry into the cell (Baker-Austin and Dopson, 2007).

In the Bacteria domain, extreme acidophiles are distributed mostly within the members of the phyla *Proteobacteria, Firmicutes, Nitrospirae, Aquificae, Acidobacteria, Actinobacteria* and

Verrucomicrobia. In the Archaea domain, the majority of cultured acidophiles, also best-studied species, belong to the two phyla *Euryarchaeota* and *Crenarchaeota*. The first isolated and characterised extreme acidophile, described by Waksman and Joffe in 1921, was *Acidithiobacillus thiooxidans* (named at that time as *Thiobacillus thiooxidans*). This sulfur-oxidising proteobacterium was isolated from enrichments containing a compost of ZVS, phosphate and soil (Dopson and Johnson, 2012). About thirty years later, another sulfur-oxidising acidophilic bacterium that was also able to oxidise ferrous iron to ferric iron at very low pH (a unique characteristic at that time), was isolated from a coal mine drainage. This bacterium, (*Acidi)thiobacillus ferrooxidans*, became the most well studied of all acidophilic microorganisms.

One well-accepted classification for acidophilic microorganisms, proposed by Johnson (2007), defined (i) acid-tolerant as species that have pH optima above 5, but which also grow at pH < 5; (ii) moderate acidophiles, which have pH optima between 3 and 5; (iii) extreme acidophiles, which have pH growth optima between 1 and 3; (iv) hyper-acidophiles, which have pH growth optima below 1.0. Organisms defined as extremely acidophilic are exclusively microbial and include prokaryotic and eukaryotic species (Johnson and Aguilera, 2019). Only a few organisms are able to grow at pH < 1 and include strains of Gram-positive (e.g. *Sulfobacillus* spp.) and Gram-negative bacteria (e.g. *At. thiooxidans*) and Archaea (e.g. *Acidianus* spp.). The archaeon species *Picrophilus oshimae* and *Picrophilus torridus* are the most acidophilic of all currently described microorganisms so far discovered. These are heterotrophs that grow optimally at pH ~0.7 and at ~60˚C, and therefore are classified as hyper-acidophilic thermophiles.

Another means of categorising acidophiles is on the basis of their temperature range in which they are active and their optimum temperature for growth. Acidophiles having temperature optima between 20˚C and 40˚C are classed as mesophiles, while moderate thermophilic grow optimally between 40˚C and 60˚C. Species that are active at temperatures above 40˚C but do not grow at ~45 - 50˚C are considered to be thermo-tolerant. No psychrophilic acidophile has yet been described, though some (e.g. *At. ferrivorans* and "*Fv. myxofaciens*") are capable of growing at temperatures below 5 ˚C, and are therefore psychrotolerant. Extreme thermophiles grow optimally at 60 - 80˚C and hyper-thermophiles have temperature optima above 80˚C, e.g. the sulfur-metabolising archaeon *Acidianus infernus*, has temperature optimum at 80 - 95˚C and is currently the most thermophilic extreme acidophile described. The majority of extremely thermophilic acidophiles are archaea, while most mesophilic, moderately thermophilic and thermo-tolerant acidophiles are bacteria. In general terms, mesophiles are mostly Gram-negative bacteria, while Gram-positive bacteria generally comprise species of

moderate thermophiles and thermo-tolerant, though there are exceptions (Johnson and Aguilera, 2019; Johnson, 2009).

Acidophilic prokaryotes, as a group, exhibit a wide diversity of metabolic capabilities, being able to use solar and chemical (inorganic and organic) energy sources, several electron acceptors (e.g. O_2, Fe^{3+}, ZVS), and organic and inorganic carbon source (and some species can use both). While the metabolic capabilities of acidophilic prokaryotes are comparable to neutrophilic species. Extremely acidophilic methanogens and denitrifiers have not yet been identified, although culture-independent analysis in extremely acidic environments have detected methanogenic archaea as members of microbial populations (Johnson, 2012; Kay *et al.*, 2013). The absence of extremely acidophilic denitrifiers can be accounted for both by the low concentrations of nitrate in most acidic environments (compared to ferric iron and sulfate), and to the fact that many acidophiles have a low tolerance to nitrate and nitrite. Fermentative and acetogenic species are rare within acidophiles, possibly due to the toxicity of small molecular weight aliphatic acids at low pH conditions (Johnson *et al*., 2012; Johnson and Aguilera, 2019). The inhibitory effects of aliphatic acids depends on their dissociation constants (pK_as). In conditions were the pH is below the pK_a value, the predominant form of the acids are the uncharged/undissociated molecules. These are often lipophilic and diffuse into the cells where the pH is ~7, therefore causing the dissociation of the acid, releasing protons and causing the acidification of cell cytoplasm and cell death (Johnson and Hallberg, 2008; Sanchez-Andrea *et al*., 2014; Koschorreck, 2008).

Many acidophiles were initially described as obligate aerobes, but shown later to be able to use electron acceptors other than oxygen, making them facultative anaerobes. This was the case for *At. ferrooxidans*, which was shown by Pronk *et al*. (1992) to grow by respiring ferric iron as well as oxygen. Strictly anaerobic extreme acidophiles species are ZVS reducers (e.g. the archaeon *Stygiolobus azoricus*). The most used electron acceptor other than molecular oxygen is ferric iron. Most bacteria and archaea that oxidise iron aerobically can also reduce ferric iron in the absence of oxygen (Johnson and Aguilera, 2019), provided they can also use electron donors other than ferrous iron. Ferric iron respiration is thermodynamically advantageous since the redox potential (E_h value) of Fe^{2+}/Fe^{3+} in acidic sulfate-rich waters is between +690 and 720 mV (Johnson *et al.,* 2012), which is close to the redox of O_2/H_2O couple +840 mV at pH 7, and considerably more positive than other potential inorganic electron acceptor such as sulfate.

Natural and anthropogenic acidic environments such as solfatara and pit lakes in abandoned mining sites are frequently oligotrophic in respect of their contents of dissolved organic carbon, much of which may also be recalcitrant to biodegradation. As a consequence, they are often

dominated by acidophilic prokaryotes that can fix inorganic carbon, i.e. autotrophs or facultative autotrophs. Other acidophiles have an absolute requirement for organic carbon sources, and are obligate heterotrophs (organic compounds act as both electron donors and carbon sources). Chemolithotrophy, i.e. the use of inorganic compounds as electron donors, is a widespread metabolism among acidophilic prokaryotes and possibly related to the fact that in low pH environments soluble inorganic compounds (e.g. reduced sulfur, Fe^{2+} and H_2) are abundant, and therefore they are often the main sources of energy available (Johnson and Aguilera, 2016). The relationship between chemolithotrophic and heterotrophic acidophiles in both oxic and anoxic zones in environments impacted by acid mine drainage (AMD) is shown in the Figure 1.2.

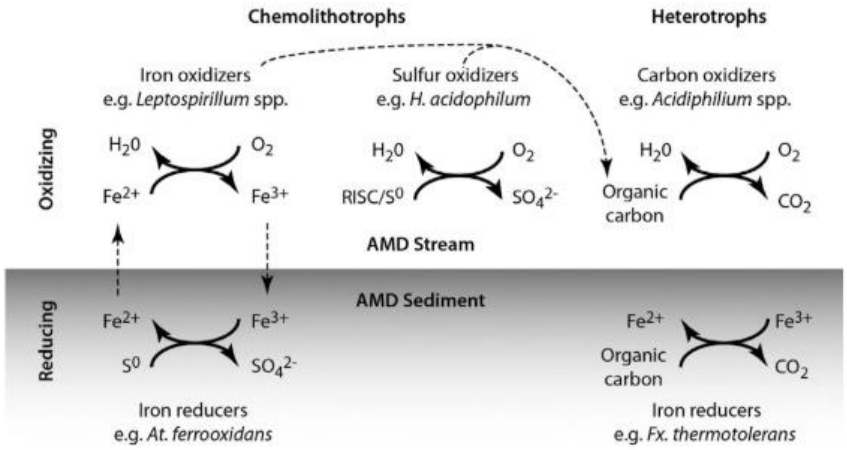

Figure 1.2. Carbon and energy sources utilised by acidophilic prokaryotes. The relationship between aerobic and anaerobic growth strategies are described as for the water column and sediment of an acid mine drainage (AMD) stream. Dotted lines denote transport processes (figure origin: Dopson, 2016).

Of the main electron donors used by acidophiles, ferrous iron oxidation generates the least amount of free energy (Table 1.2). Depending of its initial oxidation state (from -2 to +6), sulfur can transfer up to eight electrons when fully oxidised to sulfate, compared to a single electron in the oxidation of ferrous iron and two electrons for hydrogen oxidation. However, ferrous iron is often used in preference to both reduced sulfur and hydrogen. The main reasons are: (i) ferrous iron is more rapidly oxidised than zero-valent sulfur or polythionates and (ii) the machinery required to oxidise iron in some acidophiles (e.g. *At. ferrooxidans*) involves a relatively short electron transport chain (in terms of cytochromes and rusticyanin) while for reduced sulfur oxidation (e.g. tetrathionate) the synthesis of several enzymes and trans-

membrane electron transport proteins, is required which demands higher energy expenditure compared to that required for iron oxidation (Johnson *et al.,* 2012*).*

Table 1.2. Comparison of free energy changes associated with the oxidation of inorganic substrates used by chemolithotrophic acidophiles (data from Johnson and Aguilera, 2016).

Reaction	**Electrons (moles mole substrate^{-1})**	**Free energy change ΔG° (kJ mole electron donor^{-1})**
$4\ FeSO_4 + O_2 + 2\ H_2SO_4 \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2O$	1	−30 (at pH 2.0)
$H_2 + 0.5\ O_2 \rightarrow H_2O$	2	-237
$S^0 + 1.5\ O_2 + 3\ H_2O \rightarrow 2\ H_3O^+ + SO_4^{2-}$	6	-507
$H_2S + 2\ O_2 + 2\ H_2O \rightarrow 2\ H_3O^+ + SO_4^{2-}$	8	-714
$S_4O_6^{2-} + 3.5\ O_2 + 9\ H_2O \rightarrow 4\ SO_4^{2-} + 6\ H_3O^+$	14	-1,225

1.1.2.1 Autotrophic acidophilic bacteria

Many acidophiles are obligately autotrophic and obtain carbon solely from the fixation of CO_2, whereas others are facultative autotrophs and can switch between organic and inorganic carbon depending whether the former is available (the preferred source of carbon for these acidophiles). Two main groups of microorganisms carry out autotrophic metabolism in extremely acidic environments: chemolitho-autotrophs and photo-autotrophs (Johnson, 2009). They are responsible for the primary production (net assimilation of carbon) in these environments and their significance within microbial communities varies accordingly with the characteristics of the location. Although chemolitho-autotrophs are ubiquitous in acidic environments, microorganisms that use solar energy to fix CO_2 are often the predominant primary producers when sunlight is available. In acidic caves and underground abandoned mines, primary production is exclusively mediated by chemolitho-autotrophs. So far, no acidophilic phototrophic bacteria (cyanobacteria and purple/green sulfur-bacteria) have been described and all acidophilic phototrophic microorganisms identified to date are eukaryotic micro-algae (Lessmann *et al.,* 2000). A list of selected type strains of autotrophic and facultative autotrophic acidophilic bacteria is shown on Table 1.3.

Table 1.3. Characteristics of selected acidophilic autotrophic and facultative autotrophic bacteria. Data from Dopson (2016).

Species	Electron donors	Electron acceptors
Low temperature-adapted mesophiles[1]		
"Ferrovum myxofaciens"[T]	Fe^{2+}	O_2
Acidithiobacillus ferrivorans[T]	Fe^{2+}/S	O_2/Fe^{3+}
Acidithiobacillus ferriphilus[T]	Fe^{2+}/S	O_2/Fe^{3+}
Mesophiles[2]		
Acidithiobacillus ferrooxidans[T]	Fe^{2+}/S/H_2	O_2/Fe^{3+}
Acidithiobacillus albertensis[T]	S	O_2
Acidithiobacillus ferridurans[T]	Fe^{2+}/S/H_2	O_2/Fe^{3+}
Acidithiobacillus thiooxidans[T]	S	O_2
Leptospirillum ferroxidans[T]	Fe^{2+}	O_2
"Leptospirillum ferrodiazotrophum" UBA1	Fe^{2+}	O_2
Acidiphilium acidophilum[T]	S/C	O_2/Fe^{3+}
Sulfobacillus benefaciens[T]	Fe^{2+}/S/H_2/C	O_2/Fe^{3+}
Alicyclobacillus aeris[T]	Fe^{2+}/S/C	O_2
Moderately thermophilic and thermo-tolerant[3]		
Acidithiobacillus caldus[T]	S/H_2	O_2
"Acidithiomicrobium" P2	Fe^{2+}/S	O_2
Acidiferrobacter thiooxydans[T]	Fe^{2+}/S	O_2/Fe^{3+}
Leptospirillum ferriphilum[T]	Fe^{2+}	O_2
Acidimicrobium ferrooxidans[T]	Fe^{2+}/H_2/C	O_2/Fe^{3+}
Aciditerrimonas ferrireducens[T]	H_2/C	O_2/Fe^{3+}
Sulfobacillus acidophilus[T]	Fe^{2+}/S/H_2/C	O_2/Fe^{3+}
Sulfobacillus sibiricus[T]	Fe^{2+}/S/C	O_2
Sulfobacillus thermosulfidooxidans[T]	Fe^{2+}/S/H_2/C	O_2/Fe^{3+}
Sulfobacillus thermotolerans[T]	Fe^{2+}/S/C	O_2
Alicyclobacillus tolerans[T]	Fe^{2+}/S/C	O_2/Fe^{3+}
Extreme thermophiles[4]		
Hydrogenobaculum acidophilum[T]	S/H_2	O_2

[1]mesophilic but capable of growth at 5 ºC; [2]growth temperature optima 20 – 40ºC; [3]growth temperature optima 40 – 60 ºC; [4]growth temperature optima 60 ºC

Carbon dioxide fixation is a high energy-demanding process (e.g. much of the energy conserved from iron oxidation by *Acidithiobacillus* spp. is used on this single process), therefore the ability to obtain pre-fixed carbon is advantageous if the compound can be readily assimilated and metabolised (Johnson and Aguilera, 2019). Moreover, CO_2 has low solubility at low pH, being present almost entirely as gaseous CO_2 rather than soluble HCO_3^-. The solubility of CO_2 also decreases with temperature, therefore thermo-acidophilic autotrophs face a great challenge in terms of obtaining inorganic carbon (Johnson and Aguilera, 2016). Some autotrophic acidophilic bacteria (e.g. *At. ferrooxidans* and *At. thiooxidans*) have micro-compartments to facilitate this. Carboxysomes are specialised polyhedral protein bodies that encapsulate the enzymes: carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). They are used to concentrate CO_2, and therefore to enhance CO_2-fixation, possibly in response to the limited availability of CO_2 in acidic environments (Johnson and Aguilera, 2016; Blanchard and Abdul-Rahman, 2014). The pathway to fix carbon dioxide used by most of acidophilic bacteria is the Calvin-Benson-Bassham (CBB) cycle. Two forms of RuBisCO, a key enzyme in the CBB cycle, have been identified in the acidithiobacilli, and the genome analysis confirmed that species of this family contain all the genetic apparatus to fix CO_2 using the CBB cycle (Johnson and Aguilera, 2016). Only one species, *Leptospirillum ferriphilum*, is known to use the reductive tricarboxylic acid (TCA) cycle. The facultative autotroph acidophile iron- and sulfur-oxidiser *Sulfobacillus thermosulfidooxidans* also uses RuBisCO to fix CO_2 but this species appears to not synthesize carboxysomes, which possibly explains its poorer growth under autotrophic conditions than *At. ferrooxidans* (Caldwell *et al.,* 2007).

1.1.2.2 Heterotrophic acidophilic bacteria

The number of characterised obligately heterotrophic extremely acidophilic bacteria and archaea has increased greatly in the last years, but they have been much less widely studied than acidophilic chemolitho-autotrophs (e.g. *Acidithiobacillus* spp.; Johnson and Aguilera, 2016). While some acidophilic heterotrophic prokaryotes (e.g. *Alicyclobacillus disulfidooxidans*) can obtain energy by oxidising either organic or inorganic substrates such as ferrous iron or reduced sulfur (chemolitho-heterotrophs), in other cases inorganic compounds are the only electron donors used and organic materials are used only as a carbon source (e.g. the iron-oxidizer *Ferrimicrobium acidiphilum*) (Johnson, 2009). Heterotrophic acidophiles often form close associations with chemolitho-autotrophs in which they use the exudates and lysates (small molecular weight organic compounds) released by the latter as carbon or energy source. These associations are beneficial for the continued growth of chemolitho-autotrophs, since accumulation of these organic by-products inhibit their growth (Kermer *et al.,* 2012; Ñancucheo and Johnson, 2010)

Table 1.4. Characteristics of selected acidophilic obligately heterotrophic bacteria.

Species	Electron donors	Electron acceptors
Mesophiles[1]		
Acidiphilium cryptum T	C	O_2/Fe^{3+}
Acidiphilium rubrum T	C/S	O_2/Fe^{3+}
Acidithrix ferrooxidans T	Fe^{2+}/C	O_2
Acidocella facilis T	C	O_2
Acidocella aromatica T	C	O_2/Fe^{3+} mineral
Alicyclobacillus disulfidooxidans T	Fe^{2+}/S/ C	O_2
Alicyclobacillus ferrooxydans T	Fe^{2+}/S/C	O_2
Ferrimicrobium acidiphilum T	Fe^{2+}	O_2/Fe^{3+}
Acidomonas methanolica T	C	O_2
Moderately thermophilic and thermo-tolerant[2]		
Acidicaldus organivorans T	S/C	O_2/Fe^{3+}
Ferrithrix thermotolerans T	Fe^{2+}/C	O_2/Fe^{3+}
Alicyclobacillus pomorum T	C	O_2
Alicyclobacillus acidiphilus T	C	O_2
Alicyclobacillus acidocaldarius T	C	O_2
Alicyclobacillus hesperidum T	C	O_2
Alicyclobacillus contaminans T	C	O_2
Extreme thermophiles[3]		
Methylacidiphilum infernorum V4	CH_4	O_2

[1]growth temperature optima 20 – 40ºC; [2]growth temperature optima 40 – 60 ºC; [3]growth temperature optima 60 ºC.

Neutrophilic prokaryotes are known for their great versatility in terms of degradation of organic sources, metabolising small molecular weight, polymeric and synthetic organic compounds. However, acidophiles have been reported to use a more restricted selection of organic compounds, e.g. small molecular weight (simple sugars and alcohol), complex nitrogen-rich substrates (e.g. yeast extract and tryptone) and only few polymeric organic substrates (e.g. starch) (Johnson and Aguilera, 2019). Complex organic substrates are often an effective substrate for isolating and cultivating heterotrophic acidophiles, either as electron donor, carbon source or growth factor, in which the latter promotes growth of heterotroph acidophilic prokaryotes when provided in growth media with defined organic compounds (Johnson, 2009).

Gram-negative acidophiles, mostly species of the phylum *Proteobacteria*, comprise a large group of well characterised species, including several heterotrophic species such as *Acidicaldus organivorans, Acidomonas methanolica, Acidocella* spp*. and Acidiphilium* spp.. A number of species of Gram-positive acidophiles have also been fully characterized and they are distributed within two phyla: *Firmicutes* and *Actinobacteria* (Johnson and Aguilera, 2016). *Actinobacteria* are non-sporulating bacteria characterized by a high G + C contents in their chromosomal DNA (i.e. high guanine and cytosine base composition compared to adenine and tyrosine bases). The acidophilic members of this phylum are distributed within six genera (one as candidate genus), all of which at present, contain a single species. Some are moderate thermophiles (*Am. ferrooxidans, Aciditerrimonas ferrireducens, Ferrithrix thermotolerans* and the candidate genus *"Acidithiomicrobium"*) while others are mesophilic (*Acidithrix ferrooxidans* and *Fm. acidiphilum*) and share metabolic traits such as the dissimilatory oxidation of ferrous iron (except for *Atn. ferrireducens*) and reduction of ferric iron (except for *Atx. ferrooxidans* and *"Acidithiomicrobium"*). However, some members exhibit specific features such as the ability to catalyse the dissimilatory oxidation of hydrogen (*Am. ferrooxidans*) or reduced sulfur ("*Acidithiomicrobium"*) (Johnson and Aguilera, 2016). Some *Actinobacteria* can fix CO_2 such as the facultative autotroph *Am. ferrooxidans,* the facultative anaerobe *Atn. ferrireducens* (shown to fix CO_2 only under anaerobic conditions) and *"Acidithiomicrobium"*. Others are obligate heterotrophs (*Atx. ferrooxidans, Fx thermotolerans* and *Fm. acidiphilum*), obtaining carbon solely from organic sources*.* Culture-independent analysis of 16S rRNA gene showed that species of *Actinobacteria* were abundant in bacterial communities associated with species of sponge (*Xestospongia* spp.) and were assumed to form symbiotic associations with the animals (Montalvo *et al*., 2005). Species of *Actinobacteria* have been isolated in a range of environments: acidic geothermal site in Yellowstone National Park, (USA); a copper leaching operation in New Mexico (USA), solfataric field (Japan) and a geothermal site in Iceland (Johnson *et al.,* 2009).

1.1.2.2.1 Phylum *Firmicutes*

Members of the phylum *Firmicutes* are Gram-positive bacteria, characterised by having low G+C contents in their chromosomal DNA. While most species characteristically form endospores, there are numerous species, members of *Bacillales* and *Clostridiales,* that appear to be unable to do this (Seong *et al.,* 2018). Cells are mostly rod-shaped and reproduce by binary fission. Some are motile by means of flagella, and cells have rigid or semi-rigid cell walls containing peptidoglycan (Seong *et al.,* 2018). Members are very diverse physiologically, phylogenetically, chemotaxonomically, and pathogenically, and have often been classified according to the type of cell envelope (many *Firmicutes* have an additional envelope layer, such as capsules), endospore formation and aerotolerance. The hierarchical classification of

the phylum *Firmicutes* was first studied in 1872, when the genus *Bacillus* was first reported, and since then the taxonomic hierarchy has been continuously modified and updated. Currently, this phylum consists of seven classes (*Bacilli, Clostridia, Erysipelotrichia, Limnochordia, Negativicutes, Thermolithobacteria, and Tissierellia*), 13 orders, 45 families, and 421 genera (Seong *et al.,* 2018). At the genus level, *Firmicutes* are more diverse than the phylum *Actinobacteria (*1 class, 10 orders, 56 families, and 330 genera*)* and *Bacteroidetes (*4 classes, 4 orders, 21 families, and 324 genera), but less diverse than the phylum *Proteobacteria.* (Seong *et al.,* 2018).

1.1.2.2.1.1 Extremely acidophilic *Firmicutes*

The phyum *Firmicutes* currently includes two validated genera of extreme acidophiles, *Sulfobacillus* and *Alicyclobacillus*. The genus *Sulfobacillus* comprises five validated species (*Sb. thermosulfidooxidans, Sb. acidophilus, Sb. thermotolerans, Sb. benefaciens,* and *Sb. sibiricus*) and was first described in 1979 by Golovacheva and Karavaiko (Golovacheva and Karavaiko, 1979). Members of *Sulfobacillus* exhibit highly versatile metabolic capabilities and are considered to be the most generalist of all acidophiles (Johnson, 2009). They can grow (i) autotrophically using sulfide minerals, ferrous iron, sulfur and hydrogen, as electron donors while fixing CO_2; (ii) heterotrophically where organic carbon compounds act as electron donor and carbon source; and (iii) chemolitho-heterotrophically in which inorganic electron donors are oxidized and carbon is obtained from organic materials, such as yeast extract. All *Sulfobacillus* spp. share some physiological traits, such as being extremely acidophilic (pH optima < 2.5), facultatively anaerobic (use either molecular oxygen or ferric iron as electron acceptors) and in being able to tolerate elevated concentrations of many transition metals. Although some species are mesophilic, the majority are thermo-tolerant or moderately thermophilic. *Sb. benefaciens* was reported to show superior autotrophic growth than both *Sb. thermosulfidooxidans* and *Sb. acidophilus* (Johnson *et al*., 2008), while *Sb. acidophilus* grows faster and to higher cell densities in heterotrophic conditions than *Sb. thermosulfidooxidans*. *Sulfobacillus* spp. are widely distributed in acidic geothermal springs, sulfide mine heaps and dumps, coal spoil heaps and, very often are important members of microbial communities in commercial mineral bioleaching, although they tend to be less numerous than autotrophic *Leptospirillum* spp. and *Acidithiobacillus* spp. (Johnson and Aguilera, 2016; Norris *et al*.,1996; Johnson *et al*., 2008). Strains of *Sb. benefaciens* were found as the sole *Firmicutes* present in two mineral bioleaching stirred tanks (a cobaltiferous pyrite and a polymetallic nickel–copper concentrates at 40 - 45°C, in France and South Africa, respectively) and in a bioheap leaching operation of polymetallic black schist ore, in Finland (Johnson *et al*., 2008).

Quantification of microbial communities in mine tailings in Germany, Sweden and Botswana using real-time PCR detected bacteria closely related to *Sulfobacillus* in oxidised and non-oxidised tailings in Sweden (more abundant in oxidised zone) and in oxidised zone in Botswana (though in lower abundance than *Acidithiobacillus* spp.) (Schippers *et al.,* 2010). Several stirred-tank bioreactors (maintained at 45°C) of a commercial biooxidation plant in Ghana containing sulfidic-refractory gold concentrates were dominated by *Sulfobacillus* spp. (Brierley and Brierley, 2013). Interestingly, their apparent lower tolerance to transition metals compared to some other acidophiles (e.g. reported minimum inhibitory concentrations for Cu^{2+} are 5 mM for *Sb. acidophilus*, 230 mM for *Sb. thermosulfidooxidans* and 120 mM for *Sb. benefaciens*^{T} and 500 mM for *At. ferrooxidans*^{T}; Johnson *et al.,* 2008; Hedrich and Johnson, 2013) is not reflected in their wide-spread presence in bioleaching operations, and indicates that they are able to adapt to high metal concentrations in leachates, though they may also be present as inactive spores (Watling *et al.,* 2008; Brierley and Brierley, 2013).

The first *Alicyclobacillus* was isolated from an acidic thermal environment in the U.S.A by Darland and Brock in 1971, and was named at the time as *Bacillus acidocaldarius* (Darland and Brock, 1971). This bacterium was the only obligately acidophilic thermophile member of *Bacillus* family and possessed a unique fatty acid (*ω*-cyclohexane) and hopanoid. There followed reports of the *B. acidocaldarius* isolated from other thermal and acidic environments (e.g. hot springs in Japan; Oshima *et al.,* 1981). In 1984, Cerny and colleagues reported the first ω-cyclohexane fatty acid-containing *Bacillus* isolated from spoiled apple juice and was considered to be responsible for the spoilage (Chang and Kang, 2004). This bacterium was also isolated from soil samples, and was described as a new *Bacillus* species, *Bacillus acidoterrestris*. In 1992, a novel genus was established, *Alicyclobacillus,* to accommodate the *Bacillus* species containing the unusual ω-alicyclic fatty acids in their cell membrane (Wisotzkey *et al.,* 1992). The genus classification has gone through significant modifications in the last few years with the discovery of new isolates, and currently it comprises 23 species with *Alicyclobacillus montanus* being the most recent species described (Lopez *et al.,* 2018). This bacterium was isolate from enrichments containing commercial olive oil, water and sediments from an acid hot spring in Colombia, at pH 3 and 55°C. Species of *Alicyclobacillus* stain Gram-positive (or Gram variable in old cultures), though some have been reported to stain Gram-negative (*Alb. sendaiensis;* Tsuruoka *et al.,* 2003).

Alicyclobacillus spp. can grow in a wide temperature range from 20 - 70°C, and optima in most cases range from 42 - 55°C. Some species are mesophilic, e.g. *Alicyclobacillus aeris, Alicyclobacillus ferrooxydans* and *Alicyclobacillus disulfidooxidans,* and the latter is able to grow as low as 4°C and to tolerate temperatures of up to 40°C. The majority of *Alicyclobacillus* spp. are moderately acidophiles with optima pH 3.0 - 4.5, but some can grow optimally at pH

~2 (*Alicyclobacillus tolerans* and *Alb. disulfidooxidans*), and the pH range for growth of this genus as a whole ranges from 0.5 to 6.5. In terms of metabolic capabilities, *Alicyclobacillus* spp. are considered generalists rather than specialists, being able to utilise a wide range of substrates from small molecular weight (e.g. monosaccharides) to polymeric compounds (e.g. starch). Several species are obligate heterotrophs while a minority have been reported to grow autotrophically (e.g. *Alb. tolerans* and *Alb. aeris*). Some species are able to catalyse the dissimilatory oxidation of ferrous iron and sulfur (e.g. *Alb. disulfidooxidans*, *Alb. tolerans*, *Alb. aeris*, and *Alb. ferrooxydans*). Some validated species, *Alicyclobacillus pomorum, Alb. ferrooxydans*, *Alicyclobacillus macrosporangiidus*, *Alicyclobacillus contaminans* and *Alb. aeris* do not synthesize ω-alicyclic fatty acids. Some members tolerate up to 5% (w/v/) NaCl (e.g. *Alb. acidoterrestris*). Most validated species have been classified as strict aerobes, and only few species, such as *Alb. disulfidooxidans* and *Alb. tolerans*, both of which can use ferric iron as electron acceptor, have been reported to grow in absence of molecular oxygen.

Some *Alicyclobacillus* species have been isolated from geothermal environments such as hot springs (*Alicyclobacillus tengchongensis* and *Alicyclobacillus vucanalis*), solfatara soils (*Alicyclobacillus hesperidum* and *Alb. ferrooxydans*), others from soils (*Alb. macrosporangiidus, Alicyclobacillus kakegawensis, Alicyclobacillus shizuokensis*), acid mine water (*Alicyclobacillus fodiniaquatilis*), waste water sludge (*Alb. disulfidooxidans*) and fruit juices (*Alicyclobacillus dauci, Alicyclobacillus fastidious, Alb. pomorum,* and *Alicyclobacillus acidiphilus*).

Alicyclobacillus spp. were identified in enrichments for acidophilic iron- and sulfur-oxidisers supplemented with Fe^{2+} and ZVS but not organic carbon, from both water and sediments from a slag lagoon site at a smelter (Kaksonen *et al.,* 2016). Another iron-oxidizing, moderately thermophilic, acidophilic bacterium closely related to *Alb. tolerans* was isolated from mineral spoil taken from a gold mine (Yahya *et al.,* 2008). *Alicyclobacillus* spp. and *Sulfobacillus thermosulfidooxidans* were members of a bioleaching consortium and showed to be the most resistant members (in terms of decrease in cell numbers) during the solvent extraction process of a bioleaching solution when pH decreased from 2 to 1 and during contact with organic extractants (Davis-Belmar *et al.,* 2012).

The high tolerance to heat and acidity of *Alicyclobacillus* spawned research to identify extremo-enzymes with potential biotechnological applications. For instance, a novel thermostable endoglucanase from *Alb. vulcanalis* showed lignocellulose hydrolysis in extreme conditions. The purified enzyme displayed maximum activity at 80°C and pH 3.6–4.5, indicating suitability of the enzyme for industrial application in the production of cellulosic bioethanol (Boyce and Walsh, 2015).

Alicyclobacillus spp. have been associated with spoilage of commercially-pasteurized fruit juices for the last twenty years in several countries (e.g. United Kingdom, Germany, Australia, Japan and the U.S.A.). Spoilage occurred most commonly in apple juice, though other juices and diced canned tomatoes have also been reported. Off-flavours generated in spoilage juice by *Alicyclobacillus* spp. can be divided into two groups: guaiacol and the halophenols (e.g. 2,6-dibromophenol and 2,6-dichlorophenol). Guaiacol have been identified in cultures of *Alb. acidoterrestris*, *Alb. pomorum* and *Alb. acidiphilus* and this compound has been applied in the food industry as a synthetic flavouring (Chang and Kang, 2008).

1.1.2.3 Extremely acidophilic archaea

Extremely acidophilic archaea may be found in sulfur-rich zones in volcanic areas and sulfidic deposits rich in metals, in acid mine drainage systems, macroscopic growth of streamers, slimes, mats and microbial stalactites (Golyshina *et al*., 2016a), though few acidophilic archaea have been isolated from marine environments (e.g. "*Aciduliprofundum boonei"*). Extremely acidophilic archaea include aerobic and anaerobic chemolitho-autotrophs and chemolitho-organotrophs. The most commonly used electron donors for energy conservation for chemolithotrophic species are ZVS, H_2S, Fe^{2+} and H_2; O_2, Fe^{3+} and ZVS are used as electron acceptors by this group of bacteria as a whole, though individual species show more restricted metabolic versatilities. Heterotrophic metabolism is widespread among acidophilic archaea. Some archaeal species lack cell wall which affects their cell morphology making them irregular and often variable (Golyshina *et al*., 2016b). In terms of CO_2 fixation, many acidophilic facultative autotrophs use the modified 3-hydroxypropionate/4-hydroxybutyrate cycle, in which key enzymes involved in this process have been identified in *Sulfolobales* spp., *Sg. azoricus* and *Acidianus* spp. (Golyshina *et al*., 2016a). Acidophilic archaea can be sub-divided into moderate acidophiles (e.g. species from the orders *Acidilobales* and *Thermoproteales*) and extreme acidophiles (e.g. species of the orders *Sulfolobales* and *Thermoplasmatales*).

Table 1.5. Characteristics of extremely acidophilic archaea.

Species	Metabolism[a]	T °C range (optimum)	pH range (optimum)
Members of the family *Sulfolobaceae*			
Saccharolobus solfataricus	Aerobic mixo/heterotroph Oxidation of H_2 and organic C	50-87 (85)	2-5.5 (3-4.5)
Saccharolobus shibatae	Aerobic mixo/heterotroph Oxidation of H_2 and organic C	N.D-86 (81)	N.D (3)
Sulfodiicoccus acidiphilus	Aerobic, chemoheterotroph	50-70 (65-70)	1.4-5.5 (3-3.5)
Sulfolobus acidocaldarius	Aerobic mixo/heterotroph Oxidation of H_2 and organic C	55-85 (75-80)	1.5-6 (3)
Sulfolobus metallicus	Aerobic, lithoautotroph, oxidation of Fe^{2+}, sulfidic ores and ZVS	50-75 (65)	1-4.5 (2-3)
Sulfolobus tokadii	Aerobic, chemolithotroph/ heterotroph, oxidation of ZVS and C	70-85 (80)	2-5 (2.5-3)
Metallosphaera sedula	Aerobic, facultative chemolithoautotroph, oxidation of sulfidic ores, ZVS, H_2, and C	50-80 (75)	1-4.5 (2-3)
Metallosphaera tengchongensis	Aerobic, facultative chemolithoautotroph oxidation of ZVS, sulfide minerals, and C	55-75 (70)	1.5-6.5 (3.5)
Sulfurococcus yellowstonii	Aerobic, facultative chemolithoautotroph, oxidation of ZVS, Fe^{2+},sulfide minerals, C	40-80 (60)	1-5.5 (2-2.6)
Acidianus infernus	Facultative anaerobe, chemolithoautotroph, oxidation of ZVS, sulfidic ores, H_2, and C. Reduction of ZVS.	65-95 (85-90)	1-5.5 (2)
Acidianus ambivalens	Facultative anaerobe, obligate litho-autotroph, oxidation and reduction of ZVS.	ND-87 (80)	1-3.5 (2.5)
Stygiolobus azoricus	Obligate anaerobe, chemolitho-autotroph, oxidation of H_2 and reduction of ZVS.	57-89 (80)	1.5-5 (2-2.6)
"Acidianus manzaensis"	Facultative anaerobe, chemolitho-autotroph, oxidation of ZVS, H_2, C. Reduction of Fe^{3+}.	60-90 (80)	1-5 (1.2-1.5)
Members of the order *Thermoplasmatales*			
Thermoplasma volcanium	Facultative anaerobe, chemoheterotroph, reduction of ZVS.	33-67 (60)	1-4 (2)
Cuniculiplasma divulgatum	Facultative anaerobe, organotroph	10-48 (37-40)	0.5-4 (1-1.2)
Picrophilus torridus	Obligate aerobe, chemoheterotroph	45-65 (60)	0-3.5 (0.7)
Picrophilus oshimae	Obligate aerobe, chemoheterotroph	47-65 (60)	0-3.5 (0.7)
Ferroplasma acidiphilum	Facultative anaerobe, chemolithotroph, oxidation of sulfidic ores and Fe^{2+}.	15-45 (35)	1.3-2.2 (1.7)
Acidiplasma cupricumulans	Facultative anaerobe, chemomixotroph, oxidation of sulfidic ores and Fe^{2+}.	22-63 (54)	0.4-1.8 (1.1)
Thermogymnomonas acidicola	Obligate aerobe, chemoheterotroph	38-68 (60)	1.8-4 (3)

[a] ZVS, zero-valent sulfur; C, organic carbon; N.D, not determined.

1.1.2.4 Zero-valent sulfur-reducing prokaryotes

Dissimilatory reduction of ZVS has been reported for several prokaryotes, though these do not comprise a phylogenetically coherent group within the Bacteria and Archaea domains. Prokaryotic sulfur-reducing species are distributed among nine bacterial phyla (*Firmicutes, Proteobacteria, Thermodesulfobacteria, Spirochaetes, Deferribacteres, Chrysiogenetes, Aquificae, Synergistetes* and *Thermotogae*) and two archaeal phyla (*Euryarchaeota* and *Crenarchaeota*) (Florentino *et al.,* 2016b; Rabus *et al*., 2006).

Prokaryotes able to reduce ZVS can be found in freshwater and marine sediments and terrestrial environments. Several have been isolated from extreme habitats such as hot springs, hydrothermal systems in deep and shallow seas, hypersaline lakes, acidic anoxic sediments in metal-contaminated environments and solfatara (Florentino *et al.,* 2016b; Dopson and Johnson, 2012). Species of mesophilic or moderately thermophilic ZVS-reducing prokaryotes are mostly bacteria though all extreme thermophiles are archaea. Interestingly, ZVS reduction seems a characteristic of microorganisms that thrive at high temperatures. The majority of identified ZVS-reducing bacteria grow optimally at circum-neutral pH. Although some bacterial species that reduce ZVS are also able to grow in acidic conditions (pH 3.0 - 4.5), e.g. *Desulfosporosinus acidiphilus*, *Desulfosporosinus acididurans*, *Desulfurobacterium thermolithotrophum, Desulfurella amilsii, Marinitoga hydrogenitolerans and Thermanaerovibrio velox*, they all have pH optima above 5. *At. ferrooxidans*, the most widely-studied of all acidophilic bacteria and mostly cultivated as an aerobe, was reported to reduce ZVS at pH 1.3 (Ohmura *et al.,* 2002). In contrast to the rarity of extremely acidophilic ZVS-respiring bacteria, there are several species of archaea that reduce ZVS at pH values at or below 2, such as the moderately thermophile *Thermoplasma* spp., and the extreme thermophiles *Sg. azoricus*, *Sulfurisphaera ohwakuensis* and *Acidianus* spp. (Golyshina *et al.,* 2016a).

Desulfuromonas acetoxidans was the first isolate demonstrated in a pure culture to use the dissimilatory reduction of ZVS for growth (Pfennig and Biebl, 1976). This bacterium is a neutrophilic obligately anaerobic mesophile isolated from enrichments with sulfide-containing seawater supplemented with acetate and ZVS. Organic disulfide compounds (oxidized forms of cysteine and glutathione), malate or fumarate were the only other electron acceptors used by this bacterium, and ethanol or propanol were utilised as carbon sources and electron donors. *Dm. acetoxidans* does not use sulfate, sulfite, thiosulfate or nitrate as electron acceptors.

Subsequently, many other species of ZVS reducers were characterised and shown to use other compounds as electron acceptors, including ferric iron, nitrate, and inorganic sulfur

compounds, such as thiosulfate. In addition, some prokaryotes that had been initially isolated using electron acceptors such as fumarate (e.g. *Wolinella succinogenes*), or nitrate (e.g. *Ammonifex degensii*) were later shown to grow by dissimilatory reduction of ZVS (Rabus *et al*., 2006). Some ZVS-respiring prokaryotes can also grow aerobically by oxidising complex organic substrates such as yeast extract (e.g. *Ss. ohwakuensis)* or by ZVS oxidation (e.g. *Acidianus* spp.), and therefore are classified as facultative anaerobes (Golyshina *et al.,* 2016a; Dopson and Johnson, 2012; Kurosawa *et al.,* 1998). The capacity for micro-aerobic (atmospheres containing 3 - 5% oxygen) growth has been reported for some species of ZVS-reducing bacteria, e.g. *Sulfurospirillum deleyanium, Sulfurospirillum arcachonense* and *W. succinogenes*, where the oxidation of organic compounds such as fumarate was coupled to the reduction of molecular oxygen (Finster *et al.* 1997; Tanner *et al.,* 1981). In contrast, reduction of molecular oxygen seems to be a rare trait in other sulfidogens such as sulfate-reducing bacteria, which are predominantly strict anaerobes (Rabus *et al*., 2006).

Respiration of ZVS has occasionally been reported to be used on preference to electron acceptors with more favourable (i.e. positive) redox potentials. For instance, ZVS reduction at low pH coupled to H_2 oxidation has been reported as the preferable respiratory route for an extremely acidophilic archaeon, when it grew in medium where ferric iron was also supplied. This archaeon, a strain of *Acidianus*, yielded 8-fold greater biomass when growing using the H_2/ZVS couple compared to the growth using the other two couples tested: ZVS/Fe^{3+} and H_2/Fe^{3+} (where Fe^{3+} acted as electron acceptor). This result contradicted thermodynamic calculations, which indicated that ZVS/Fe^{3+} and H_2/Fe^{3+} could yield three- and four-fold more energy per mole of electrons transferred, respectively, than H_2/ZVS. This study suggested that the biochemical machinery necessary to metabolise the H_2/ZVS couple required less energy to be assembled compared to that for the other two redox couples, therefore the preferable substrate was the one that demanded less energy for the electron transfer reactions, rather than the substrate that could potentially supply greater energy (Amenabar *et al.,* 2017).

Enrichment cultures from which the first ZVS reducers were isolated contained acetate as electron donor and ZVS as electron acceptor. Later on, these isolates were shown to also oxidise propanol and fumarate (Pfennig and Biebl 1976; Rabus *et al.,* 2006). Other organic substrates such as organic acids (e.g. lactate, propionate, pyruvate), alcohols (e.g. methanol, ethanol) and carbohydrates (e.g. glucose, cellulose, rhamnose, starch, molasses) have also been reported to be used as electron donors for sulfur-reduction. Litho-autotrophic growth of ZVS-reducers using hydrogen as electron donor has been described in some species of bacteria and archaea (Rabus *et al.,* 2006). The neutrophilic ZVS-reducing archaeon *Pyrobaculum neutrophilus* is a facultative autotroph which is able to use either CO_2 or acetate as carbon source during growth on ZVS and H_2. Several ZVS-reducing archaea grow solely

on complex organic sources such as yeast extract. Only a few isolates, such as the two hyperthermophilic neutrophiles *Thermoproteus tenax* and *Pyrococcus glycovorans,* utilize defined organic compounds (e.g. glucose) for growth by sulfur respiration. Oxidation of organic compounds coupled to ZVS-respiration may be complete, generating CO_2 as the final product (e.g. *Desulfuromonas* spp.*, Dsf. amilsii, Thermoproteus tenax)* or incomplete generating equimolar concentrations of acetic acid and CO_2 (e.g. *Sulfospirillum* spp.*, Wolinella* spp.*, Shewanella* spp. and *Pseudomonas mendocina*) (Tanner *et al.,* 1981; Rabus *et al*., 2006; Pfennig and Biebl, 1976).

The hyperthermophilic neutrophilic archaeon *Pyrococcus furiosus* grows on substrates including starch, maltose, peptone by fermentation; however, when media are amended with ZVS growth yields increase and H_2S is generated. Another archaeon, the acidophilic hyperthermophile *Acidilobus aceticus* also can grow by fermentation (using yeast extract) stimulated by ZVS, producing H_2S and acetate as by-products. The role of ZVS in this metabolism has been hypothesized as an additional electron sink that facilitates fermentation, and this phenomenon has been demonstrated in several organotrophic hyperthermophilic archaea (Prokofeva *et al*., 2000).

Zero-valent sulfur is a solid substrate with poor solubility in water, and is less accessible than soluble electron acceptors, such as sulfate. Utilisation of ZVS as electron acceptor can occur by two possible mechanisms: (i) conversion of sulfur into more hydrophilic compounds such as polysulfides (which can be formed at high temperatures, therefore favouring growth of thermophilic and hyperthermophilic species); (ii) physical attachment of microbes to ZVS, resulting in a direct conversion of ZVS to sulfide. In low pH conditions, some soluble sulfur compounds such as polysulfides and thiosulfate are unstable, decomposing into ZVS nanocrystals, sulfide and sulfur oxides. Therefore, it has been hypothesised that physical attachment to solid phase ZVS is the only possible mechanism for ZVS-respiration by acidophilic prokaryotes (Florentino *et al.,* 2016b).

Prokaryotes play a significant role in the cycling of sulfur compounds in natural and man-made environments. In anoxic conditions they catalyse both the dissimilatory reduction and the disproportionation of sulfur species (Finster, 2008; Findlay*,* 2016). Bacterial disproportionation of ZVS, thiosulfate, sulfite have been reported to have a major impact on the sulfur cycle of marine sediments (Böttcher *et al.*, 2005) and to occur in environments such as brackish and fresh water sediments, soda lakes and shallow-sea hydrothermal vents (Rabus *et al*., 2006; Frolova *et al*., 2018). Sometimes also referred as “inorganic fermentation” (e.g. by Bak and Pfenning, 1987), disproportionation occurs when sulfur species with intermediate oxidation states both donate and accept electrons. These are energy-generating redox reactions that

generate sulfate and sulfide (Bak and Cypionka, 1987). Bacteria able to carry out this metabolism are abundant in nature and distributed across the Bacteria domain, e.g. *Desulfovibrio desulfuricans* CSN; *Thermosulfurimonas marina; Desulfurella amilsii* (Frolova *et al*., 2018; Bak and Cypionka, 1987; Finster, 2008).

Disproportionation of ZVS at pH 7 is not a thermodynamically favourable reaction, having a positive ΔG^0 value (Eq. 1.7). In contrast, the disproportionation of thiosulfate and sulfite have $\Delta G^0 < 0$ at pH 7, Eq. 1.8 and 1.9, respectively (Rabus *et al*., 2006). Sulfur disproportionation was reported in cultures at pH 7 that contained iron (III) or manganese (IV) oxides, in which the oxidized metals acted as sulfide scavengers, making the reaction energetically favourable (Thamdrup *et al.* 1993; Lovley and Phillips, 1994). There are relatively few reports describing disproportionation of sulfur at low pH, e.g. Hardisty *et al.,* 2013; Florentino *et al.,* 2016a.

$$4\ S^0 + 9\ H_2O \rightarrow SO_4^{2-} + 3\ HS^- + 5\ H_3O^+ \qquad \Delta G^0 = +\ 41\ kJ\ mol^{-1} \qquad \text{Eq.1.7}$$

$$S_2O_3^{2-} + 2\ H_2O \rightarrow SO_4^{2-} + HS^- + H_3O^+ \qquad \Delta G^0 = -\ 22\ kJ\ mol^{-1} \qquad \text{Eq.1.8}$$

$$SO_3^{2-} + H_3O^+ \rightarrow SO_4^{2-} + HS^- + H_2O \qquad \Delta G^0 = -\ 59\ kJ\ mol^{-1} \qquad \text{Eq.1.9}$$

1.1.2.4.1 Acidophilic and acid-tolerant ZVS-reducing prokaryotes

While there are no reports of ZVS-reducing bacteria that grow optimally at pH < 3, dissimilatory reduction of ZVS at extremely low pH has been reported for numerous archaea species and most of them are hyperthermophiles. The thermo-acidophilic family *Sulfolobaceae* (*Crenarchaeota*) has several acidophilic species that grow by ZVS respiration. Species of the genus *Acidianus* (e.g. *Ad*. *brierleyi*, *Ad*. *infernus*, *Ad*. *ambivalens* and *Ad*. *sulfidivorans*) isolated from solfatara environments and acidic thermal springs (*Ad*. *brierleyi*) are able to grow at pH as low as 1, except for *Ad*. *sulfidivorans* where the lowest pH for growth is 0.35 (Golyshina *et al.,* 2016a). They grow optimally at pH ~ 2.0 and at temperatures ranging from 70 °C to 85 °C. While most species are facultative anaerobes that oxidise a number of substrates such as ZVS, H_2, Fe^{2+}, sulfidic ores and organic carbon, some are obligately chemolitho-autotrophs (e.g. *Ad*. *ambivalens* and *Ad*. *sulfidivorans*). Other ZVS-reducers of the family *Sulfolobaceae* belong to the genera *Stygiolobus* (*Sg. azoricus*) and *Sulfurisphaera* (*Ss. ohwakuensis*). The former is an obligate anaerobe chemolitho-autotroph that can couple reduction of ZVS to the oxidation of H_2, while the latter is a facultative anaerobe that can couple ZVS-reduction to the oxidation of complex carbon sources. The lowest and the optimum pH for growth of *Sg. azoricus* and *Ss. ohwakuensis* are 1-1.5 and 2.0-2.6, while their optimum temperatures are 80 °C and 84 °C, respectively. The family *Thermoplasmataceae* of the phylum *Euryarchaeota* contain two species of ZVS reducers: *Thermoplasma acidophilum and Thermoplasma volcanium.* They were isolated from a coal refuse pile and a submarine

solfatara field, respectively. Both are chemolithoheterotrophs, facultative anaerobes and moderate thermophiles (maximum temperature for growth 63-67 °C). The lowest pH for growth is 0.5 (*Tp. acidophilum*) and 1.0 (*Tp. volcanium*) and the optimum is 2.0. They have an absolute requirement for complex organic carbon sources. These species are also widely distributed in solfataric volcanic areas around the world (e.g. Indonesia, Italy and USA; Golyshina *et al.,* 2016a).

1.1.2.5 Sulfate-reducing prokaryotes

Sulfate is a thermodynamically stable compound and is the most abundant form of sulfur on the surface of planet Earth. Sulfate-reducing bacteria (SRB) are ubiquitous in anoxic environments and couple the dissimilatory reduction of sulfate to the oxidation of organic compounds or molecular hydrogen; they are important players in the geochemical sulfur and carbon cycles (Vigneron *et al.,* 2018; Muyzer and Stams, 2008). Early studies estimated that more than half of the organic carbon in marine sediments were mineralised *via* dissimilatory reduction of sulfate (Jørgensen and Fenchel, 1974). However, more recent reports calculated that sulfate reduction instead accounts for 12 - 29% of the oxidation of the organic carbon flux to the sea floor (Bowles *et al.,* 2014)

Sulfur compounds may be utilised by microorganisms either for dissimilative reactions (i.e. for energy conservation) or for assimilative purposes (i.e. for the synthesis of biomolecules). A variety of inorganic sulfur compounds can be assimilated into prokaryotic cells for protein synthesis. For instance, high sulfate concentrations in seawater ensures that sulfur is never a limiting factor for the growth of marine microorganisms. Sulfate must be reduced before incorporation into the protein biosynthetic pathway and very often the requirement of sulfur-containing amino acids for protein synthesis is satisfied by direct reduction of sulfate rather than uptake of pre-existing precursors. Many species of bacteria are able to reduce sulfate for biosynthetic purposes, a process that has been widely studied (e.g. Faou *et al.,* 1990). Some bacteria, however, are unable to assimilate sulfate (e.g. *Francisella* spp.) and either require thiol compounds, such as cysteine, or assimilate less oxidised inorganic sulfur compounds such as thiosulfate (e.g. the photosynthetic *Chlorobium* spp.; Faou *et al.,* 1990).

Biological dissimilatory sulfate reduction was first demonstrated in the laboratory in 1886 by Hoppe-Seyler. The first species isolated, from enrichments containing mud from sewers using malate and aspartate in 1895, was *Desulfovibrio desulfuricans* and later this strain was shown to oxidise lactate or ethanol incompletely, generating acetate (referenced in Rabus *et al.,* 2006).

Sulfate-reducing prokaryotes are distributed in several phyla within the Bacteria domain (e.g. *Proteobacteria*, *Nitrospirae*, *Firmicutes*, *Thermodesulfobacteria*) and two phyla in the Archaea

domain (*Euryarchaeota*, *Crenarchaeota*) (Müller *et al.,* 2015). Sulfate-reducing bacteria comprise a diverse group of microorganisms in terms of morphology (e.g. cocci, rods, vibrios, sarcina-like cell aggregates and multicellular gliding filaments), and exhibit very versatile redox metabolism (Rabus *et al.,* 2006; Krekeler and Cypionka, 1995). They are predominantly strict anaerobes, though many species may tolerate exposure to air for certain periods and there are also reports that some strains are able to reduce molecular oxygen; e.g *Dv. desulfiricans* strain CSN (Krekeler and Cypionka, 1995; Rabus *et al.,* 2006). Some sulfate-reducing prokaryotes may utilise sulfite, thiosulfate or ZVS generating sulfide, and sometimes alternative electron-acceptors such as nitrate, nitrite, sulfonates, iron (III), uranium (VI) and chromium (VI). Sources of electron donors for sulfate-reducers vary greatly: hydrogen, low-molecular weight organic compounds (e.g. mono- and dicarboxylic aliphatic acids and alcohols), polar aromatic compounds and hydrocarbons (e.g. selective degradation of *n*-alkanes in crude oil was detected by the thermophilic *Desulfothermus naphtae* strain TD3; Rueter *et al.,* 1994). However, sulfate reducers do not usually use polysaccharides and proteins as electron donors (Muyzer and Stams, 2008; Rabus *et al.,* 2006)

Sulfate-reducing bacteria are active in a variety of geochemical conditions such as low pH (e.g pH ~3.5, *D. acididurans*) or high pH (pH > 9.5, e.g. *Desulfonatronovibrio hydrogenovorans*), extremely low or high temperature (e.g *Thermodesulfobacterium hveragerdense*), high salt concentrations (e.g. *Desulfovibrio salinus*) and high pressure (e.g. *Pseudodesulfovibrio indicus*). Marine and freshwater ecosystems represents an important habitat for sulfate-reducing bacteria; sediments in most aquatic environments offer suitable growth conditions for sulfate-reducing microorganisms. Psychrophilic species of SRB have been isolated from cold Arctic marine sediments (e.g., *Desulfofrigus* spp., *Desulfotalea* spp.) and were attributed to be a significant fraction of the indigenous cold-adapted populations in the Arctic Ocean, where rates of sulfate reduction were comparable to measurements made in marine sediments in temperate climate areas (Knoblauch *et al.,* 1999; Sagemann *et al*., 1998). A variety of environments may offer adequate growth conditions for sulfate-reducing microorganisms including flooded soils (e.g. rice paddies), hypersaline environments (e.g. solar salterns), mud volcanos, deep subsurface environments (e.g. oil reservoirs) and industrial processes (e.g. sludge digesters, oil production plants; Rabus *et al.,* 2006). Sulfate-reducing bacteria have been extensively used in bioremediation for treatment of sulfate- and/or metal-rich effluents, as described in sections 5.1 and 6.1. They also inhabit living organisms, such as ruminants and in the human intestinal tract. In marine worms, form an intimate relationship with other microorganisms such as aerobic sulfide-oxidizing bacteria (Muyzer and Stams, 2008).

Sulfate-reducing archaea are less phylogenetically diverse than their SRB counterparts. They often inhabit hydrothermal vents, hot springs and deep, warm oil reservoirs, but fewer species have been characterised. For instance, the hyperthermophile *Archaeoglobus fulgidus,* which was isolated from a shallow submarine hot vent in the Mediterranean Sea, utilises unsaturated aliphatic hydrocarbons as electron donors coupled to dissimilatory reduction of sulfate (Khelifi *et al*., 2014). Less is known of the metabolic capabilities of sulfate-reducing archaea, but they seem less versatile than SRB, capable of coupling the reduction of sulfate (and other sulfur compounds as thiosulfate and sulfite, but not ZVS) to the oxidation of H_2, starch, peptides and aliphatic hydrocarbons.

1.1.2.5.1 Acidophilic and acid-tolerant sulfate-reducing prokaryotes

Sulfidogenesis has been reported to occur in anoxic sediments and microbial mats in low-pH environments, in which dissimilatory sulfate reduction is, in most cases, the main source of sulfide. Sulfate is usually present in elevated concentrations in acidic environments associated with the oxidation of ZVS and sulfide minerals, and can diffuse into anoxic sediments (Johnson and Hallberg, 2008). Studies have found that in many acidic lakes (pH ~3) sediments are less acidic than the overlying water bodies, probably due at least in part to biosulfidogenesis. This also allows sulfate reduction to be mediated in more neutrophilic microsites (Koschorreck, 2008). However, in metal-rich sediments such as found in abandoned mining sites, hydrogen sulfide generated by sulfate reduction results in the precipitation of chalcophilic metals such as copper and zinc that have small solubility products, and this generates protons and helps to maintain the acidity of anoxic zones (Johnson, 2016).

Sulfate reduction becomes more thermodynamically favourable as pH decreases. The redox potential E^0 of SO_4^{2-}/HS^- at pH 7.0 is - 217 mV and it becomes more positive as pH decreases, e.g. at pH 3.0 E^0 is +75 mV, and therefore a more favourable electron sink (Johnson and Hallberg, 2008). Although there is no thermodynamic impediment for sulfate reduction at low pH, other factors may affect this metabolism in acidic conditions. Metabolic products of dissimilatory sulfate reduction, such as H_2S and small molecular weight aliphatic acids, are potentially inhibitors at low pH. Sulfide is toxic to all microorganisms because it reacts with metal ions and other functional molecules in the cell (Koschorreck, 2008). In addition, acidic waters are frequently characterised by having high concentrations of sulfate and dissolved iron but being carbon limited, and thus sulfate reducers have to compete with other microorganisms for electron donors (e.g. heterotrophic iron-reducing prokaryotes). When acidic systems are supplemented with organic substrates (e.g. H_2, acetate, lactate, ethanol, glucose and complex substrates) sulfate reduction can be initiated or enhanced (Koschorreck, 2008).

The genus *Desulfosporosinus* comprises a group of strictly anaerobic, rod-shaped, spore-forming Gram-positive sulfate-reducing bacteria. Several reports have shown that acidophilic strains of this genus were readily enriched and often isolated from sediments from acidic environments, e.g. Tinto River sediments and coal mine-derived acidic mine drainage (Sanchez-Andrea *et al*., 2013; Senko *et al.,* 2009). One such example is strain *Desulfosporosinus* sp. I2, which was isolated from low pH (pH 2) enrichments containing sediments from oxic layers of a pool located in tailings of an abandoned gold mine in Siberia. Strain I2 was reported to grow at 142 mM Cu^{2+} (initial concentration) and at pH ranging from 1.7 to 7.0, with maximum cell numbers and the shortest lag phase at pH 2.6 (initial pH). It is important to note that because the medium used was not buffered, growth of strain I2 increased the culture pH (sulfate reduction is a proton-consuming reaction at pH < 7) and lowered Cu^{2+} concentrations (due precipitation of CuS). Therefore, final pH values of cultures of strain I2 ranged from 4 to 5 (Mardanov *et al.,* 2016).

The earliest description of an acidophilic sulfate-reducing bacterium, isolate M1, was by Sen and Johnson (1999). This was later categorised as the type strain of *Desulfosporosinus acididurans* (Sanchez-Andrea *et al.,* 2015). A co-culture of *D. acididurans*^{T} and the acetate-consumer *Acidocella aromatica* was grown bioreactor at a fixed pH (3.7) with glycerol as electron donor and zinc as a sink for H_2S. Acetate produced from the incomplete oxidation of glycerol by *D. acididurans* was metabolised by *Ac. aromatica*, which in return produced H_2 that was consumed by *D. acididurans* (Kimura *et al*., 2006). Another moderate acidophile, *D. acidiphilus,* isolated from a decantation pond in a mining site in France, had a pH growth minimum of 3.6 and optimum of pH 5.2. This bacterium can use H_2 and organic compounds such as glycerol and pyruvate as electron donors coupled to the reduction of sulfate (Alazard *et al.,* 2010). To date, *D. acididurans* and *D. acidiphilus* are the only species of acidophilic SRB to have been fully described and validated.

Sulfate reduction mediated in low pH bioreactors has been used to remediate acid mine drainage at laboratory-scale (described in section 5.1). Besides *D. acididurans*, two other acidophilic SRB, coded CEB3 and CL4, were isolated from these (Rowe, *et al.,* 2007; Ñancucheo and Johnson, 2012). Both isolates belong to the family *Peptococcaceae* and share 97% similarity of their 16S rRNA genes. *Peptococcaceae* sp. CEB3 and *D. acididurans* were usually the SRB detected in greatest abundance inthese studies. For instance, in a continuous flow low pH sulfidogenic bioreactor operated at pH values between 4 and 5 and temperatures 30 and 45°C, *D. acididurans* was the predominant sulfidogen detected when bioreactor was operated at pH 5 and 30°C while *Peptococcaceae* sp. CEB3 dominated at pH 4 and 45 °C (Santos and Johnson, 2016). However, it remains the case that relatively few SRB that are

metabolically active in acidic (pH < 5) liquors have been isolated and described, and those that have been are moderately rather than extremely acidophilic.

1.2 Application of acidophilic prokaryotes in biohydrometallurgy

Modern metal mines are mainly characterised by large footprints (especially opencast mines), consumption of large amounts of energy (hauling rocks, comminution, smelting), use of noxious chemicals such as in froth flotation to concentrate target metals, large-scale production of carbon dioxide (smelting) and generation of a vast amounts of wastes (rock dumps and mineral tailings; Johnson, 2018). With the intensive mining of metals in the last 50 years, most of the accessible high-grade ore bodies have been exploited and the demand for each of the seven major metals (iron, manganese, aluminium, copper, nickel, zinc, and lead) is anticipated to continue to increase over the next 30 years (Elshkaki *et al.,* 2018). This is driving the use of lower grade primary ores by the mine industry and serious consideration of the need to the exploit alternative sources of metals, such as marine and deep buried deposits (Johnson, 2018). Therefore, technologies that offer lower "environmental costs", but still are economically viable and effective to meet future demand, are required.

Biomining is a technology that uses microorganisms (mainly acidophilic prokaryotes) to promote the extraction and recovery of metals from ores and metal-containing waste (Johnson, 2010). It is a general term that includes both bioleaching and biooxidation processes, which are well-established technologies for extracting base and precious metals mainly from sulfidic primary ores (reduced minerals; Brierley and Brierley, 2013; Johnson, 2010). These biotechnologies are a subset of hydrometallurgy, and compete with hydrometallurgical processes (e.g. pressure oxidation) and pyrometallurgy (roasting and smelting). Biomining has important advantages over conventional mineral extraction and processing in situations such as: (i) applications where pyrometallurgical processes cannot be applied due the nature of the mineral concentrate (e.g. large amounts of arsenic-bearing minerals and complex polymetallic ores); (ii) processing low-grade/ run-of-mine ores (chiefly copper) and (iv) biooxidation of refractory gold ores (Johnson, 2018). Biomining operations currently account for an estimated global production of 15% of copper and 5% of gold; other metals such as nickel and zinc are also targeted in biomining operations though they are currently produced in lower amounts (Brierley and Brierley, 2013). Biotechnologies that use acidophilic prokaryotes to process electronic waste and to recover metals from process waters and waste streams in some active and in abandoned mines are currently being investigated (Bryan *et al.,* 2015; Hedrich and Johnson, 2014; Santos and Johnson, 2016).

1.2.1 Bioprocessing of mineral ores

1.2.1.1 Oxidative bioprocessing

Oxidative mineral bioprocessing involves the solubilisation of sulfide minerals in acidic liquors by acidophilic microorganisms. Bioleaching and biooxidation operate under this principle and use similar consortia of microorganisms. However, while in bioleaching the target metal(s) is solubilized during bio-processing, in biooxidation the oxidative dissolution is used to remove minerals that obstruct the target metal, therefore a second process using chemical extractants is required to solubilise the metal (e.g. recovery of gold from refractory gold ores; van Aswegen *et al.,* 2007)

The first bioleaching operations were developed to extract copper from mine waste, a process termed “dump leaching”. This process usually involves using waste (run-of-mine; large rocks) stacked into very large piles (> 100 m high), irrigated with diluted sulfuric acid. The acid is used to stimulate indigenous iron- and sulfur-oxidizing prokaryotes, since in these operations usually do not use direct inoculation. Copper-rich liquors (termed as pregnant leach solutions; PLS) draining the dumps are collected in ponds and copper is recovered, usually by cementation. Heap bioleaching was a later development of this engineering process (Fig. 1.3) that consist of uncrushed, crushed or crushed and agglomerated ores stacked into smaller height piles (heaps) which are usually 2-10 m height, and can be stacked on top of each other (“lifts”) such as at Escondida coper mine in Chile. Like dumps, heaps are also irrigated, but in addition are often inoculated with acidophilic microorganisms and aerated (oxygen and carbon dioxide are required for the bioleaching microorganisms). Impermeable membranes are placed underneath heaps to avoid infiltration of PLS to the underlying ground. Heap bioleaching operations often use solvent extraction coupled to electrowinning (SX-EW) to recover the target metal(s) from the PLS. The metal-depleted raffinate solutions generated after SX-EW are recirculated through the heaps via a network of pipes. These solutions may be combined with liquor from the inoculating pond containing acidophilic consortia of mineral-oxidisers. Bio-heaps are usually covered with a waterproof thermal blankets to maintain heat and moisture (Fig. 1.3).

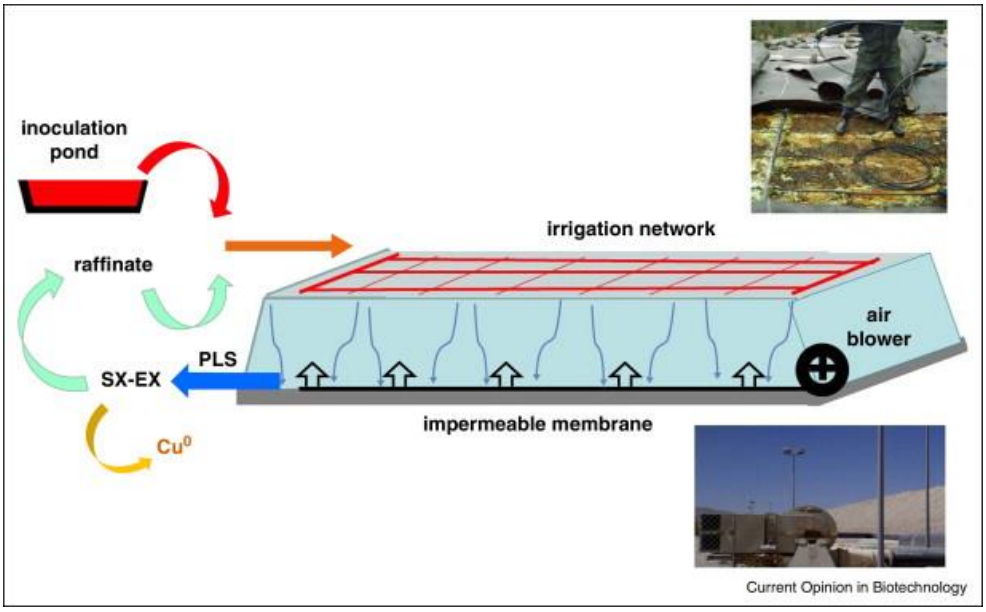

Figure 1.3. Schematic of a heap bioleaching operation. The photograph images show examples of an irrigation system and a thermal cover (top) and an air blower fan (bottom) used in heap bioleaching operations (figure origin: Johnson, 2014).

The reactions that underline the (bio)chemistry of bioleaching have been the focus of much research during the past 30 years and the sulfur chemistry involved in the leaching mechanism has been resolved (Schippers *et al.,* 1996; Schippers and Sand, 1999). Sulfide minerals can be categorised as either "acid-insoluble" (e.g. FeS_2) or 'acid-soluble' (e.g. $Fe_{(1-x)}S$, where x= 0 - 2), sphalerite (ZnS), chalcopyrite ($CuFeS_2$); Vera *et al.,* 2013). Ferric iron is the primary oxidant for both acid-soluble and acid-insoluble sulfide minerals, while H_3O^+ degrades only the former.

In bioleaching, the oxidation of the sulfur moiety in metal sulfides occurs by an "indirect mechanism" (a direct enzymatic oxidation has not yet been demonstrated; Sand *et al.,* 1995). This mechanism consists of the attack of ferric iron (an abiotic reaction producing ferrous iron and soluble sulfur intermediates) combined with the biological oxidation of both ferrous iron (regenerating Fe^{3+}, Eq. 1.5) and sulfur intermediates (which are also attacked by Fe^{3+}) (Sand *et al.,* 1995). The oxidative dissolution of "acid-insoluble" sulfide minerals is thought to proceed by "the thiosulfate pathway". In this, after the initial attack of Fe^{3+}, the sulfur moiety is oxidised first to thiosulfate (Eq. 1.4), which is unstable in the bioleaching conditions, and is oxidised to tetrathionate (Eq.1.10), and then is decomposed in a series of reactions (Eq. 1.11 -1.15) to other sulfur compounds such as ZVS, sulfite, trithionate and pentathionate (Schippers *et al.,* 1996). The sulfur compounds generated are oxidised either chemically or biologically (coupled to the reduction of oxygen or ferric iron) to sulfate. The thiosulfate pathway is shown in the Figure 1.4 and summarised in Eq. 1.16:

$$2\ Fe^{3+} + 2\ S_2O_3^{2-} \leftrightarrow 2\ FeS_2O_3^{+} \rightarrow 2\ Fe^{2+} + S_4O_6^{2-} \qquad \text{Eq.1.10}$$

$$S_4O_6^{2-} + 2\ H_2O \rightarrow HSSSO_3^{-} + SO_4^{2-} + H_3O^{+} \qquad \text{Eq.1.11}$$

$$S_3O_3^{2-} + S_2O_3^{2-} + 0.5\ O_2 + H_3O^{+} \rightarrow S_5O_6^{2-} + 2\ H_2O \qquad \text{Eq.1.12}$$

$$S_3O_3^{2-} + S_4O_6^{2-} \rightarrow S_2O_3^{2-} + S_5O_6^{2-} \qquad \text{Eq.1.13}$$

$$S_3O_3^{2-} + 1.5\ O_2 \rightarrow S_3O_6^{2-} \qquad \text{Eq.1.14}$$

$$4\ S_3O_3^{2-} \rightarrow S_8 + 4\ SO_3^{2-} \qquad \text{Eq.1.15}$$

$$S_2O_3^{2-} + 8\ Fe^{3+} + 15\ H_2O \rightarrow 8\ Fe^{2+} + 2\ SO_4^{2-} + 10\ H_3O^{+} \qquad \text{Eq.1.16}$$

The sulfur moiety in “acid-soluble” sulfide minerals (“MS”) is oxidised to ZVS *via* polysulfides (H_2S_n, $n \geq 2$) and this mechanism is referred to as “the polysulfide pathway” (Eq. 1.17 and Eq. 1.18; Vera *et al.,*2013; Schippers and Sand, 1999). Zero-valent sulfur generated is chemically inert under bioleaching conditions and it can either accumulate around the oxidising mineral or be oxidised to sulfate by acidophilic prokaryotes (Eq. 1.1; aerobically or anaerobically).

$$MS + Fe^{3+} + H_3O^{+} \rightarrow M^{2+} + Fe^{2+}\ 0.5\ H_2S_n + H_2O \quad (n \geq 2) \qquad \text{Eq.1.17}$$

$$0.5\ H_2S_n + Fe^{3+} + H_2O \rightarrow S_8 + Fe^{2+}\ H_3O^{+} \qquad \text{Eq.1.18}$$

The mineralogy, the presence of oxidants and the pH affects the formation of sulfur compounds intermediates in the two reaction pathways (Vera *et al.,* 2013). Prokaryotes can catalyse oxidative dissolution of sulfide minerals in two ways: non-contact (carried out by planktonic cells) and contact (the oxidative dissolution occurs within the extracellular polymeric substance, i.e. in the interface of the cell with the mineral; Sand *et al.,* 2001).

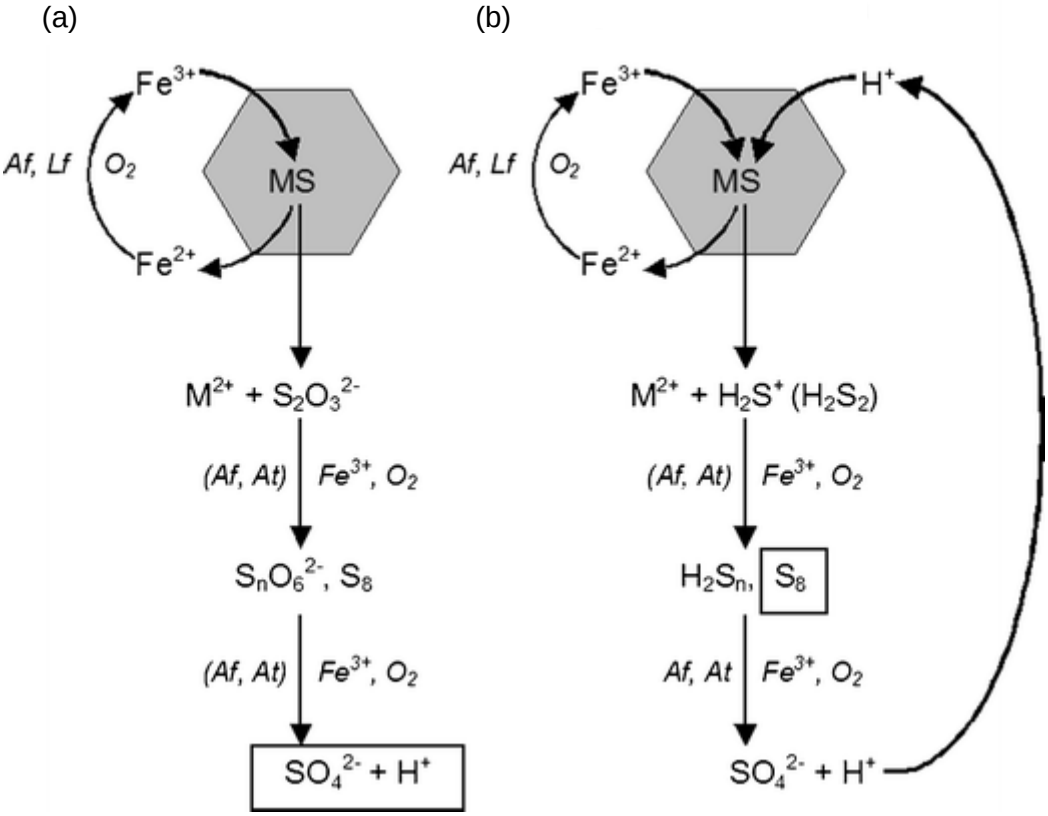

Figure 1.4. Mechanisms involved in the oxidative dissolution of metal sulfides, as mediated by two well-characterised acidophiles, *At. ferrooxidans* and *L. ferrooxidans*: (a) "the thiosulfate pathway" and (b) "the polysulfide pathway". "*Af*" refers to *Acidithiobacillus ferrooxidans*, "*Lf*" to *Leptospirillum ferrooxidans* and "*At*" to *Acidithiobacillus thiooxidans. Af* and *Lf* catalyse the dissimilatory oxidation of Fe^{2+} (recycling Fe^{3+}), while *At* and *Af* can oxidise the sulfur compounds generated in the reactions of both pathways. For reactions that are predominantly abiotic, sulfur-oxidising bacteria are indicated in brackets due to their minor contribution. The main reaction products of each mechanisms are indicated in boxes: (a) sulfuric acid and (b) ZVS. Equations displayed in each mechanism are not stoichiometric (figure origin: Vera *et al*., 2013).

1.2.1.2 Reductive mineral bioprocessing

Reductive bio-processing of minerals, sometimes referred to as "biomining in reverse gear" (Johnson and du Plessis, 2015), involves using acidophilic bacteria to catalyse the solubilisation of oxidised iron and manganese minerals, such as goethite and asbolane. This process operates on the opposite principle to conventional bioleaching operations, though both operate at low pH (Hallberg *et al.,* 2011). Currently, commercial-scale bioprocessing of minerals is mainly limited to sulfidic ores (reduced ores). However, several metals with commercial value are also found in oxidised ores, and these represent very large and more accessible mineral reserves for some metals such as nickel (Johnson and du Plessis, 2015).

Oxidised ore bodies (e.g. laterites) are not amenable to oxidative dissolution processing, and thus they are usually processed using pyrometallurgy or hydrometallurgical pressure leaching methods. Limonitic laterite ores represent important reserves of nickel and cobalt due its widely distribution and abundance on Earth lithosphere, especially in low latitude countries (e.g. Brazil, Philippines; Johnson, 2018). These ores contain predominantly oxidised iron and manganese minerals, and generally contain 1–1.5% (by weight) of Ni, mostly associated with goethite (FeO(OH)).

Reductive mineral dissolution can be catalysed by facultatively anaerobic chemolithotrophic bacteria in acidic liquors and the main reaction involved is the dissimilatory reduction of ferric iron present in the mineral phase of the ore that results in the degradation of the ferric mineral and release of the associated metal (Hallberg *et al.,* 2011). The ferrous iron generated can also promote the reductive dissolution of manganese (IV) minerals at low pH, leading to the release of associated metals such as cobalt, but bacteria such as *At. ferrooxidans* can also directly reduce manganese (IV) to manganese (II) (Smith *et al*., 2017). An important difference between oxidative and reductive dissolution processes is that in the former the mineral phase itself is the energy source (reduced sulfur and/or ferrous iron) for prokaryotes catalysing the process, which is not the case in the latter. Reductive mineral dissolution requires the addition of an external energy source (electron donor) which can be either organic or inorganic, depending on the bacteria carrying out iron reduction (Hallberg *et al.,* 2011). Acidophilic bacteria that couple the oxidation of ZVS to the reduction of ferric iron in the absence of oxygen (e.g. *Acidithiobacillus ferrooxidans*) have been applied successfully in the reductive dissolution of limonitic nickel ores (Fig. 1.5; Hallberg *et al.,* 2011). Extraction of nickel from this ores involves two main reactions: (i) abiotic acidic dissolution of goethite (Eq. 1.20) and (ii) reduction of ferric iron catalysed by acidophilic bacteria (Eq. 1.19). Studies have also demonstrated the effectiveness of anaerobic reductive bioleaching in extracting cobalt, which is mainly associated with the manganese (IV) minerals (Smith *et al*., 2017).

$$S^0 + 6\ Fe^{3+} + 12\ H_2O \rightarrow SO_4^{2-} + 6\ Fe^{2+} + 8\ H_3O^+ \qquad \text{Eq. 1.19}$$

$$FeO(OH) + 2\ H_3O^+ \rightarrow Fe^{3+} + OH^- + 3\ H_2O \qquad \text{Eq. 1.20}$$

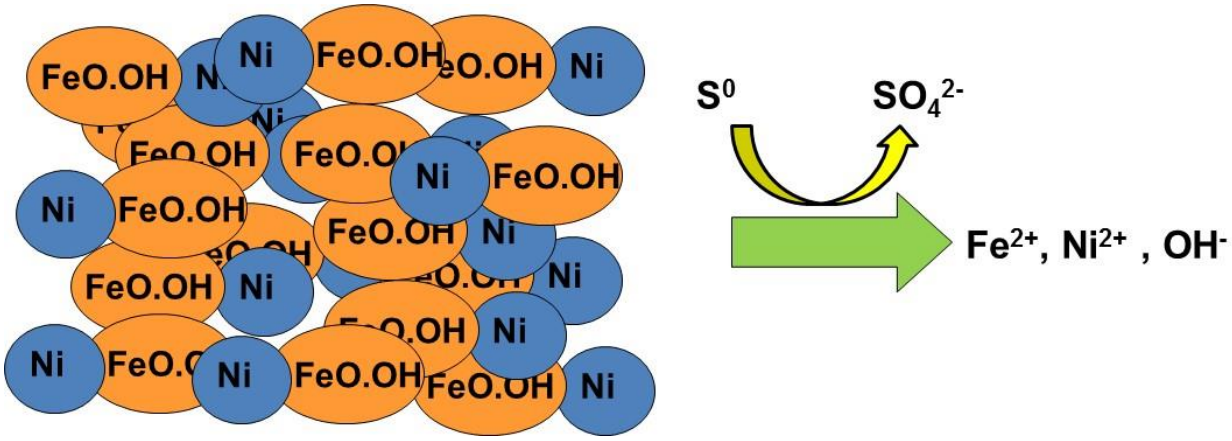

Figure 1.5. Schematic representation of the reductive dissolution of a nickel limonite in which sulfur acts as the electron donor for acidophilic bacteria (figure origin: Johnson and du Plessis, 2015)

Reductive mineral dissolution is an alkali-generating reaction, as shown in equation 1.20, and as a consequence one of the main operational cost is the sulfuric acid required to maintain the pH within optimum range for the process. Since some acidophilic bacteria can generate sulfuric acid by the oxidation of ZVS (Eq. 1.1) in the presence of oxygen, the use of alternated cycles of active aeration (to promote acid production) and anoxic conditions (to enable reduction of ferric iron) has been suggested to avoid this cost (Johnson, 2018).

A study of Marrero *et al*. (2017) used a different approach to the reductive dissolution of laterite overburden by operating a bioreactor supplemented with ZVS and inoculated with *At. thiooxidans* at pH 0.8 in aerobic conditions. Results from this study showed that the aerobic reactors (inoculated or not with *At. thiooxidans*) exhibited higher ferric iron release from the laterite overburden than the corresponding anaerobic bioreactors. In addition, the aerobic reductive dissolution using *At. thiooxidans* was more effective in extracting total and ferrous iron, manganese, cobalt and nickel than the anaerobic reductive dissolution using *At. ferrooxidans*. The study suggests that the higher rates of ZVS oxidation together with higher turnover of reduced inorganic sulfur compounds (in particular higher thiosulfate concentration generated by chemical reaction with oxygen or biological ZVS oxidation) explain the higher dissolution rate of the laterite overburden in aerobic conditions, considering that ferric iron is chemically reduced by thiosulfate and other sulfur compounds.

1.2.2 Remediation of mine-impacted waters

Microbially-catalysed dissolution of metal sulfides (as described in section 1.1.1.2) occurs in abandoned mine sites throughout the world. Metal-rich drainage waters, commonly known as acid mine drainage (AMD) due to their characteristic low pH (which can be < pH 3), is a significant source of pollution and the removal of metals from these effluents combined with neutralisation of their acidity, is required to protect the environment. Total acidity in AMD derives from both protons (H_3O^+) and as a result of hydrolysis of soluble metals (iron, aluminium and manganese). Alkalinity can be generated where minerals are hosted in carboniferous strata, forming mainly bicarbonate (HCO_3^-) due to dissolution of basic minerals (e.g. calcium carbonates), and the effluents draining those mining sites can develop neutral-to-alkaline pH if net alkalinity exceeds net acidity. Potentially valuable base metals dissolved in AMD have the potential to be recovered and recycled, though this has not traditionally been a priory in AMD remediation.

Different technologies have been developed to remediate the impact of metal-rich effluents on receiving streams and surrounding environment, referred to as “active” and “passive” treatments. The former usually require intensive maintenance and input of consumables, while the latter require, in theory, lower maintenance and other costs. Both of these approaches can also subdivided into biological and abiotic systems.

1.2.2.1 Passive remediation systems

Anoxic limestone drains (ALD) are sometimes used for passive treatment of AMD. These consist of buried beds of limestone ($CaCO_3$) constructed to receive anoxic acid mine drainage, in which alkalinity is progressively added through dissolution of the limestone (Johnson and Hallberg, 2005). The advantages of this system is the low cost of construct and maintenance; in terms of disadvantages, its effectiveness depends on the geochemistry of the drainage waters (Watzlaf *et al.,* 2000). Presence of high concentrations of dissolved oxygen, iron (III) and aluminium reduces greatly the neutralising capacity of the system, due to the build-up of hydroxide precipitates that progressively impair the permeability of ALDs (Watzlaf *et al.,* 2000). An ALD system was used to remove Zn as smithsonite ($ZnCO_3$) from hard, circum-neutral pH mine water was reported to have low efficiency, with only 22% of zinc removal (Nuttall and Younger, 2000)

Aerobic wetlands are used to treat net alkaline iron-rich mine waters and are based on the oxidation of ferrous iron (Eq. 1.5) and subsequent hydrolysis of ferric iron which is proton-generating reaction (Johnson and Hallberg, 2005). Aerobic wetlands may be also used to remove iron from AMD in combination with another treatment (e.g. ALD) that generate alkalinity. Precipitated metals and metalloids accumulate in the sediments of aerobic wetlands,

and as time progresses these systems reach their maximum capacities to retain metals and require excavation and disposal in suitable landfill sites.

Some passive bioremediation technologies to treat AMD rely on the abilities of microorganisms to generate alkalinity and immobilise metals, although sometimes limestone also is incorporated into the system. Iron and sulfate are often highly abundant in mine drainage, and while the reduction of soluble iron does not generate alkalinity, the dissimilatory reduction of solid phase (crystalline and amorphous) ferric iron does (Eq. 1.21). These and the reduction of sulfate (Eq. 1.22) are the main alkali-generating biological processes in passive systems (Johnson and Hallberg, 2005). Example of these systems are anaerobic compost bioreactors and permeable reactive barriers that have been used in full-scale treatment systems (Younger *et al.,* 2003). Important aspect of this systems is that they are affected by seasonal variation, which makes their performance less predictable. In addition, their requirement for land area may not be feasible.

$$Fe(OH)_3 + 3\ H_3O^+ + e^- \rightarrow Fe^{2+} + 6\ H_2O \qquad \text{Eq. 1.21}$$

$$SO_4^{2-} + 10\ H_3O^+ + 8\ e^- \rightarrow H_2S + 14\ H_2O \qquad \text{Eq. 1.22}$$

Compost bioreactors and permeable reactive barriers (PRBs) are based on the same reductive principles and differ in terms of their construction: the former are established close to the land surface and the latter are placed underground in order to intercept and treat contaminated ground waters. These system use an organic matrix that donate electron donors to the reductive reactions occurring (iron and sulfate reduction), therefore their life-span is limited by the availability of electron donor. The organic material used is usually a mixture of biodegradable materials (e.g. manure, spent mushroom compost; composted municipal waste) and recalcitrant constituents (e.g. sawdust, peat), in which the latter are applied to act as a long-term provider of substrates for microbially-catalysed reactions (Johnson and Hallberg, 2005). Limestone can be added to both systems to enhance alkalinity generation and metals are removed as sulfides, hydroxides and carbonates by adsorption to the organic matrix. Compost bioreactors that incorporate limestone gravel beds are referred to as reducing and alkalinity producing system (RAPS; Fig 1.6) (Younger *et al*., 2003).

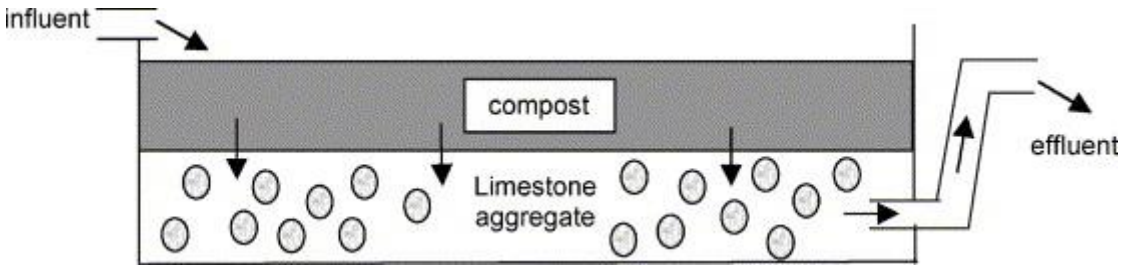

Figure 1.6. Design of a reducing and alkalinity producing system (RAPS) (figure origin: Johnson and Hallberg, 2005). When AMD flow through the compost matrix dissolved oxygen is removed and alkalinity is generated in both compost and limestone gravel bed.

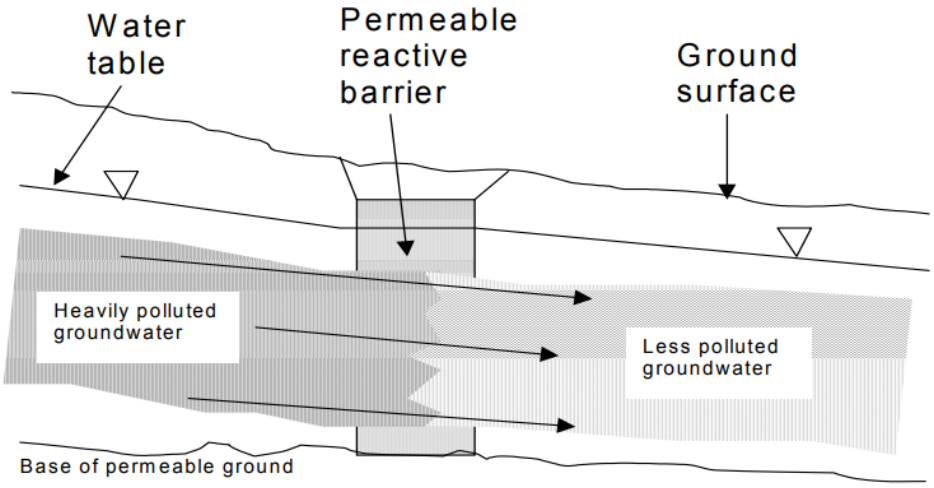

Figure 1.7. Schematic representation of a permeable reactive barriers (PRBs) (figure origin: Younger *et al*., 2003)

1.2.2.2 Active remediation systems

Abiotic active remediation approaches often involve adding an alkaline compound such as lime (CaO) in combination with intense aeration to promote oxidation of ferrous to ferric iron. The metal precipitates formed occur mainly as carbonates and hydroxides, and to precipitate all transition metals present (including manganese) the pH may have to be increased to > 8. The precipitates are usually coagulated with a chemical flocculating agent partially dewatered to form high density sludge (HDS). While this approach is both proven and effective, it is expensive, and immobilised metals with commercial value cannot be recycled and the HDS generated (oxidised mixture of potentially toxic transition metals, metalloids and often arsenic) need to be transferred to landfill sites that are designated to receive hazardous wastes (Johnson and Hallberg, 2005).

Active biological treatment systems utilise sulfidogenic bioreactors and iron bio-oxidation and precipitation bioreactors to remediate mine waters and, in some cases, to recover dissolved metals. In the latter case, mine waters are considered to be potential resources rather than pollution problems.

Bioremediation of extremely acidic (pH ~2) metal-rich wastewater using a modular system to promote the bio-oxidation and selective precipitation of iron was reported by Hedrich and Johnson (2012). The modular system consisted of three units: (i) a bioreactor inoculated with the autotrophic ferrous iron-oxidiser "*Fv. myxofaciens"*, (ii) a schwertmannite precipitation reactor (where alkali and a flocculating agent were added) and (iii) a packed-bed reactor also inoculated with "*Fv. myxofaciens"* where the remaining ferrous iron was oxidised (Fig. 1.8). The mine water containing initially 280 mg L^{-1} of Fe^{2+} and at end of the treatment, the processed water contained < 1 mg L^{-1} soluble iron, while the other metals present in the mine water (aluminium, copper, manganese and zinc) did not precipitate, therefore a "pure" schwertmannite was produced.

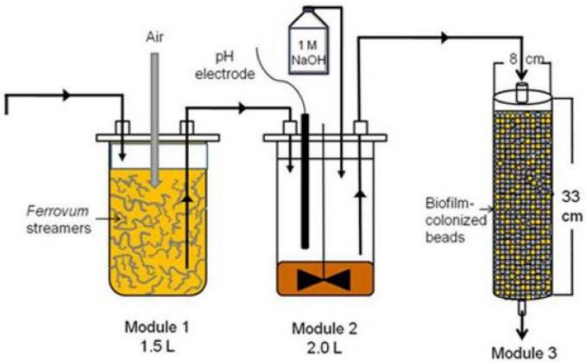

Figure 1.8. Representation of a modular system for bioremediation of mine water by selective precipitation iron as schwertmannite (figure origin: Hedrich and Johnson, 2012).

Sulfidogenic bioreactors are engineered systems based on biogenic production of hydrogen sulfide that usually use a consortium of neutrophilic sulfate- or ZVS-reducing bacteria. Biogenic generation of H_2S is also used in compost bioreactors and PRB, however sulfidogenic bioreactors have several advantages over these passive systems: (i) they are operated to optimise generation of H_2S while geochemical parameters are readily controlled, (ii) performance is more predictable and (iii) selective recovery of base metal is possible.

Currently, full-scale biosulfidogenic systems are operating in many locations around the world. The BioSulphide® process (https://www.bqewater.com/technology-solutions/metals/) is operated by the Canadian company BioteQ Environmental Technologies Inc., with commercial plants in Canada, the U.S.A, Mexico, Australia, Turkey and China. The process consists of a chemical stage and a biological stage that operate separately. Sulfide is generated in a

bioreactor (biological stage) by the reduction of ZVS using electron donors such as acetic acid, and the H_2S is transfer to an anaerobic agitated contactor containing metal-rich wastewater, comprising the chemical stage (Bratty *et al.,* 2006). At this point, selective metal precipitation can be achieved by careful manipulation of pH and sulfide concentration by harnessing the differences in solubility products of transition metal sulfides, which results in high-grade metal sulfide precipitates that are further recovered (by clarification and filtration) and then sent to a smelters to produce pure-grade metals (Fig. 1.9).

Another example of full-scale biosulfidogenic system is the THIOTEQ™ process (https://en.paques.nl/products/other/thioteqmetal) operated by Paques B.V. (The Netherlands) using similar operation mode to that of BioSulphide® consisting of two stages process (chemical and biological), that aim to recover metal from effluents with low metal concentrations. THIOTEQ™ has been implemented at the gold mine Pueblo Viejo, in the Dominican Republic, and this technology has successfully recovered high-grade copper concentrate (up to 12,000 ton per year) from the waste stream derived from gold/silver extraction. Paques B.V. operates another system, SULFATEQ™ (https://en.paques.nl/products/other/sulfateq) that removes sulfate and soluble metals from wastewater (with pH range of 2 – 8) using a two stage process, both based on microbial catalysed reactions. In the first stage a consortium of SRB reduce sulfate to sulfide (mostly as HS^-) coupled to the oxidation of H_2 or an organic compound (e.g. ethanol) and metal precipitation occurs either in the same or in a separate reactor depending of the toxicity of the wastewater (in terms of pH and metal concentration). The second stage converts the excess sulfide produced into ZVS using autotrophic sulfide-oxidising prokaryotes, which is separated from the final effluent.

Sulfidogenic bioreactors based on sulfate reduction have been applied successfully for treatment of sulfate- and metal-rich wastewater with various pH values. This process decreases concentrations of sulfate, remove metals and generates alkalinity, which are necessary to eliminate the three major issues of AMD. However, neutrophilic SRB are highly sensitive to even moderate acidity, therefore these bacteria need to be shielded against direct contact with very acidic waters. Consequently, systems used to treat very acidic mine waters include a neutralization step before the wastewater is subjected to bacterial sulfate reduction. To offset this problem, laboratory-scale continuous flow low pH sulfidogenic bioreactors containing consortia of novel acidophilic SRB have been developed to remediated AMD in a single stage process, i.e. without the need for a neutralisation step, and thus a minimising operation cost and complexity of engineered systems (Ňancucheo and Johnson, 2012; 2014; Hedrich and Johnson, 2014; Santos and Johnson, 2016). Ňancucheo and Johnson (2012) reported that a low pH bioreactor was able to selectively precipitate chalcophilic metals from

different synthetic AMD by adjusting the pH of the bioreactor pH from 3.6 to 2.2 and lowering the concentration of glycerol (the electron donor) from 5 mM to 0.7 mM. Copper sulfide was efficiently precipitated (>99%) and ~8% of zinc precipitated inside the reactor, while aluminium and ferrous iron remained in solution. Santos and Johnson (2016) reported that a low pH sulfidogenic bioreactor effectively removed copper, zinc, nickel and cobalt from a synthetic mine water while the bioreactor was operated under different pH (4 – 5) and temperatures (30 - 45 °C), confirming that the aSRB consortium used in the bioreactor was both robust and adaptable.

An integrated system was developed by Hedrich and Johnson (2014) to simultaneously ameliorate and selectively recover transition metals from acidic mine water with complex chemical composition. The process aimed to recover zinc and iron as potentially saleable products, since theses metals were present in higher concentrations. The system consisted of one module for iron bio-oxidation and precipitation (as schwertmannite) and a second module (a low pH sulfidogenic bioreactor) for selective precipitation of zinc as zinc sulfide, while other transition metals (cooper, nickel and cadmium) which were present in minor concentrations, were co-precipitated in a separate off-line vessel also as metal sulfides. In addition, arsenic was removed by co-precipitation using part of the schwertmannite, as the first step of the integrated process. The results obtained in this study showed that transition metals present in AMD with complex composition can be effectively recycled into valuable products by the integration of modular biomineralization units.

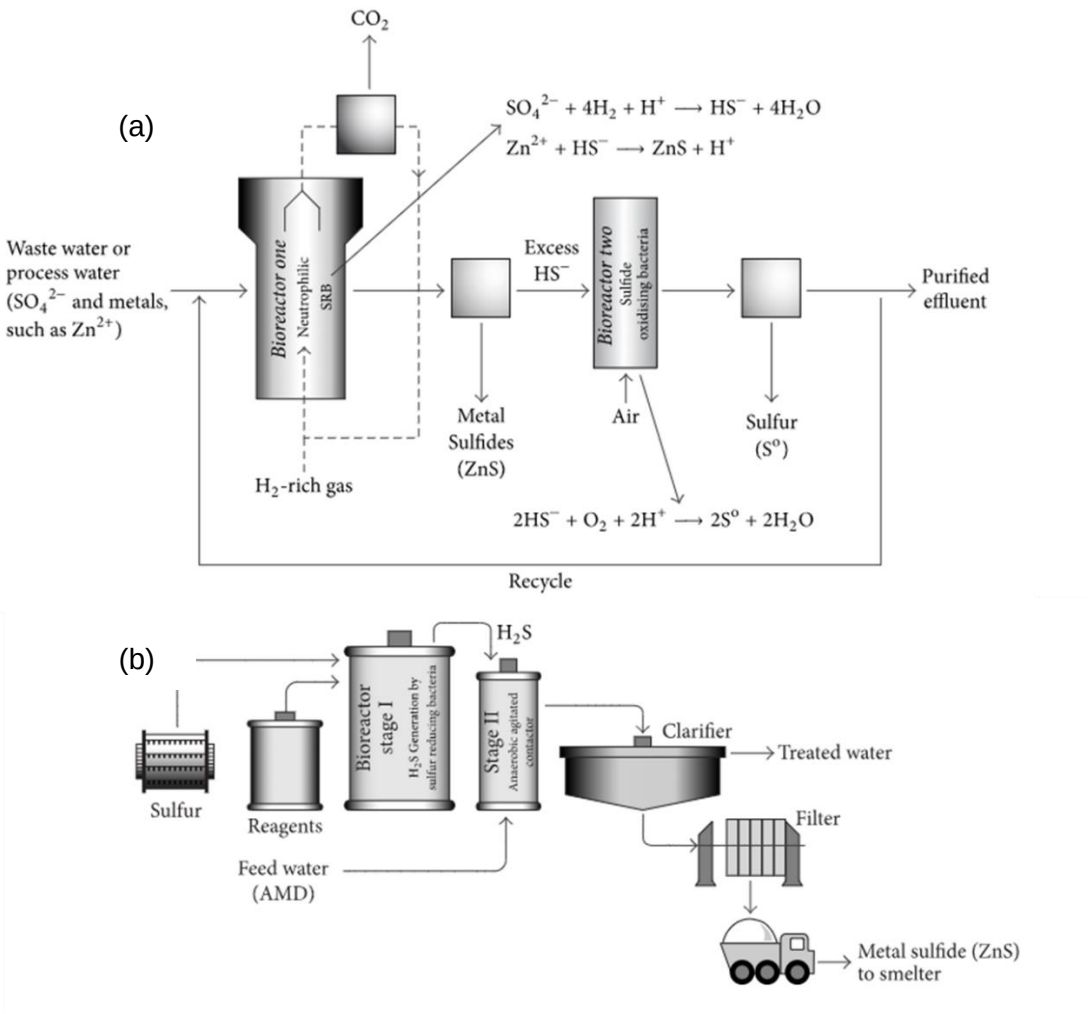

Figure 1.9. Schematic representation of (a) the SULFATEQ™ and (b) BioSulphide® processes (figure origin: Ñancucheo *et al.,* 2017).

1.3 Scope and objectives of the project described in this thesis

The current project studied the potential of novel acidophilic isolates of the phylum *Firmicutes* for applications in biohydrometallurgical processes. This involved examining physiological characteristics and using them in laboratory-scale bioreactors for remediating and recovering metals from mine waters. While some known species of acidophilic *Firmicutes* have been identified in some commercial biohydrometallurgical processes, the discovery of new genera that were the subject of research reported in this thesis, some of which have novel physiological traits, suggests that bacteria of this phylum could have important roles in new and modified biomining and bioremediation practices.

The objectives of this study were:

- To characterise a novel genus of extremely acidophilic *Firmicutes* isolated from a variety of low pH environments prior to the present study. To assess their potential for bioleaching operations.

- To isolate and characterise a novel acidophilic ZVS-reducing *Firmicute,* and to assess the dissimilatory reduction of zero-valent sulfur at low pH by selected strains of acidophilic sulfate-reducing bacteria.

- To development of a new variant of a low pH sulfidogenic bioreactor to remove transition metals from circum-neutral pH wastewaters using a consortium of novel acidophilic sulfidogens of the phylum *Firmicutes.*

- To assess the performance of the low pH sulfidogenic bioreactor for removing zinc from wastewaters from two sites draining mine tailings from abandoned lead/zinc mines in U.K.

- To examine the abiotic generation of H_2S in a low pH reactor containing ZVS and ZVI.

Chapter 2. Materials and Methods

This chapter describes materials and methods used routinely during the course of this study. Modifications to the listed methods used for specific experiments are described in corresponding chapters. Water used for microbial cultivation-based techniques and most of analytical methods was reverse osmosis (RO)-grade (RiOs^{TM}, Millipore, UK). Ultrapure-grade water (Milli-Q® A10, Millipore, USA) was used for ion chromatography, dissolved organic carbon and biomolecular analyses. All chemicals and reagents used in this project were of analytical grade quality and supplied by Fisher Scientific (UK) or Sigma-Aldrich (UK). Molecular biology reagents were supplied by GenomeLab (USA) and Promega (USA).

2.1 Microorganisms

The *Acidophile Culture Collection* at Bangor University (Bangor, Wales) provided all microorganisms used in this study, which are listed in the Table 2.1.

Table 2.1. List of bacteria used in the current research project, and corresponding national culture collection numbers.

Name (strain)	Culture Collection number
Acidithiobacillus ferrooxidans^{T}	ATCC 23270
Acidithiobacillus thiooxidans (HN)	ND
Sulfobacillus thermosulfidooxidans^{T}	DSM 9293
Acidocella aromatica^{T}	DSM 27026
Acidiphilium cryptum (SJH)	NCIB 12826
Acidibacillus ferrooxidans^{T}	JCM 31945
Acidibacillus ferrooxidans (SLC40)	ND
Acidibacillus ferrooxidans (BSH1)	ND
Acidibacillus ferrooxidans (ITV01)	ND
Acidibacillus ferrooxidans (GS1)	ND
Acidibacillus ferrooxidans (G1)	ND
Acidibacillus sulfuroxidans^{T}	JCM 31946
Acidibacillus sulfuroxidans (Y0010)	ND
Desulfosporosinus acididurans^{T}	DSM 27692
Peptococcaceae sp. (CEB3)	ND
Desulfobacillus acidavidus (CL4)	ND
Firmicute (I2511)	ND

ND: not deposited in any collection at the time of writing.

2.2 Microbial cultivation-based techniques

Most media and culture glassware used in this study were sterilized by autoclaving at 120°C for 30 minutes and stored at room temperature. Glass dispensing pipettes were sterilized by dry heat 160°C for 4 hours. Heat-labile stock solutions, i.e. 1 M ferrous sulfate (pH adjusted to 2.0 with sulfuric acid), 0.2 M potassium tetrathionate and 0.2 M sodium thiosulfate were filter-sterilised using 0.2 µm (pore size) Fisherbrand™ sterile polyethersulfone (PES) syringe filter (Fisher Scientific, UK) and stored at 4 °C.

2.2.1 Basal salt solutions

Liquid and solid medium used for cultivating acidophilic bacteria during this study were amended with either acidophile basal salts (ABS) or chloride basal salts (CBS) (Table 2.2).

Table 2.2. Composition of acidophile basal salts (ABS) and chloride basal salts (CBS) used to supplement liquid and solid medium in this study. All concentrations are shown in g L^{-1} of 50x stock solutions.

ABS salts (g L^{-1})		**CBS salts (g L^{-1})**	
$Na_2SO_4.10H_2O$	7.5	NH_4Cl	18
$(NH_4)_2SO_4$	22.5	KCl	2.5
KCl	2.5	$MgCl_2.6H_2O$	2.0
$MgSO_4.7H_2O$	2.5	Na_2HPO_4	0.3
KH_2PO_4	2.5	KH_2PO_4	2.5
$Ca(NO_3)_2.4H_2O$	0.7	$Ca(NO_3)_2.4H_2O$	0.7

2.2.2 Trace elements (TE)

Liquid and solid medium were also occasionally amended with trace elements (TE) prepared as a stock solution, concentrated 1,000-fold (Table 2.3). Each component listed was added consecutively to 800 mL of (RO)-grade water (previously adjusted to pH 2.0 with H_2SO_4), then the solution volume was made up to 1000 mL and autoclaved.

Table 2.3. Composition of trace elements (TE) stock solution (1000x concentrated).

Compounds	Concentration ($g\ L^{-1}$)
$ZnSO_4 \cdot 7H_20$	10
$CuSO_4 \cdot 5H_2O$	1.0
$MnSO_4 \cdot 4H_2O$	1.0
$CoSO_4 \cdot 7H_2O$	1.0
$Cr_2(SO_4)_3 \cdot 15H_2O$	0.5
H_3BO_3	0.6
$NaMoO_4 \cdot 2H_2O$	0.5
$NiSO_4 \cdot 6H_2O$	1.0
$Na_2SeO_4 \cdot 10H_2O$	1.0
$Na_2WO_4 \cdot 2H_2O$	0.1
$NaVO_3$	0.1

2.2.3 Zero-valent sulfur (ZVS) suspensions

Some of the experiments described in this thesis utilised sources of ZVS that were exposed to sulfur-oxidizing bacteria (microbially pre-wetted ZVS) or generated biologically (biosulfur). Oxidation of sulfur species could generate compounds such as polysulfides (S_x^{2-}) and polythionates ($S_nO_6^{2-}$) that can potentially increase the redox reactivity of ZVS (Pronk *et al.,* 1990; Findlay*,* 2016). Polysulfides and some polythionates (e.g. trithionate) are considerably unstable at low pH which affects their detection in acidic systems. In this study, the presence of those sulfur compounds was not assessed. However, polysulfides concentration can be measured, for instance, by reversed-phase liquid chromatography using an Alltech reversed-phase C18 column (Kamyshny *et al*., 2004) and polythionates by ion chromatography using an anion exchange column (Dionex IonPac AS16; Jeffrey and Brunt, 2007), in both cases using a UV detector.

Microbially pre-wetted ZVS

To overcome the hydrophobicity of powdered ZVS, a liquid medium was prepared and inoculated with sulfur-oxidizing bacteria. During growth on sulfur, bacteria generate wetting agents that allow ZVS to become more hydrophilic (evidenced by changing from floating particulate matter to sediment material; Steudel and Holdt, 1988).

One liter of medium containing ~ 300 g of sterile ZVS powder, ABS and TE, pH adjusted to 3.0 with sulfuric acid, was inoculated with *At. thiooxidans,* and incubated aerobically, shaken

at 50 rpm and 30 ºC, until all the ZVS had accumulated in the bottom of the flask. The culture was decanted and the remained ZVS was washed with RO-grade H_2O, pH 3.0 (adjusted with hydrochloric acid). The washing step was repeated several times until the concentration of SO_4^{2-} had been lowered to below detection limits (< 1.5 mg L^{-1}). The wetted ZVS was weighed and a suspension was prepared by adding RO-grade H_2O, pH 3.0 adjusted with hydrochloric acid, to a final concentration of 1 – 10% (w/v). The suspension was then placed in a borosilicate glass bottle and sterilized (110 ºC for 60 minutes).

Biosulfur

Biosulfur is a hydrophilic fine-grain sulfur "cake" produced by bacterial oxidation of H_2S to ZVS during the remediation of H_2S-rich waters and gasses, by the Dutch company Paques b.v. (Cline *et al.,* 2003).

Biosulfur "cake" (provided by Paques) was broken into small pieces and washed several times with RO-grade H_2O, pH 3.0 (adjusted with hydrochloric acid) until the concentration of SO_4^{2-} had been decreased to below detection limits (< 1.5 mg L^{-1}). Next, the biosulfur was weighed and a suspension was prepared by adding RO-grade H_2O, pH 3.0, to a final concentration of 1 – 10% (w/v). The suspension was placed in borosilicate glass bottle and sterilized (110 ºC for 60 minutes).

2.2.4 Liquid media

The liquid media used routinely during this study are listed in Table 2.4. All media were heat-sterilised and allowed to cool before the addition of heat-labile components (e.g. $FeSO_4$, $K_2S_4O_6$).Some media contained zero-valent sulfur (powder, Sigma-Adrich, UK) which was supplemented prior sterilisation (110 °C for 60 min). Likewise, pyrite powder (0.85 mm, iron disulfide, 95%, Strem Chemicals, USA) was added to selected media before sterilisation at 120°C for 30 min.

Table 2.4. Composition and pH of liquid media used in this study.

Liquid medium (code)	Components (final concentration)	pH
Fe^{2+} (**Fe**)	ABS, TE, $FeSO_4$ (0.1 - 25 mM)	1.8 - 2.0
Pyrite (**Py**)	ABS, TE, Py (1%, w/v), $FeSO_4$ (5 mM)	1.8 - 2.0
Fe^{2+}/Yeast Extract (**FeYE**)	ABS, YE (0.02%, w/v), $FeSO_4$ (0.1 - 10 mM)	1.8 - 2.0
Pyrite/Yeast Extract (**PyYE**)	ABS, YE (0.02%, w/v), Py (1%, w/v), $FeSO_4$ (5 mM)	1.8 - 2.0
ZVS/Fe (**Sm**)	ABS, TE, Fe (0.5 mM), ZVS (5%, w/v)	3.0
Tetrathionate/Fe^{2+} (**Tm**)	ABS, TE, Fe (0.5 mM), $K_2S_4O_6$ (2.5 mM)	2.0
ZVS/Yeast Extract (**Sr**)	ABS, TE, YE (0.005 - 0.01%, w/v), ZVS (1 %, w/v)	(3.0 - 4.0)[a]
ZVS/Yeast Extract (**Src**)	CBS, TE, YE (0.01%, w/v), ZVS (1 %, w/v)	(3.0 - 5.0)[b]
Glycerol/ zinc sulfate (**aSRB**)	ABS, TE, YE (0.01%, w/v), glycerol (4 mM) and $ZnSO_4$ (7 mM)	(3.5 - 4.0)[a]

[a]pH was adjusted with H_2SO_4; [b]pH adjusted with HCl;

2.2.5 Solid media

Solid media were used for microbial isolation and characterization (Table 2.5). Agarose (Type I, Sigma-Aldrich) was used at 0.5 % (w/v) as gelling agent in all solid media.

This study used only solid overlay medium, which consisted of doubled-layered gels, where the lower layer was inoculated with an acidophilic heterotroph and the top layer of sterile gelled medium (Johnson and Hallberg, 2007; Ňancucheo *et al.,* 2016). Medium solutions and agarose suspensions were autoclaved separately and combined when cooled to ~50°C. The combined medium was split into two sterile containers (~70:30, by volume). One container was inoculated with 5 mL of active culture, gently mixed, and poured immediately as a thin underlay (~ 13 mL) into Petri plates. After setting, the sterile medium from the second container (kept in a water bath at 50°C) was then poured with ~ 27 mL on the top of the underlayer. Fresh prepared overlay plates were allowed to “mature” for 24 h before inoculation. Plates were stored at 4°C and used within 2 months.

2.2.6 Growth of bacteria under different oxygen concentrations

To incubate cultures under anaerobic conditions, inoculated liquid and solid medium were placed in 2.5 L sealed jars (Oxoid™ AnaeroJar™, Oxoid Ltd., UK) containing Anaero*Gen*™ AN25 sachets (Oxoid, UK). The sachets contain activated charcoal which convert O_2 to CO_2, producing an atmosphere containing < 0.1% O_2 (by volume). To grow microorganisms under microaerobic conditions, inoculated liquid and solid medium were placed in 2.5 L sealable jars

containing Campy*Gen*™ CN25 sachets (Oxoid, UK), which resulted in atmospheres containing ~ 6% (by volume) of O_2.

Table 2.5. Composition of solid overlay media used in this study.

Medium code	Components	pH[a]	Underlay organism
iFe<u>o</u>	ABS, TE, $FeSO_4$ (20 mM)	2.5	*Acidiphilium cryptum* (SJH)
FeS<u>o</u>	ABS, TE, $FeSO_4$ (20 mM), tryptone soy broth (0.025%, w/v) and $K_2S_4O_6$ (2.5 mM)	2.5	*Acidiphilium cryptum* (SJH)
YE3<u>o</u>	ABS, YE (0.02%, w/v) and $FeSO_4$ (0.1 mM)	3.0	*Acidiphilium cryptum* (SJH)
aSRB	ABS, TE, glycerol (4 mM), YE (0.01%, w/v), $FeSO_4$ (0.1 mM), $ZnSO_4$ (7 mM) and $MgSO_4$ (4 mM).	3.5 and 4.0	*Acidocella aromatica*[T]
S^0<u>o</u> / $ZnCl_2$	CBS, TE, glycerol (5 mM), YE (0.01%, w/v), $ZnCl_2$ (1 mM) and 50 µL microbially pre-wetted ZVS (10%, w/v)[c]	4.0[b]	*Acidocella aromatica*[T]
S^0<u>o</u>	CBS, TE, glycerol (5 mM), YE (0.01%, w/v) and 50 µL microbially pre-wetted ZVS (10%, w/v)[c]	3.0[b]	*Acidocella aromatica*[T]
Sc<u>o</u>	CBS, TE, glycerol (5 mM), YE (0.01%, w/v), $FeSO_4$ (0.1 mM) and colloidal sulfur (0.5%, w/v)[d]	3.0[b]	*Acidocella aromatica*[T]

[a] pH of the liquid components before combining with the agarose suspensions (or addition of ferrous sulfate and tetrathionate); [b]pH was adjusted with HCl; [c] sterile microbially pre-wetted ZVS was spread evenly on the top of the overlay 24 h after pouring the plate; [d] colloidal sulfur (powder, Sigma-Aldrich, UK) was dissolved directly in the medium prior to sterilisation at 110 ºC for 60 minutes.

2.3 Analytical Methods

2.3.1 Electrochemical and colorimetric analysis

2.3.1.1 pH and redox potential measurements

Values of pH were measured in the laboratory using an Accumet 50 pH meter coupled to a pHase combination glass electrode (VWR®, Avantor, UK) and were calibrated regularly with standards pH 7.0, 3.0 and 1.679. Oxidation-reduction potential (redox) was measured using a combined platinum-Ag/AgCl glass redox electrode (Orion™ Metallic Combination Electrode, Thermo Scientific™, USA). The redox electrode was also coupled to the Accumet 50 pH meter, and calibrated using +440 ± 5 mV standard (ZoBell's solution: 4.22 g $K_4Fe(CN)_6$ and 4.65 g $K_3Fe(CN)_6$ dissolved in (RO)-grade water, made up to 100 mL).

2.3.1.2 Total iron and ferrous iron concentrations

The Ferrozine assay (Stookey, 1970) is a colorimetric assay used routinely to determine total and ferrous iron concentrations. Ferrozine reagent was prepared mixing 50 mL of 0.5 M HEPES Buffer (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid, adjusted to pH 7.0 with KOH) and 0.5 g of Ferrozine reagent (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine) to 500 mL of RO-grade water and stored in the dark at room temperature. Samples (50 µL) were added to 950 µL of Ferrozine reagent, mixed and absorbance was measured at 562 nm against a reagent blank using a Cecil CE1011 spectrophotometer (Cecil Instruments, U.K.). A calibration curve was constructed (Fig. 2.1) using $FeSO_4$ standards solutions at different concentrations (0.2 – 1 mM) and performing the method described above.

Total iron concentrations were measured by adding excess L-ascorbic acid (in powder) to a sample to reduce Fe^{3+} to Fe^{2+}. After the L-ascorbic acid was completely dissolved, the ferrozine assay was performed as described above. Concentrations of Fe^{3+} were calculated by subtracting from total iron the Fe^{2+} concentrations.

2.3.1.3 Copper concentrations

A colorimetric assay was used to determine soluble copper concentrations (Anwar *et al.,* 2000). The assay is based on the measurement of cuprous ion (Cu^+) concentrations, and required adding a reducing agent (hydroxylamine) to reduce Cu^{2+} ions in liquid samples to Cu^+. The assay consisted of preparing: (a) tartrate buffer: a solution of 0.5M sodium potassium tartrate (14.11 g $C_4H_4KNaO_6.4H_2O$ in 100 mL RO-grade water) adjusted to pH 5.5 with HCl; (b) bicinchoninic acid solution: the solution obtained from Sigma-Aldrich, UK (0.2%, v/v) was diluted to 0.1% (v/v) with tartrate buffer; (c) hydroxylamine solution was prepared by dissolving 10 g of hydroxylamine·HCl in 100 mL of RO-grade water. Fifty microliters of sample were

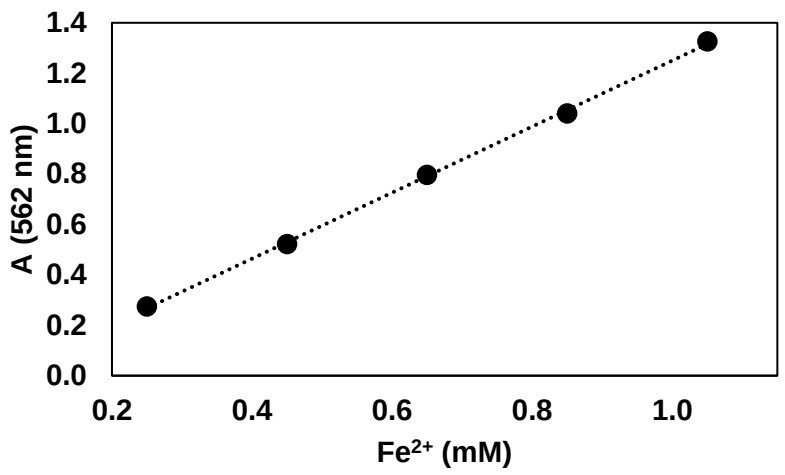

Figure 2.1. Representative calibration curve for the Ferrozine assay (Stookey, 1970). Linear correlation: *r=0.99*.

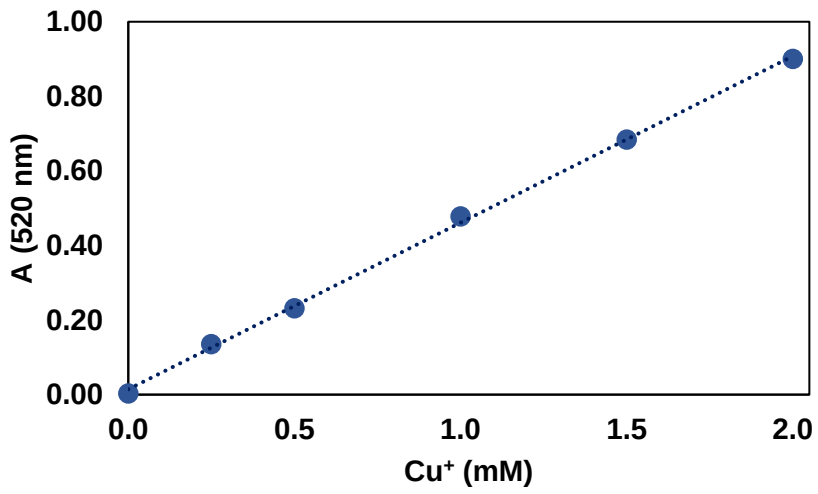

Figure 2.2. Representative calibration curve for the colorimetric copper assay used (Anwar *et al* 2000). Linear correlation: *r=0.99*.

mixed with 200 µL of hydroxylamine solution and left at room temperature to react for 10 minutes. Next, 650 µL of tartrate buffer was added to and mixed with the sample. Finally 50 µL of bicinchoninic acid was added to the mixture and vortexed for a few seconds. The absorbance at 562 nm against reagent as a blank was recorded using a Cecil CE1011 spectrophotometer (Cecil Instruments, U.K.). A calibration curve was constructed using $CuSO_4$ solution with concentrations ranging from 0 to 2 mM and performing the method described above (Fig 2.2).

2.3.1.4 Manganese concentrations

To determine concentrations of soluble manganese, a colorimetric assay was used based on that described by Mariner *et al.* (2007). The following solutions were prepared: (i) FAD solution (5 mL formaldehyde solution 36% v/v mixed to 10 g analytical grade hydroxylamine·HCl (Sigma-Aldrich, UK) and RO-grade water to a final volume of 45 mL). (ii) NH_4-buffer pH 9 (7 g of ammonium chloride, 60 mL of ammonium hydroxide 35% v/v and 40 mL RO-grade water. (iii) 2.2 M hydroxylamine·HCl solution and (iv) 0.1 M EDTA adjusted to pH 8 with NaOH in (RO)-grade water. Samples were pre-filtered through 0.2 µm (pore size) sterile PES membrane filter units. The assay was performed by thoroughly mixing 2 mL of filtered sample (diluted in RO-grade water if necessary) with 0.3 mL FAD solution and 0.4 mL NH_4-buffer, and left to react for 3 min at room temperature. Next, 0.3 mL hydroxylamine·HCl solution and 0.4 mL EDTA were added to the mixture and allowed to react for 4 min. Absorbance was recorded at 420 nm against the reagent as blank. Calibration curves were prepared (Fig. 2.3), using known concentration of soluble manganese (generally between 0.25 and 1.0 µg Mn^{2+} mL^{-1}).

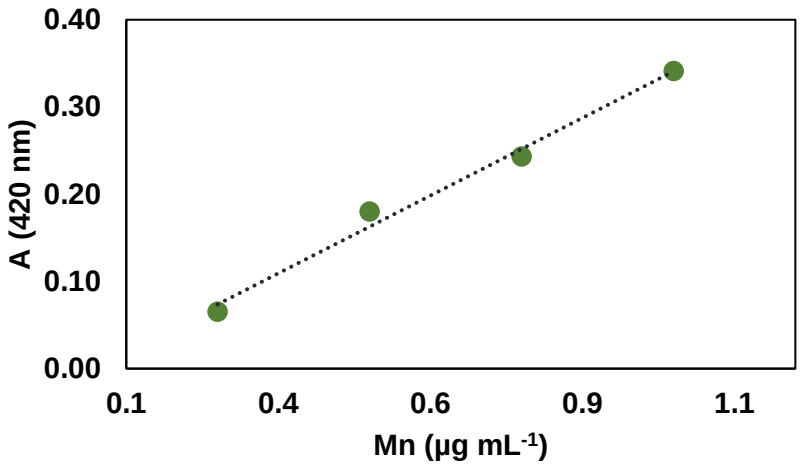

Figure 2.3. Representative calibration curve for the colorimetric manganese assay used (based on Mariner *et al.,* 2007). Linear correlation: *r=0.99*.

2.3.1.5 Protein concentrations

The Bradford assay was used to determine protein concentrations (Bradford, 1976). Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G-250 (100 mg L^{-1}) in (RO)-grade water containing 5% (v/v) ethanol and 10% (v/v) phosphoric acid, and stored in the dark at 4°C. Cells were pelleted and re-suspended in 0.5 mL of 0.5 M NaOH and incubated at room temperature for 15 min, diluted with 0.5 M NaOH when necessary. Volumes of cell suspension

(100 µL) were mixed with 1 mL of Bradford reagent and left to react for 2 min. Absorbance was measured at 595 nm, using the Bradford reagent as blank. A calibration curve was constructed using bovine serum albumen (BSA; prepared by dissolving 0.05 g BSA powder in 5 mL of RO-grade water for a 10 mg mL^{-1} stock solution) with concentrations ranging from 0 to 50 µg mL^{-1}(Fig. 2.4) Cells suspension and standard solutions were treated in similar conditions.

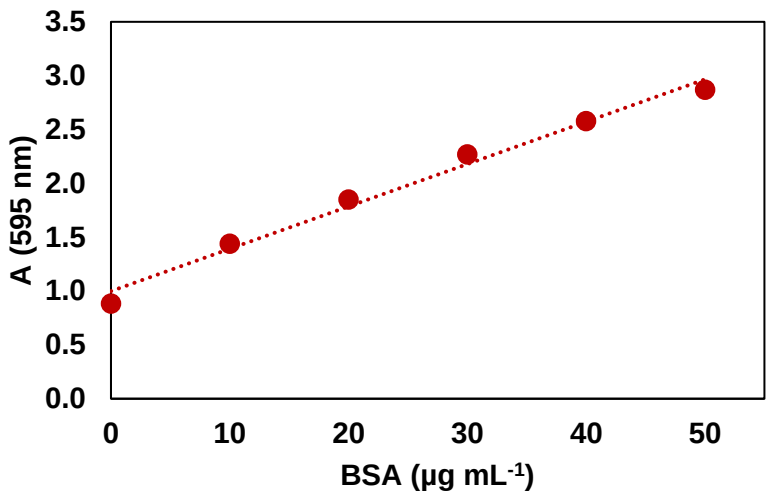

Figure 2.4. Representative calibration curve for the colorimetric determination of proteins using the Bradford assay (Bradford, 1976). Linear correlation: *r=0.99*.

2.3.1.6 Ion chromatography

Concentrations of transition metal (zinc), anions (sulfate and acetate) and non-ionic organic compounds (glucose and glycerol) were determined by ion chromatography (Dionex, USA) and data were analysed using Chromeleon software (version 6.4 SP2 Build 731, Dionex, USA). All reagents and standards used were prepared with ultrapure-grade Milli-Q® water, and all samples, standards and reagents were filtered with a 0.2 µm (pore size) sterile PES membrane filter units.

2.3.1.6.1 Zinc analysis

Soluble zinc was determined by a Dionex ion chromatography system contained an IonPac™ CS5A column, an AD25 absorbance (520 nm) detector and an IP25 isocratic pump. The flow rate was set to 1.2 mL min^{-1}, column pressure, *ca*. 1600 p.s.i. and 250 µL of sample was injected. The eluent for this analysis contained PDCA (pyridine-2,6-dicarboxylic acid; 1.4 mM), KOH (13.2 mM), K_2SO_4 (11.2 mM) and formic acid (14.8 mM) pH adjusted to 4.2 with formic acid. PDCA forms anionic complex with zinc and this complex are differentially retained on the

column. The post column reagent was prepared with 0.12 g 4-(2-pyridylazo) resorcinol (PAR) dissolved in 1 L of diluent solution (1 M 2-dimethylaminoethanol, 0.5 M ammonium hydroxide and 0.3 M sodium bicarbonate at pH 10.4). Standard solutions (0.5, 1.0 and 5 ppm) were used at the beginning and end of each run. Samples were diluted with a solution containing 1% (v/v) of HNO_3 and 0.05% (v/v) of H_2O_2; where samples were not diluted, they were acidified with a solution of 25% (v/v) of HNO_3.

2.3.1.6.2 Analysis of anions

Concentrations of sulfate and acetate were measured with an IonPac™ AS11 column, an ASRS ULTRA 4 mm suppressor and an IC25 ion chromatograph fitted with a DS11 conductivity detector. The pressure was maintained around 1,500 p.s.i. (lower limit was set to 200 p.s.i. and upper limit was set at 3,000 p.s.i.), the flow rate was set at 1 mL min^{-1} and sample injection volume was 250 µL. The column was maintained at room temperature and the suppressor current was set at 50 mA. For the acetate analysis, the eluent (KOH) concentration was 15 mM and for sulfate analysis the concentration of KOH was 20 mM. A range of standards (0.1, 0.5 and 1.0 mM for acetate and 0.5, 2.0 and 5.0 mM for sulfate) was used at the beginning and end of each run. Chromeleon software was used to construct the calibration curves.

2.3.1.6.3 Organic compounds

Concentrations of non-ionic organic compounds (glucose and glycerol) were determined using a Dionex AP autosampler with ICS-3000 pump and ED50 electrochemical detector, ED 40 amperometric detector and a CarboPac™ MA1 BioLC™ column. Flow rate was 0.4 mL min^{-1}, temperature of the column was maintained at room temperature and sample injection was 250 µL. The eluent used was NaOH (0.5 M) and column pressure was maintained 1500 p.s.i (minimum 200 p.s.i and maximum 2.000 p.s.i). Standards solutions of glucose and glycerol (0.5, 1.0 and 2.0 mM) were used at the start and end of each run.

2.3.2 Determination of dissolved organic carbon (DOC) concentrations

Dissolved organic carbon concentrations (DOC) were determined by oxidising organic carbon with sodium persulfate (5%, w/v; acidified with 0.5%, v/v nitric acid) in the presence of UV light, and measurements of the CO_2 produced (LABTOC; Pollution & Process Monitoring Ltd., U.K.). Standard solutions (containing 0 to 10 mg DOC L^{-1}) were prepared using potassium hydrogen phthalate. Samples and standards were prepared with ultrapure-grade water, filtered through 0.2 µm (pore size) sterile PES membrane filter units.

2.3.3 Titratable alkalinity

The alkalinity of waste water influent liquors (Chapter 6) was measured by titrating 100 mL of liquor with 0.1 M HCl until the pH decreased from ~7.0 to 4.0. The pH was measured as described in section 2.3.1.1, and prior to the titration, calibration was performed with pH 7 and pH 4 standard solutions. The volume of HCl used to decrease the pH of the solution to 4 and the volume of sample (V, in mL) were used to calculate the alkalinity using Equation 2.1:

$$\text{Alkalinity (mg of } CaCO_3\ L^{-1}) = (A \times M \times 50{,}000) / V \qquad \text{Eq.2.1}$$

where:

A = volume of HCl in mL

M = molarity of HCl

2.4. Microscopic techniques

2.4.1 Stereomicroscopy

Colonies grown on solid media were visualised using a stereomicroscope (Wild M3Z, Heerbrugg, Switzerland) at x50 to x400 magnification. Images were captured with a GXCAM-5 (GT Vision Ltd.) digital camera attached to the microscope.

2.4.2 Cell counts

Microbial cells were counted using a Thoma bacterial counting chamber (Hawksley, U.K). A sample (20 µL) of homogeneous cell suspension was withdrawn and placed onto the chamber and covered with a cover slip. The chamber was placed on a Leitz Labolux phase contrast microscope fitted with a Phaco 2 x40 objective lens (x400 magnification) to perform cell counts. To calculate cell numbers (cells per 1 mL), the number of cells in a square unit and the number of squares were recorded, and Equation 2.2 was used.

$$\text{Cells} = \text{number of cells counted} \times (2.5 \times 10^7) / \text{Number of square unit} \qquad \text{Eq. 2.2}$$

2.5 Field analysis

2.5.1 On-site analyses

On-site measurements of temperature, pH, dissolved oxygen (DO) and oxidation-reduction (redox) potentials were performed using a YSI 556 MPS multi-meter (Xylem Inc., USA). Calibrations were performed prior to using the multi-meter: (i) pH and redox electrodes, as described in section 2.3.1.1, and (ii) DO calibration was performed with water-saturated air (97.8%; 8.73 mg/L, at 23°C).

2.5.2 Sample collection for DNA extraction

Mine water samples were collected for DNA extraction and cells were harvested by passing the water through sterile 0.2 µm (pore size) cellulose nitrate membrane filters (Whatman, UK). The filter membranes were transferred to laboratory (~ 4 h transit time) and stored at -20°C until DNA was extracted.

2.5.3 Mine water collection

Mine-impacted waters were collected on sites using clean non-sterile low-density polyethylene containers (20 L). The containers with collected water were transferred to laboratory (~ 4 h transit time), stored at room temperature and used as influent liquors of the "hybrid" sulfidogenic bioreactor (Chapter 6).

2.6 Biomolecular techniques

2.6.1 DNA extraction

Pure cultures – Lysis method

DNA was extracted from colonies grown on solid media by removing 1-3 representative colonies with a sterile wire, re-suspending in 20 µL cell-lysis solution (0.05 M NaOH plus 0.25 %, w/v of sodium dodecyl sulfate) and heating at 95ºC for 15 minutes using a PCR thermocycler Techne® TC-312 (Midwest Scientific, USA). Lysates were allowed to cool and 80 µL of sterile ultrapure Milli-Q® water was added. Cells from liquid cultures were concentrated by centrifugation (16,000 x g for 10 minutes) until sufficient biomass was obtained and pellets were re-suspended in cell-lysis solution. DNA from these samples was then extracted as described above. Cells from iron-containing cultures were washed with sterile ultrapure-grade water pH 1.7 before addition of cell-lysis solution.

Pure cultures – CTAB method

Cells from liquid cultures were concentrated by centrifugation (16,000 x g for 10 minutes) in sterile 1.5 mL micro-centrifuge tubes until sufficient biomass was obtained. The pellet was washed in TE (10 mM Tris and 1 mM EDTA, pH 8.0) until the pH of the biomass had been increased to ~ 7.0. Then the pellet was re-suspended in 450 µL of lyzozyme solution (1 – 2 mg mL^{-1}, dissolved in TE) and incubated at 37ºC for 1 hour. Five microliters of RNAse A (1 mg mL^{-1}), 5 µL of proteinase K (18 mg mL^{-1}) and 15 µL of SDS (10%, w/v) were added and mixed with the lysozyme mixture, and incubated at 37ºC for 1 hour. Next, 100 µL of 5 M NaCl and 80 µL of warm CTAB solution (10%, w/v dissolved in 0.7 M NaCl) were added, mixed and incubated at 65ºC for 10 minutes. An equal volume of chloroform:isoamyl alcohol (24:1) were added and centrifuged (16,000 x g for 5 minutes), then the upper phase transferred to a 1.5

mL sterile micro-centrifuge tubes. An equal volume of isopropanol was added, mixed by inverting and incubated at 4ºC for 10 minutes. The tube was centrifuged (16,000 x g for 10 minutes) and the supernatant was discarded. The pellet was washed with 70% (v/v) ethanol (centrifuged at 16,000 x g for 10 minutes), and left to dry at room temperature. A subsample was withdrawn to assess DNA concentration with NanoDrop 2000 (Thermo Scientific™, USA) and the remaining DNA was frozen at -20ºC.

Enrichments, mine water and bioreactor samples

Biomass from enrichment cultures (section 4.2.1), mine water (section 2.5.2) and bioreactor samples (sections 5.2.2, 6.2.2.3 and 6.2.3.3) were collected by filtering samples through sterile 0.2 µm (pore size) cellulose nitrate membrane filter. DNA from cells attached to the filter membranes were extracted using PowerSoil UltraClean Microbial DNA Isolation Kit (QIAGEN, Denmark), following manufacturer's instructions.

2.6.2 Polymerase chain reaction (PCR) and gel electrophoresis

PCR was used to amplify bacterial or archaeal small subunit ribosomal RNA genes (16S rRNA genes); primers used are listed in Table 2.6. One microlitre of sample was added to PCR master mix, which was prepared with the following reagents: GoTaq® Flexi Buffer (5x colourless, 4 µL), dNTPs (2 mM, 2 µL), $MgCl_2$ (25 mM, 2 µL), forward primer (10 pmol μL^{-1},0.4 µL), reverse primer (10 pmol μL^{-1},0.4 µL), dimethyl sulfoxide (0.4 µL), GoTaq® Hot Start Polymerase (0.2 µL), ultra-pure water (9.6 µL), for a final volume of 19 µL. Thermal cycling was performed in a Techne® TC-312 (Midwest Scientific, USA) thermocycler. The PCR program for bacterial 16S rRNA gene used was 5 min at 95ºC, 30 cycles at 95ºC (30 s), 30 s at 55ºC, 1.5 min at 72ºC and final extension at 72ºC (10 min). For archaeal 16S rRNA genes, the PCR program used was 5 min at 95ºC, 30 cycles at 95ºC (30 s), 30 s at 62ºC, 1 min at 72ºC and final extension at 72ºC (10 min).

Agarose gel electrophoresis was used to examine PCR products. Agarose gels (0.7%, w/v) were prepared with 30 mL of 0.5x TBE buffer (5.4 g tris-hydroxymethyl-methylamine, 2.75 g boric acid and 2 mL of 0.5 M EDTA per 100 mL of buffer; pH adjusted to 8.0 with NaOH) by heating the mixture of agarose and TBE in a microwave oven. After the agarose had cooled to ~ 50ºC, SafeView® (NBS Biologicals Ltd., UK) was added to a final concentration of 0.1 µL mL^{-1} and the gel was poured in a gel casting tray. Five microliters of PCR products (mixed with 1 µL of DNA loading dye, 6x Blue/Orange, Promega, USA) and a DNA ladder (1kb, Promega, USA) were placed into the wells of the agarose gel. Electrophoresis was carried out for ~20 minutes at constant voltage (90 mV) using Bio-Rad PowerPac™ 300 power supply (Bio-Rad, USA). Gel-Doc EQ (Quantity One software, Bio-Rad, USA) was used to obtain an

image of the gel under UV light. PCR products were purified with SureClean® Plus (Bioline Reagents Ltd., UK) following manufacturer's instructions, and re-suspended in 20 µL sterile ultrapure-grade water.

Table 2.6. Primer oligonucleotides used in this study and their respective target genes. F = forward, R = reverse.

Primer	Sequence (5'→3')	Target gene	Reference
27F (Cy5-labelled)[a]	AGAGTTTGATC(A/C)TGGCTCAG	Bacterial 16S rRNA	
27F (unlabeled)	AGAGTTTGATC(A/C)TGGCTCAG	Bacterial 16S rRNA	Lane *et al* (1991)
1387R	GGGCGG(A/T)GTGTACAAGGC	Bacterial 16S rRNA	Marchesi *et al (*1998)
Arch 20F	TTCCGGTTGATCC(T/C)GCC(A/G)G	Archaeal 16S rRNA	Orphan *et al* (2000)
Arch 915R	GTGCTCCCCCGCCAATTC	Archaeal 16S rRNA	Orphan *et al* (2000)

2.6.3 Terminal restriction enzyme fragment length polymorphism (T-RFLP) analysis

Following amplification of 16S rRNA genes and purification of PCR products (section 2.6.2), semi-quantitative analysis was performed using terminal restriction enzyme fragment length polymorphism (T-RFLP). This fingerprinting technique uses a 27F primer, labelled with Cy5 dye, (5′-AGT GTT TGA TCCTGG GTC AG-3′) and unlabelled 1387R primer (5′- GGG CGGWGT GTA CAA GGG-3′). Digestion of PCR products was performed in a 10 µL reaction mix containing 6.0 µL ultrapure-grade water, 1.0 µL restriction enzyme 10x buffer, 1.0 µL bovine serum albumin (10 mg mL^{-1}), 1.0 µL restriction enzyme (HaeIII, CfoI or AluI; Promega, UK) and 1.0 µL purified PCR product. The digestion mix was incubated at 37°C for 1 h. To analyse the 16S rRNA gene fragments (up to 600 nucleotides in length), Genome Lab DNA size standard kit - 600 (SLS 600, Beckman Coulter, USA) was used. SLS 600 (28 µL) and digestion mix (2 µL) were added into a 96-well conical bottom plate and a drop of mineral oil was placed on the top of the sample. Terminal restriction fragments (T-RFs) were separated on a capillary sequencer CEQ8000 Genetic Analysis System (Beckman Coulter, USA) and assigned by comparison to database of acidophilic microorganisms held at Bangor University. The relative abundances of individual T-RFs was calculated by comparing their peak areas with that of the summated T-RF peak area.

2.6.4 DNA sequence analysis

Purified PCR products of isolates were sequenced by Macrogen Inc. (South Korea). Raw sequences were analysed using Chromas Lite version 2.1.1, aligned using ClustalW software and compared with gene sequences deposited in the GenBank database (National Centre of Biotechnology Information, NCBI).

Multiple sequence alignment can be performed using other available programs such as SILVA (Quast *et al.,* 2012). SILVA is a (web)aligner and database of quality-controlled and up-to-date fully aligned gene sequences of the small and large subunit of ribosomal RNA (rRNA) from the Bacteria, Archaea and Eukaryota domains. This program use a phylogenetic tree-guided manual curation approach for the taxonomy, which generate accurate alignments and thus, improved phylogenetic analysis and classification. Alignment software such as ClustalW perform alignments considering only the primary structure of the 16S rRNA gene sequence, while SILVA integrates in the alignment a global secondary structure of the ribosomal RNA molecule, which increases the confidence that the positional homology within the sequences is conserved (Quast *et al*., 2012; Schloss, 2013).

MEGA software using Neighbour-joining method with Kimura 2-parameter (as the nucleotide substitution model) was selected to reconstruct the phylogenetic trees described in this thesis. Other methods can be applied for constructing phylogenies. For instance, Maximum Likelihood method using programs such as PhyML, RAxML and PHYLIP; and Bayesian method using software such as MrBayes and BEAST (which also can infer time-measured phylogenies using molecular clocks).

The result of a phylogenetic analysis might vary greatly depending of the nucleotide substitution model applied; therefore, the use of statistical model selection has become a critical step for phylogenetic assessment from DNA sequences alignments (Posada, 2008). Programs such as jModelTest (Darriba *et al.,* 2012) provide the best-fit evolutionary model for a given alignment by implementing distinct statistical criteria such as “Akaike Information Criterion” (AIC), “Akaike Information Criterion” corrected for small sample sizes (AICc), Bayesian Information Criterion (BIC), and a decision-theoretic performance-based approach (DT), that ranks and selects the best model of the 88 available options. These can be classified as simpler models (e.g. Jukes-Cantor and Kimura 2-parameter) or more complex (e.g. General Time Reversible).

2.7. Statistical Analysis.

One-way ANOVA and t-tests were used (when specified) to evaluate any significant (95% level) effect of changing treatment conditions in experiments performed in this study. The statistical software package IBM S.P.S.S. (version 24) was used and Post-Hoc Tukey tests were applied in the ANOVA to examine if there was significant differences between conditions tested.

In this thesis, for the calculations of mean values represented by two data points, it was used data range to indicate the data variation. When mean values were obtained from three or more data points, it was used standard deviation to indicate the variation in the data set.

Chapter 3. Characterisation of novel acidophilic mineral-oxidising *Firmicutes*: "*Acidibacillus ferrooxidans*", gen. nov. sp. nov., and "*Acidibacillus sulfuroxidans*", gen. nov. sp. nov..

3.1 Introduction

Acidophilic microorganisms comprise a large variety of different species that are widely distributed in all three domains of known life-forms (Johnson and Aguilera, 2016). While the greatest number (and earliest isolates) of known extremely acidophilic bacterial species are members of the phylum *Proteobacteria* other phyla, including the *Firmicutes*, *Nitrospirae*, *Actinobacteria* and *Aquificae*, all include species that grow optimally at pH < 3. Currently, the *Firmicutes* (endospore-forming eubacteria with low G+C contents) include two genera of extreme acidophiles, *Sulfobacillus* and *Alicyclobacillus*, most species of which are moderate thermophiles (growth temperature optima of 40ºC – 60ºC) though some are mesophilic. *Sulfobacillus* spp. are typically found in mineral- and sulfur-rich acidic environments, such as solfatara fields and biomining operations, and are characterised by having far greater metabolic versatilities than many of the more specialised bacteria (such as *Leptospirillum* and *Acidithiobacillus* spp.) with which they frequently cohabit. Species such as *Sb. thermosulfidooxidans, Sb. acidophilus* and *Sb. beneficiens* can grow autotrophically by oxidizing inorganic electron donors (sulfur, ferrous iron and hydrogen) and fixing carbon dioxide, heterotrophically using organic carbon as both energy and carbon source, and chemolitho-heterotrophically whereby they obtain most of their energy from oxidizing inorganic electron donors but use organic materials, such as yeast extract, as carbon sources. *Sulfobacillus* spp. are facultative anaerobes that use either molecular oxygen or ferric iron as terminal electron acceptors. In contrast, the genus *Alicyclobacillus* includes species of moderate (pH growth optima 3 - 5) as well as extreme acidophiles. The earliest isolates were obligately heterotrophic moderate thermophiles that were isolated from pasteurised fruit juices which they had contaminated. Later isolates (e.g. *Alb. disulfidooxidans* and *Alb. ferrooxydans*) more resembled *Sulfobacillus* spp., both in terms of the environments they inhabited and in their metabolic capabilities (e.g. in catalysing the dissimilatory oxidation of sulfur and iron). *Alicyclobacillus* spp. tend, however, to be generally more "heterotrophically inclined" than *Sulfobacillus* spp., and grow more successfully using defined organic compounds such as glucose (e.g. Watling *et al.*, 2015).

For a number of years, some acidophilic *Firmicutes* isolated from mineral-rich terrestrial or acidic aquatic environments have been found, from sequencing of their 16S rRNA genes, to be affiliated to neither *Sulfobacillus* nor *Alicyclobacillus* (e.g. Johnson *et al.*, 2001; Breuker *et*

al., 2009; Lu *et al.*, 2010), though these have not been fully characterized. Clones related to these isolates have also been found in diverse locations (e.g. He *et al.*, 2008; Fujimura *et al.*, 2012; Urbieta *et al.*, 2012). This Chapter describes the characteristics of eight such strains, isolated from a variety of low pH environments from different global locations prior to the present study, and show that they comprise two distinct species of a proposed novel genus of *Firmicutes*, "*Acidibacillus*".

3.2 Bacteria and culture conditions

The eight bacterial strains studied were isolated prior to this study from a variety of mine-impacted and geothermal sites from different global locations over a twenty-year period and maintained within the *Acidophile Culture Collection* at Bangor University (U.K.; Table 3.1).

Table 3.1. Sites of origin of bacteria identified as *"Acidibacillus"* spp.

Isolate	Origins and dates of isolation	Reference
SLC66^T & SLC40	Experimental system used to accelerate the oxidation of mine waste (pH 2.9, 25 °C); Utah, USA (1994)	Johnson *et al.* (2001)
GS1	Sediment in a pit lake at an abandoned copper mine (pH 3.8, 23 °C); Spain (2015)	Falagan *et al.* (2014)
BSH1	Constructed wetland receiving coal mine drainage (pH 7.0, 14 °C); England, UK (2014)	K.B. Hallberg and D. B. Johnson (unpublished)
ITV01	Stream draining waste rock at a copper mine (pH 4.9, 32 °C); Brazil (2013)	Dall'Agnol *et al.* (2016)
Gal-G1	Geothermal area (pH 3.0, 80 °C); Soufriere Hills, Montserrat, W.I. (1996)	Atkinson *et al.* (2000)
Y002^T & Y0010	Geothermal area (pH 2.7, 30-60°C) Yellowstone National Park, Wyoming, USA (2000)	Johnson *et al.* (2003)

The eight isolates were cultivated routinely on FeSo solid medium (section 2.2.5). Plates were incubated at 30º or 45ºC and grown colonies of strains SLC66^T and Y002^T were visualized using a stereomicroscope (section 2.4.1). Single colonies were transferred into and maintained in FeYE liquid medium (section 2.2.4), which contained 10 mM ferrous sulfate and 0.02% (w/v) yeast extract adjusted to pH 2.0 with sulfuric acid. Cells from liquid cultures were visualised using Leitz Labolux phase contrast microscope (section 2.4.2).

Bacterial colonies on FeSo medium had "fried egg" morphologies (Fig. 3.1) typical of heterotrophic iron-oxidizing acidophiles such as *Sulfobacillus* spp. (Johnson *et al*., 2008), the orange coloration of the colony centres resulting from the accumulation of oxidized iron. Cells of all the "*Acidibacillus*" strains listed in Table 3.1 were motile rods, and formed oval endospores, which were located at the cell termini. It was noted that numbers of individual cells of Y002^T increased during the early phases of incubation (up to 2 days) but declined subsequently; this appeared to be related to cells aggregating as incubation progressed, a feature that was much less apparent in cultures of SLC66^T.

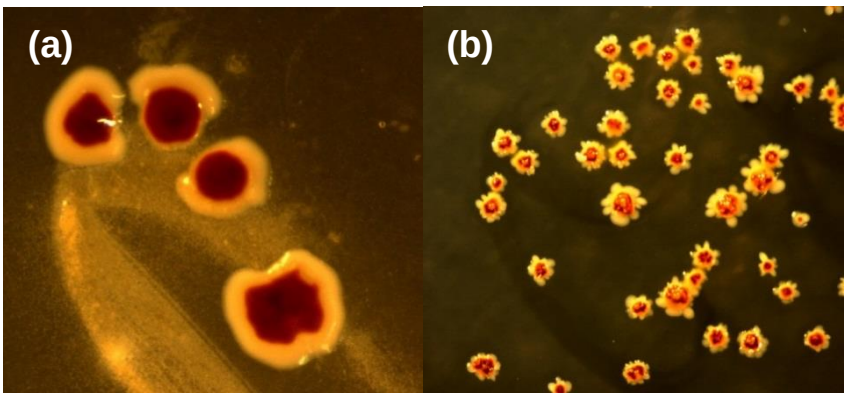

Figure 3.1. Colonies of (a) *"Ab. sulfuroxidans"* Y002^T, and (b) *"Ab. ferrooxidans"* SLC66^T grown on FeSo overlay medium. Colony sizes are ~2 – 5 mm diameter.

3.3 Phylogenetic analysis

3.3.1 Methods

16S rRNA gene sequences of the eight strains (retrieved from the NCBI database; Table 3.2) were analysed using BLAST (http://ncbi.nlm.nih.gov/BLAST). The sequences of "*Acidibacillus*" strains were aligned with those of related type strains using Muscle (Edgar, 2004) *via* MEGA7 software (Kumar *et al.,* 2015), followed by manual editing to remove gaps. Phylogenetic trees were constructed by neighbour-joining analyses with the Kimura 2-parameter method (Kimura,

1980) using MEGA7. Tree topologies reliability was confirmed by bootstrap analysis using 1000 replicates.

Table 3.2. Accession numbers and lengths of 16S rRNA gene sequence of "*Acidibacillus"* isolates.

Strain	Accession number	16S rRNA partial gene (bp)
SLC66^T	AY040739	1467
SLC40	KP860947	1268
BSH1	KP852500	1224
GS1	KP779998	1168
ITV01	KP860946	1272
Gal-G1	AY529492	1286
Y002^T	KP860945	1257
Y0010	AY140235	1333

3.3.2 Results

Analysis and comparison of 16S rRNA gene sequences confirmed that all eight isolates were members of the phylum *Firmicutes* (order *Bacillales*, family *Alicyclobacillaceae)*. Figure 3.2 shows that they clustered into three closely-related groups: (i) Group IA comprising isolates SLC66^T, SLC40, BSH1 and GS1; (ii) Group IB represented by the isolates ITV01 and Gal-G1, and (iii) Group II corresponding to isolates Y002^T and Y0010. Groups IA and IB shared >99% similarity of their 16S rRNA genes and all of these were proposed to be strains of the novel species *"Ab. ferrooxidans"*. Group II isolates shared >99% 16S rRNA gene similarity but were more distantly related (94% gene similarity) to both Groups IA and IB and considered to be strains of a different species, *"Ab. sulfuroxidans"*. Following this study, two novel isolates closely-related to "*Acidibacillus*" spp. have been identified, S^{0}AB and Huett2, isolated from commercial zero-valent sulfur in the United Kingdom and from acid mine drainage of a former minefield in Germany, respectively (Appendix 3B). Isolate S^{0}AB and "*Ab. sulfuroxidans*" Y002^T shared 96% similarity of their 16S rRNA gene sequence and the strain S^{0}AB clustered with

Group II but in a separate branch. Isolate Huett2 showed 99% identity of 16S rRNA gene sequence to "*Ab. ferrooxidans*" SLC66^T and clustered in the same branch of strains SLC40 and BSH1.

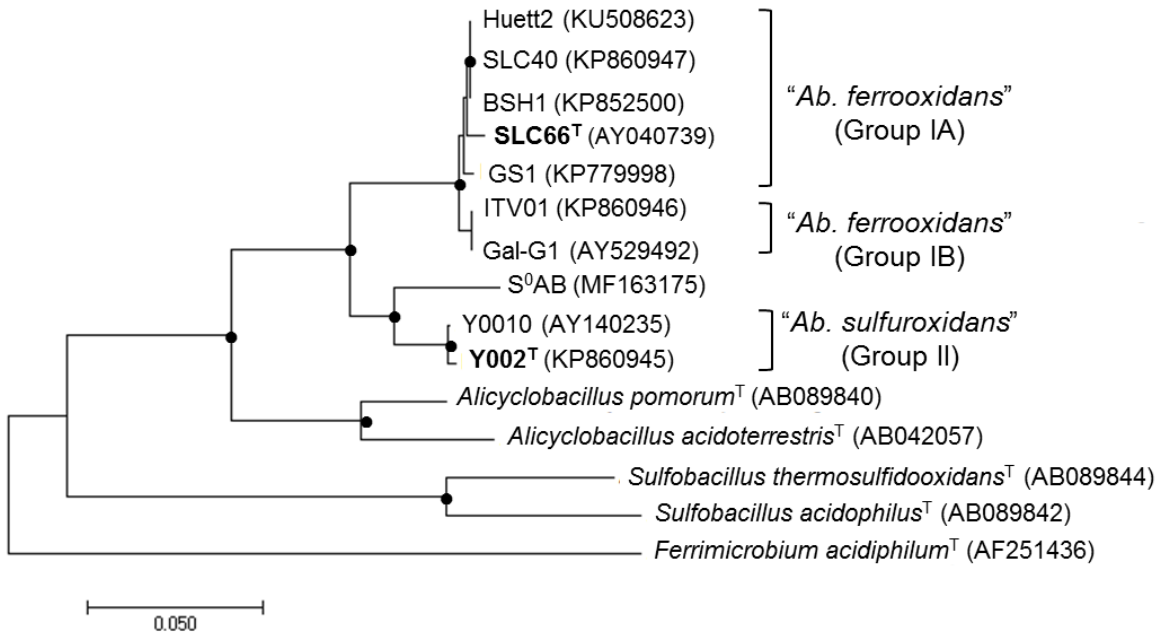

Figure 3.2. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the relationship of "*Acidibacillus ferrooxidans" and "Ab. sulfuroxidans"* to other Gram-positive acidophiles. GenBank accession numbers are given in parentheses for each strain. The support in bootstrap analysis (1000 replicates) with values ≥70% are indicated by "●". The sequence of *Ferrimicrobium acidiphilum*^{T} (AF251436) was used as the outgroup.

3.4 Physiological analyses

3.4.1 Effect of pH and temperature on growth of "*Acidibacillus*" spp.

3.4.1.1 Methods

Growth rates and optimum pH values and temperatures for growth of the two proposed type strains, SLC66^T (*"Ab. ferrooxidans"*) and Y002^T (*"Ab. sulfuroxidans"*) were determined by growing the bacteria in pH- and temperature-controlled 2 L bioreactors (FerMac 231/260, Electrolab, UK). The liquid medium used FeYE (section 2.2.4) contained 0.02% (w/v) yeast extract and 0.1 mM (SLC66^T) or 1 mM (Y002^T) ferrous sulfate. The bioreactor was stirred at 100 rpm and aerated with ~1.5 L min^{-1} of sterile atmospheric air. At the end of the exponential phase, the bioreactors were partly drained, leaving ~200 ml of spent medium, and fresh medium was added to make up the culture volume to ~1 L, prior to starting a new experimental run. For isolate SLC66, growth rates were tested at temperatures between 21ºC and 35ºC,

cultures maintained at pH 2.3, and at pH between 1.9 and 4.2, at 30 ºC. For isolate Y002, the temperature range was 30ºC to 50ºC, the medium maintained at pH 1.8, and the pH range 1.5 to 2.0, at 45 ºC. Culture doubling times were calculated for each experiment from semi-logarithmic plots of optical density (OD; at 600 nm) against time

Additional tests were carried out in replicate flasks with FeYE liquid medium containing 10 mM Fe^{2+} to assess growth of all eight strains: (i) at minimum pH (carried out at 30 ºC for Group IA, 40 ºC Group IB and 45 ºC Group II) and (ii) at maximum temperature (at pH 2.0 medium adjusted with sulfuric acid).

3.4.1.2 Results

Relationships between cell numbers and optical density at 600 nm of the isolates SLC66^T and Y002^T are shown in Figure 3.3. Figures 3.4 and 3.5 show the effects of pH and temperature on the culture doubling times (t_d's) of "*Acidibacillus"* type strains. Both bacteria were confirmed to be extreme acidophiles, though Y002^T was more acidophilic with a pH optimum and minimum for growth of 1.8 and 1.5, respectively, while corresponding values for SLC66^T were

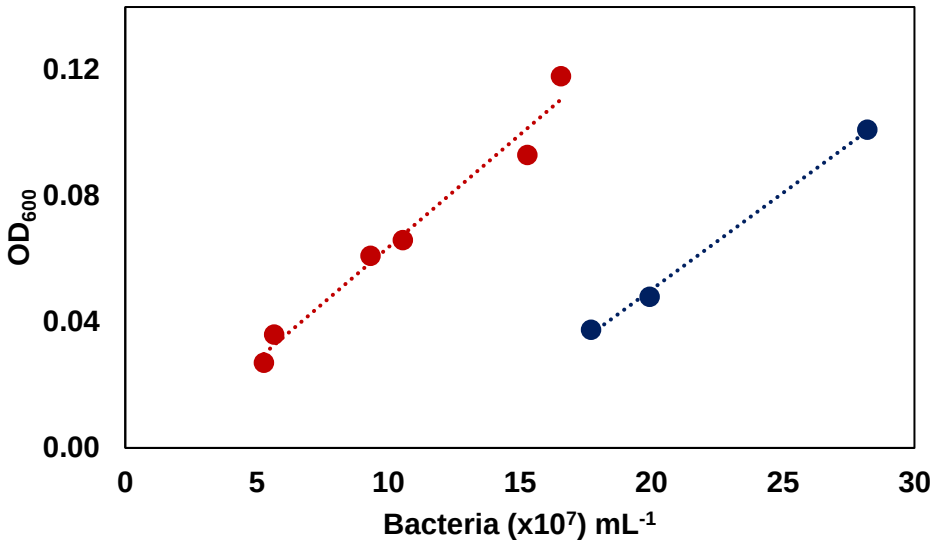

Figure 3.3. Relationship between optical density at 600 nm and cell numbers of "*Acidibacillus"* type strains. Key: *"Ab. ferrooxidans"* SLC66^T (●; *r* = 0.99; at 30°C); "*Ab. sulfuroxidans*" Y002^T (●; *r* = 0.97; at 45°C).

2.9 and 1.9. The two isolates also displayed contrasting temperature-related growth: SLC66^T was mesophilic (temperature optimum and maximum of ~30ºC and 37.5ºC) while Y002^T was a moderate thermophile with an optimum growth temperature of ~43ºC and a maximum of 50ºC. When grown at optimum conditions of pH and temperature, SLC66^T had a culture doubling time (t_d) of 6.7 h (corresponding to a growth rate, μ, of 0.10 h^{-1}), while the moderate

thermophile Y002^T grew much more rapidly (minimum t_d of 2.1 h, corresponding to a μ of 0.33 h^{-1}).

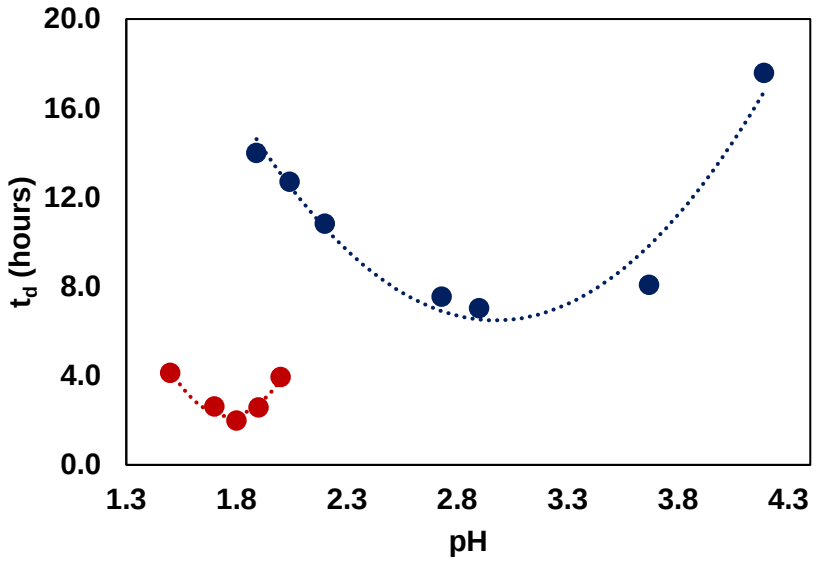

Figure 3.4. Effect of pH on the culture doubling times (t_d) in hours. Key: *"Ab. ferrooxidans"* SLC66^T (●; at 30°C); "*Ab. sulfuroxidans*" Y002^T (●; at 45°C); using polynomial regression.

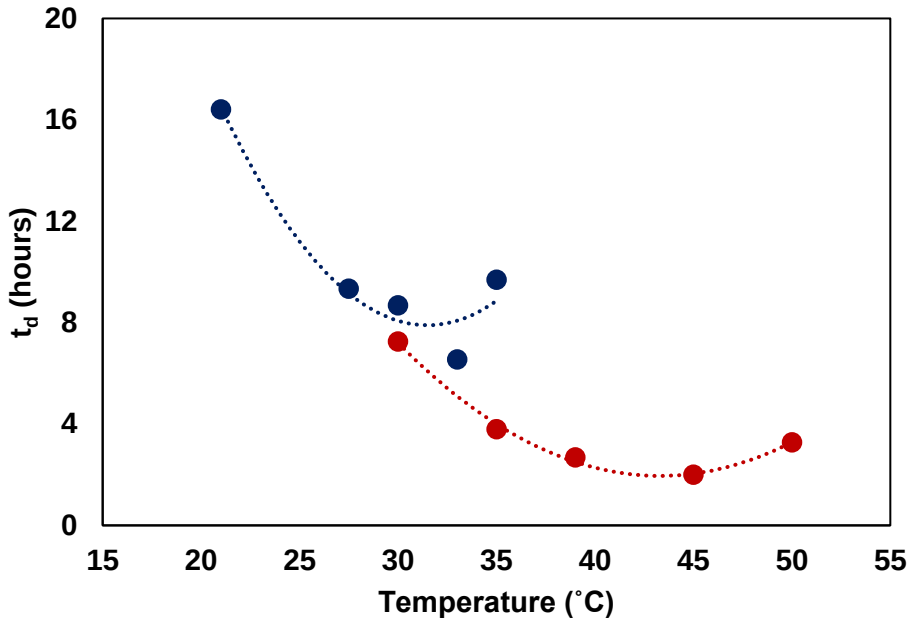

Figure 3.5. Effect of temperature on the culture doubling times (t_d) in hours. Key: *"Ab. ferrooxidans"* SLC66^T (●, at pH 2.3); "*Ab. sulfuroxidans*" Y002^T (●, at pH 1.8); using polynomial regression.

Tests carried out in shake flasks confirmed that none of the "Group I" isolates (*"Ab. ferrooxidans"*) grew at pH 1.5, though one Group IA strain (BSH1) and both Group IB strains (ITV01 and Gal-G1) grew at pH 1.75. None of the four Group IA strains grew at 40ºC, in contrast to both Group IB strains (though neither of these grew at 45ºC). Strains of *"Ab. sulfuroxidans"* (Y002^T and Y0010) grew at 45ºC, though Y0010 was less acidophilic than Y002^T and grew at pH 1.75 though not at pH 1.5 (Table 3.3).

Table 3.3. *"Acidibacillus"* spp maximum temperature and minimum pH for growth of cultures in FeYE liquid medium.

Isolate	$T_{maximum}$ (ºC)	$pH_{minimum}$
SLC66^T	37.5	1.90
SLC40	<40[a]	1.90
BSH1	<40[a]	1.75
GS1	<40[a]	1.90
ITV01	40	1.75
Gal-G1	40	1.75
Y002^T	50	1.50
Y0010	45[a]	1.75

[a] Maximum temperature was not assessed.

3.4.2 Dissimilatory redox transformations of inorganic electron donors and acceptors

3.4.2.1 Oxidation of ferrous iron

3.4.2.1.1 Methods

Dissimilatory oxidation of ferrous iron was determined by monitoring changes in ferrous iron concentrations in FeYE liquid medium (section 2.2.4) containing 10 – 25 mM Fe^{2+}. To determine whether bacteria were able to utilize the energy available from oxidizing iron, strains SLC66^T and Y002^T were grown in replicate flasks containing 0.005% (w/v) yeast extract and different concentrations of ferrous iron (1, 10 and 25 mM, at an initial pH of 1.9 for SLC66^T, and 1, 25 and 50 mM, at an initial pH of 1.7 for the more acidophilic isolate Y002^T). Culture media were designed to maximize the amount of ferrous iron oxidation without causing hydrolysis (and precipitation) of the ferric iron generated, which would have impaired the accuracy of cell counts. Cultures were incubated (at 30ºC for SLC66^T and 45ºC for Y002^T),

shaken at 100 rpm, and residual ferrous iron (section 2.3.1.2) and cell numbers (section 2.4.2) determined daily for up to 6 days.

3.4.2.1.2 Results

All eight of the isolates catalysed the oxidation of ferrous iron in acidic media, and this was found to be correlated (r = 0.97) with growth of the bacteria. Figure 3.6 shows correlations for type strains SLC66^T and Y002^T. In cultures containing 0.005% (w/v) of yeast extract, cell yields of both SLC66^T and Y002^T increased in parallel with the amount of ferrous iron oxidized (Fig. 3.7), although the increased in cell numbers was less marked in Y002^T cultures due to cell aggregation.

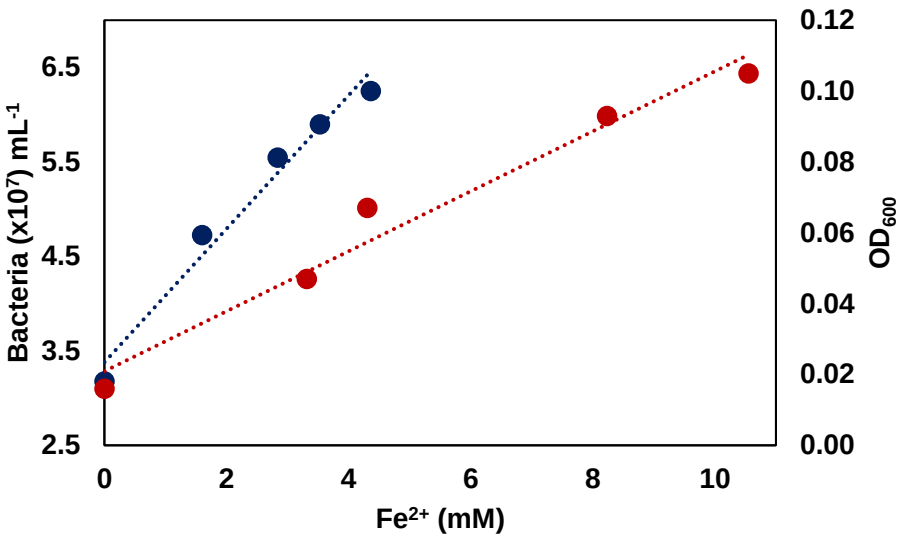

Figure 3.6. Relationship between bacterial growth and ferrous iron oxidation in cultures of the proposed type strains of "*Acidibacillus*". Bacteria were grown in FeYE liquid medium containing 10 mM Fe^{2+}, 0.02% (w/v) yeast extract pH 2 (SLC66^T) or pH 1.8 (Y002^T). Growth was assessed by cell counts for strain SLC66^T (●; r = 0.97) and by optical density for Y002^T (●, r = 0.97).

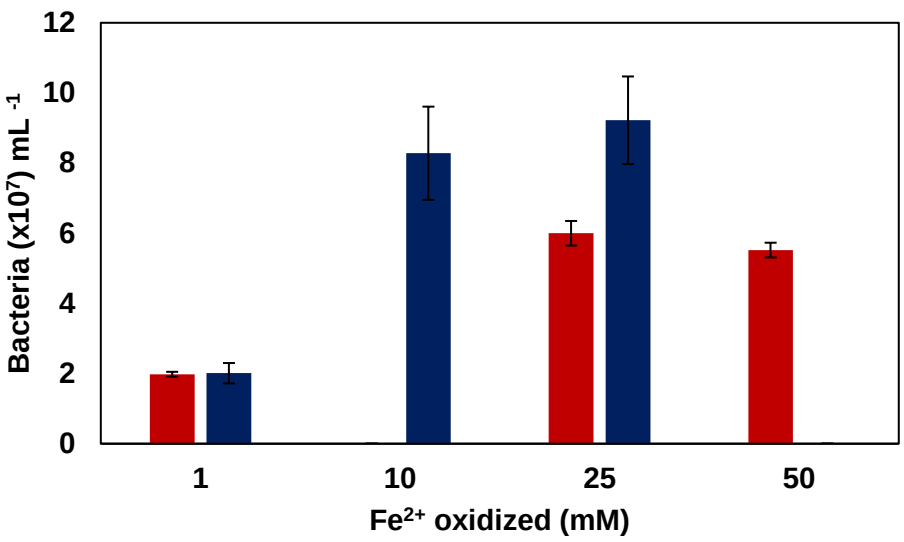

Figure. 3.7. Effect of ferrous iron concentrations on cell numbers of *"Ab. ferrooxidans"* SLC66^T (■) and *"Ab. sulfuroxidans"* Y002^T (■). Cultures were grown at yeast extract concentration of 0.005%, w/v and variable concentrations of ferrous iron. Bars indicate mean values of maximum cells numbers recorded (after 2 days incubation in cultures of Y002^T, and 4 days in cultures of SLC66^T) and the error bars data range (n = 2).

3.4.2.2 Specific rates of iron oxidation

3.4.2.2.1 Methods

Specific rates of ferrous iron oxidation by *"Ab. sulfuroxidans"* Y002^T were determined based on a technique described previously (Johnson *et al.,* 2012). The isolate was grown in FeYE liquid medium (pH 1.8 and 45ºC) in a stirred (100 rpm) and aerated (1.5 L min^{-1}) bioreactor (FerMac 231/260, Electrolab, UK). Biomass from 20 mL of culture was harvested at late exponential/early stationary phase by centrifugation, resuspended in 2 mL of acidophile basal salts (ABS, section 2.2.1) pH 1.8 and gently mixed to obtain a homogenous cell suspension. From this homogeneous biomass 0.5 mL was used to measure protein concentration (section 2.3.1.5) and 0.5 mL aliquots added to 9.5 mL aliquots of "reaction mixture" (1 mM ferrous sulfate and ABS, pH adjusted to pH 1.8 with sulfuric acid) in 20 mL universal bottles. Cell-free controls were set up in parallel. Tests were incubated at 45ºC in a water bath and aliquots were withdrawn at regular intervals for up to 1 h to determine residual ferrous iron concentrations (section 2.3.1.2). Specific rates were calculated as mg ferrous iron oxidized per minute per mg of protein. Analyses were carried out in triplicate.

To determine specific rates of ferrous iron oxidation by "*Ab. ferrooxidans"* SLC66^T a modified protocol was used. The isolate was initially grown in FeYE medium (25 mM Fe^{2+}, 30ºC and pH 2.0) in a bioreactor. When Fe^{2+} concentrations had declined to <0.5 mM, additional ferrous

sulfate (~3 mM) was added and samples were withdrawn regularly to determine residual Fe^{2+} concentrations over the following 90 minutes. Concentrations of bacterial proteins were measured at the start and end of these experiments to determine whether there had been any significant increase in biomass during the time span of the experiments. Specific rates of ferrous iron oxidation were calculated as described for *"Ab. sulfuroxidans"* Y002^T.

3.4.2.2.2 Results

The specific rates of ferrous iron oxidation obtained for SLC66^T at 30ºC and pH 2.0 were 36.1 ± 3.4 mg min^{-1} mg $protein^{-1}$ and for Y002^T at 45ºC and pH 1.8 were 48.5 ± 1.3 mg min^{-1} mg $protein^{-1.}$

3.4.2.3 Pyrite leaching tests

3.4.2.3.1 Methods

The oxidative dissolution of pyrite by the novel *Firmicutes* was tested by inoculating the two proposed type strains (SLC66^T and Y002^T) into a pyrite liquid media supplemented (PyYE) or not (Py) with 0.02% (w/v) yeast extract (section 2.2.4). Replicate shake flask cultures were incubated at either 30ºC (SLC66^T) or 45ºC (Y002^T). Both non-inoculated cultures and others inoculated with the moderately thermophilic *Firmicute, Sulfobacillus* (*Sb.*) *thermosulfidooxidans*^{T} were incubated in parallel, to act as negative and positive controls (*Sb. thermosulfidooxidans* cultures were grown only in PyYE liquid medium and were incubated at 45ºC). Samples were withdrawn at regular intervals to measure pH, redox potentials (E_H values), and concentrations of ferrous iron and total soluble iron (section 2.3.1).

3.4.2.3.2 Results

"*Ab. ferrooxidans*" SLC66^T catalysed the oxidative dissolution of pyrite, as evidenced by increasing concentrations of total soluble iron and cultures developing more positive E_H values with incubation time, though again this was only observed with yeast extract-containing cultures (Fig. 3.8a). Cultures of Y002^T, in contrast, initially failed to show any evidence of pyrite oxidation in liquid media that had been prepared under identical conditions. It was also noted that this isolate was unable to oxidize the ferrous iron released during sterilization of pyrite when the mineral was autoclaved in the presence of yeast extract. However, when sterile yeast extract solution was added subsequent to autoclaving pyrite/ABS, oxidative dissolution of pyrite proceeded, as shown in Figure 3.8b. As with SLC66^T, it was found that pyrite oxidation by strain Y002^T was negligible in yeast extract-free medium, though adding yeast extract to

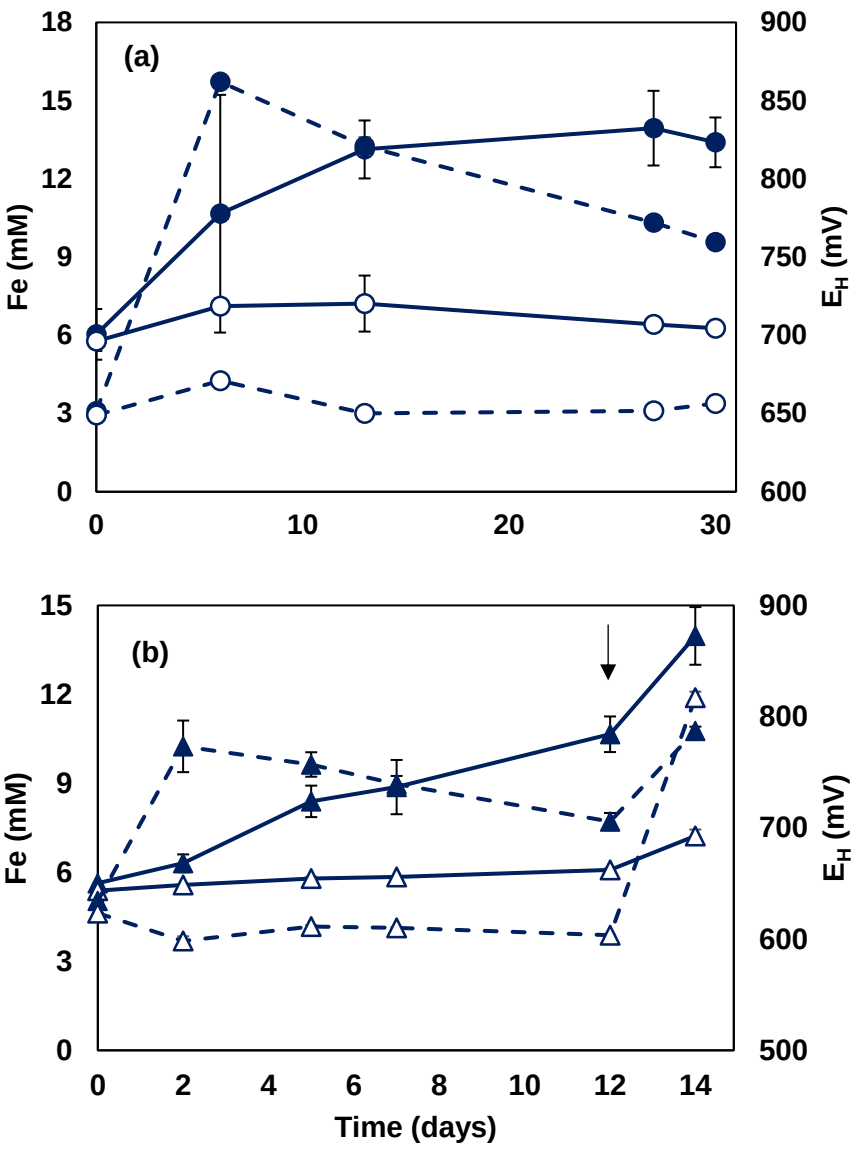

Figure 3.8. Oxidative dissolution of pyrite by (a) *"Ab. ferrooxidans"* SLC66^T (at 30°C) and (b) *"Ab. sulfuroxidans"* Y002^T (at 45°C) in the presence and absence of 0.02% yeast extract. Key: SLC66^T with (●) and without (O) yeast extract; Y002^T with (▲) and without (Δ) yeast extract. Solid lines show total soluble iron concentrations and broken lines redox potentials (E_H) values (symbols show mean values and error bars range values of replicate cultures). The arrow in (b) shows the point at which (on day 12) sterile yeast extract solution was added to both the yeast extract-free and yeast extract-containing cultures. Non-inoculated control cultures showed little change in total soluble iron and redox potential during the time course of the experiments. Data points indicate mean values and error bars data range (n = 2).

“inorganic” cultures of Y002^T at day 12 resulted in rapid oxidation of the ferrous iron present, as indicated by a mean increase in redox potential of >200 mV during the following two days, and the initiation of pyrite dissolution (Fig. 3.8b). The addition of further yeast extract (at day 12) to cultures of Y002^T that had yeast extract added at the start of the experiment also resulted in more positive E_H and accelerated pyrite oxidation. Pyrite dissolution by *“Ab. sulfuroxidans”* Y002^T was noted to be about 40% less extensive than that observed in cultures of *Sb. thermosulfidooxidans*^{T} grown under identical conditions.

3.4.2.4 Tests for the reduction of ferric iron and zero-valent sulfur (ZVS)

3.4.2.4.1 Methods

Dissimilatory ferric iron reduction was assessed by growing cultures in FeYE liquid medium containing 10 mM Fe^{2+}, adjusted to either pH 2.0 (isolates of Group IA and IB; incubated at 30ºC) or 1.8 (isolates Group II; incubated at 45ºC). Shake flask cultures were incubated aerobically, until ferrous iron concentrations had fallen to <0.5 mM, at which point 20 mL aliquots were withdrawn from each and placed in 25 mL sterile bottles, further yeast extract added (to 0.02% w/v) and the replicate bottles placed in sealed jars under either anaerobic or micro-aerobic atmospheres (section 2.2.6). Samples were withdrawn after 2 and 4 days (moderate thermophiles) and 6 and 10 days (mesophiles) and concentrations of ferrous iron determined (section 2.3.1.2). Dissimilatory reduction of zero-valent sulfur (ZVS) was tested in cultures incubated anaerobically in FeYE media amended with 5 mM glucose and 0.5% (w/v) ZVS.

3.4.2.4.2 Results

All strains of “*Acidibacillus*” tested were able to catalyse the dissimilatory reduction of ferric iron under anaerobic conditions (Fig.3.9), though no reduction was observed in parallel cultures incubated under micro-aerobic conditions. The two strains of *“Ab. sulfuroxidans”* displayed the greatest propensity for iron reduction, and the two Group IB strains the least. None of the isolates were found to reduce ZVS.

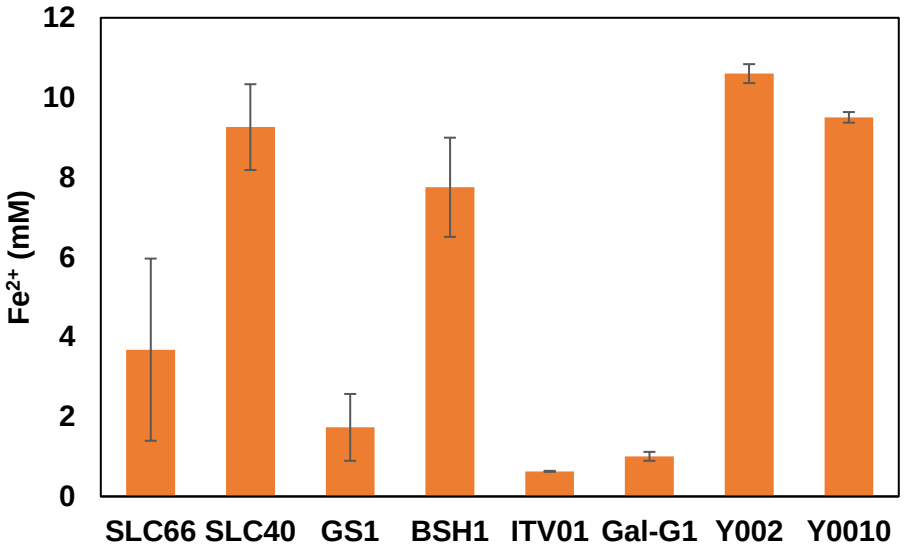

Figure 3.9. Dissimilatory reduction of ferric iron by strains of "*Acidibacillus*" spp. Cultures were grown aerobically in the presence of ferrous iron until near completion of iron oxidation, supplemented with yeast extract and placed into an anaerobic jar. The bar graphs show mean values of ferrous iron concentrations after 2 days (Y002^T and Y0010) or 6 days (all other cultures), and error bars depict range values of replicate cultures.

3.4.2.5 Oxidation of zero-valent sulfur (ZVS)

3.4.2.5.1 Methods

Dissimilatory oxidation of ZVS was tested by inoculating active cultures into Sm liquid medium (section 2.2.4) with or without 0.02% (w/v) yeast extract, and poised initially at ~pH 3.0. Since the end product of the reaction is sulfuric acid, both changes in pH and sulfate concentrations, as well as increases in cell numbers were used to monitor growth. Oxidation of tetrathionate was assessed by growing isolates Y002^T and Y0010 in Tm medium (section 2.2.4) pH 3 with (0.02%, w/v) or without yeast extract. Growth was monitored by enumerating cells and measuring changes in pH and sulfate concentrations.

3.4.2.5.2 Results

None of the six Group I (*"Ab. ferrooxidans"*) strains oxidized ZVS (Fig. 3.10). In contrast, pH declined and sulfate concentrations increased as a result of the dissimilatory oxidation of sulfur to sulfuric acid, in yeast extract-containing cultures of both Group II isolates (Y002^T and Y0010; Fig. 3.10 and Fig 3.11). Cell numbers of Y002^T and Y0010 did not correlate with oxidation of sulfur, which was thought to be due to attachment of cells to particulate ZVS. In contrast, numbers of both Y002^T and Y0010 increased in tetrathionate-containing media, paralleling changes in sulfate concentrations and culture pH (and was more pronounced in cultures of Y002^T), confirming that the two strains of *"Ab. sulfuroxidans"* can oxidize

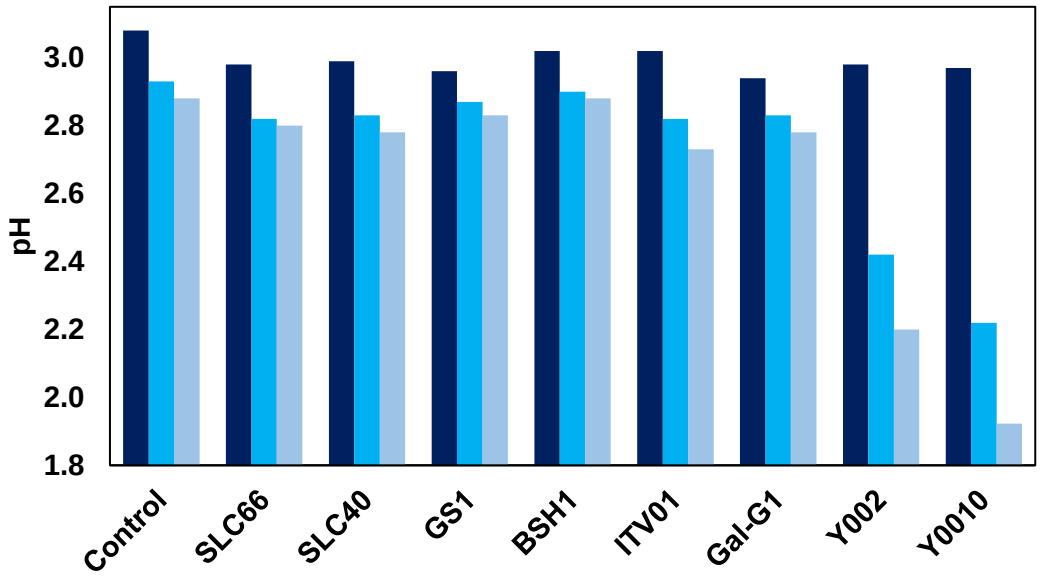

Figure 3.10. Acid production due to dissimilatory oxidation of zero-valent sulfur (ZVS) of "*Acidibacillus*" spp. Cultures were grown aerobically in Sm liquid medium supplemented with yeast extract. The bar graphs show pH values at time zero (■), after 20 days (■) and 28 days (■).

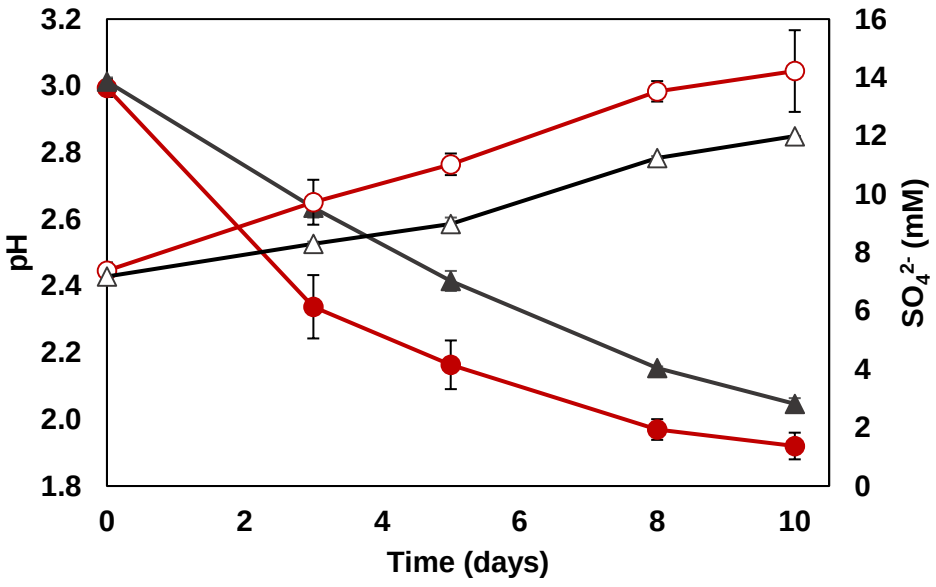

Figure 3.11. Acid and sulfate production due to dissimilatory oxidation of zero-valent sulfur by strains of *"Ab. sulfuroxidans"* grown in Sm liquid medium containing 0.02% (w/v) yeast extract. Key: changes in pH (solid symbols) and sulfate (open symbols) in cultures of Y002^T (●, o) and Y0010 (▲, Δ). Each data point shows the mean value of replicate cultures and error bars (where visible) denote ranges in the parameters measured.

tetrathionate as well as ZVS. No significant changes in pH and sulfate concentrations were found in in cultures of Y002^T and Y0010 that did not contain yeast extract or in non-inoculated controls.

3.4.3 Carbon metabolism

3.4.3.1 Tests for the ability of *"Acidibacillus"* spp. to grow by assimilation of inorganic carbon

3.4.3.1.1 Methods

To assess growth using CO_2 as a carbon source, an experiment was set up in a sealed jar with *"Ab. sulfuroxidans"* Y002^T incubated under elevated concentrations of CO_2 (CO_2-enhanced air), generated by placing a 20 mL universal bottle containing sodium bicarbonate (1.3 g), citric acid monohydrated (0.1 g) and water (10 mL) into an Oxoid™ AnaeroJar™ immediately before closure. Replicates containing Fe medium (25mM Fe^{2+}, section 2.2.4) amended with 0.005% (w/v) yeast extract were inoculated with active culture. Replicate cultures of *Sb. thermosulfidooxidans*^{T} in the same medium described previously were placed alongside the Y002^T cultures. Replicate cultures were set up in a separate jar containing a CO_2 sorbent solution (100 mL of 1 M NaOH). Both jars were shaken 100 rpm at 45ºC and residual ferrous iron and cell numbers were determined after 24 h.

A second experiment was set up using the cultures of *"Ab. sulfuroxidans"* Y002^T and *Sb. thermosulfidooxidans*^{T} from the first experiment as inoculum (Fig. 3.12). Two sealed jars (CO_2-enhanced air and air stripped of CO_2) were set up with replicates cultures containing 25 mM Fe^{2+} liquid medium, and no yeast extract. Both sealed jars were incubated at 45˚C and shaken at 100 rpm. Ferrous iron concentrations and cell numbers were determined for up to 4 days.

3.4.3.1.2 Results

In the first experiment, where all media were supplemented with yeast extract, cell numbers and oxidation of ferrous iron were similar in cultures incubated under elevated concentrations of CO_2 or in the absence of CO_2 (Fig. 3.13, Appendix 3D).

In the second experiment, where yeast extract-free medium was used, cell numbers and oxidation of ferrous iron were greater in cultures of *Sb. thermosulfidooxidans*^{T} incubated under elevated concentrations of CO_2 than both Y002^T cultures (CO_2-enhanced air and in the absence of CO_2) and in cultures of *Sb. thermosulfidooxidans*^{T} incubated in the absence of CO_2 (Fig. 3.14).

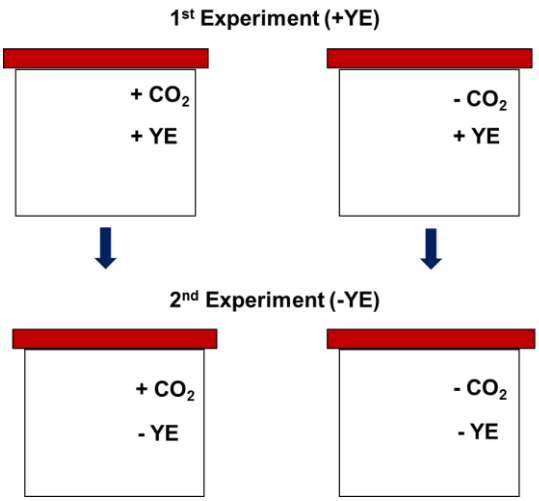

Figure 3.12. Schematic representation of experiments for growth by assimilation of inorganic carbon of *"Acidibacillus"* spp. In the first experiment, cultures were supplemented with yeast extract (+YE). The second experiment used yeast extract-free medium (-YE).

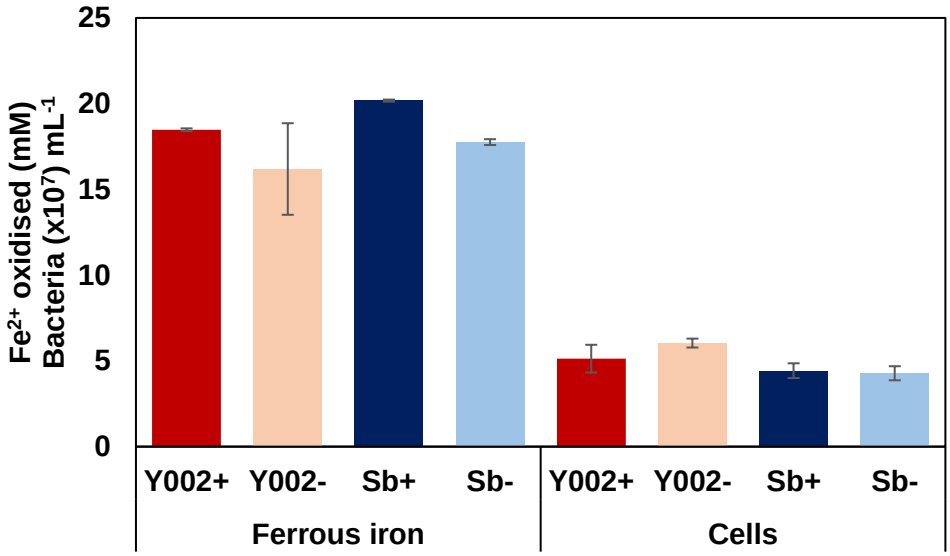

Figure 3.13. Tests for the ability of *"Ab. sulfuroxidans"* Y002^T to grow by assimilation of inorganic carbon. Cultures were grown in Fe liquid medium amended with 0.005% (w/v) yeast extract. The bar graphs show mean values of Fe^{2+} oxidised (mM) and cell numbers. "Y002+" refers to cultures of *"Ab. sulfuroxidans"* Y002^T grown under elevated concentrations of CO_2; "Y002-" refers to cultures incubated in the absence of CO_2 ; "Sb+" refer to cultures of *Sb. thermosulfidooxidans*^{T} incubated under elevated concentrations of CO_2 and "Sb-" refers to cultures incubated in the absence of CO_2. The bar graphs show mean values and error bars depict range values of replicate cultures.

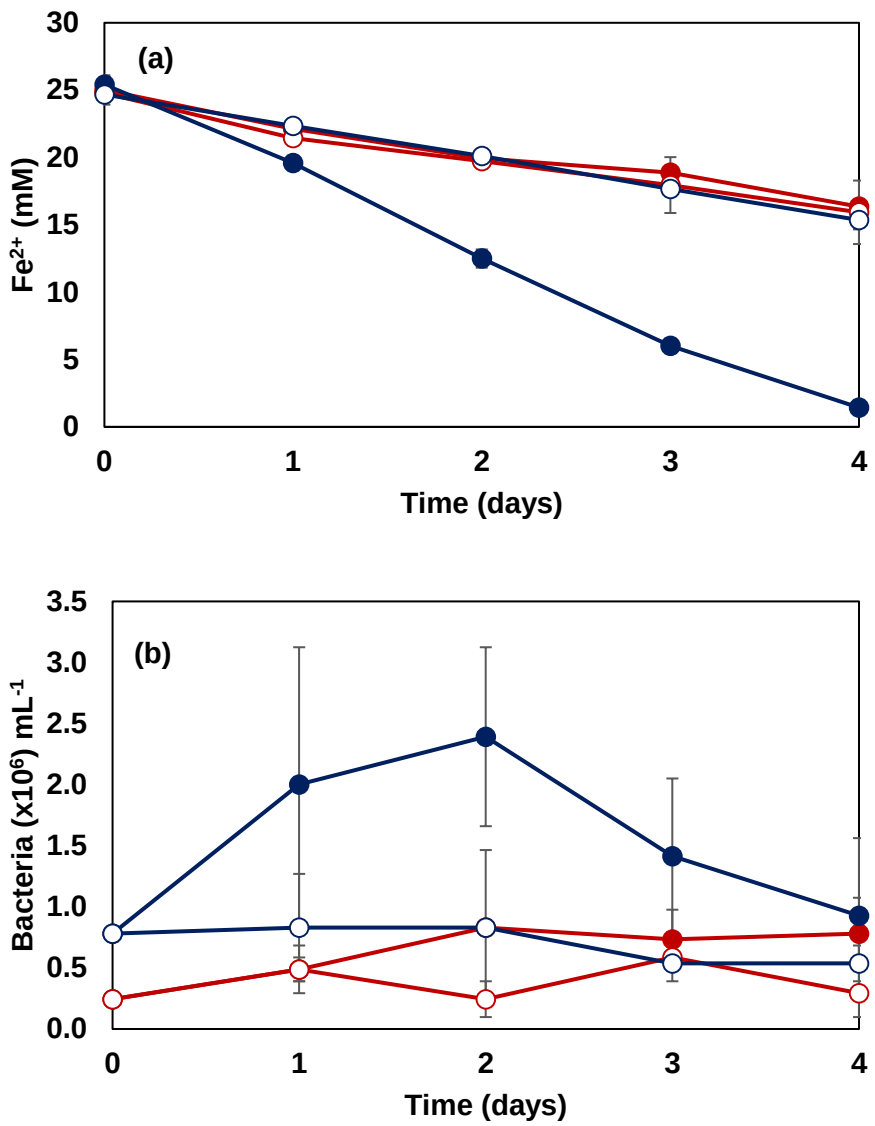

Figure 3.14. Tests for the ability of *"Ab. sulfuroxidans"* Y002^T to grow by assimilation of inorganic carbon. Data points indicate mean values of Fe^{2+} concentrations (a) and cell numbers (b) of cultures grown in Fe liquid medium in the absence of yeast extract. Key: *"Ab. sulfuroxidans"* Y002^T cultures incubated under elevated concentrations of CO_2 (●) or in the absence of CO_2 (o); *Sb. thermosulfidooxidans*^{T} cultures incubated under elevated concentrations of CO_2 (●) or in the absence of CO_2 (o). Error bars depict range values of replicate cultures.

3.4.3.2 Test for the potential requirement of reduced sulfur compounds for growth of "*Acidibacillus*" spp.

Previous work with strains of *Sulfobacillus* (Marsh and Norris, 1983; Wood and Kelly, 1983; 1984) have shown that autotrophic growth on ferrous iron required a source reduced sulfur. An experiment was set up to ascertain whether the requirement for yeast extract by the two nominated type cultures of "*Acidibacillus*" was due to the latter providing a source of reduced sulfur. Test were carried out using both an inorganic (tetrathionate) and organic (cysteine) sulfur source.

3.4.3.2.1 Methods

The potential requirement of reduced sulfur compounds for growth of "*Acidibacillus"* spp. was determined by growing cultures in Fe liquid medium (section 2.2.4) at pH 1.8 or 2.0, containing 10 mM Fe^{2+}, in the absence of yeast extract. Replicate flasks amended with 0.02% (w/v) yeast extract were set up to act as ~~a~~ positive controls. In the 3 days after the start of the experiment, one of the replicate flasks containing no yeast extract was amended with potassium tetrathionate (final concentration, 0.1 mM) and growth monitored. After a further 4 days of incubation, the other replicate flask containing no yeast extract was supplemented with 0.1 mM L-cysteine. Finally, at the eleventh day of experiment, flasks amended previously with tetrathionate and L-cysteine were also amended with 0.02% (w/v) yeast extract. Cultures were incubated at 30ºC for SLC66^T and 45ºC for Y002^T, shaken at 100 rpm and residual ferrous iron and cell numbers were determined (section 2.3.1).

3.4.3.2.2 Results

Cultures not containing yeast extract of both nominated type strains of "*Acidibacillus"* were able to oxidise ferrous iron, although much slower than those containing yeast extract (Fig 3.15 and 3.16). However, no increase in cell numbers occurred in cultures where no yeast extract had been added, and addition of neither inorganic (tetrathionate) nor organic (cysteine) reduced sulfur resulted in any changes in iron oxidation or cell growth. In cultures of *"Ab. sulfuroxidans"* Y002^T that had been amended with tetrathionate, addition of 0.02% (w/v) yeast extract at day 11 resulted in accelerated iron oxidation and increased cell numbers, although cell numbers in cultures initially amended with yeast extract were much greater.

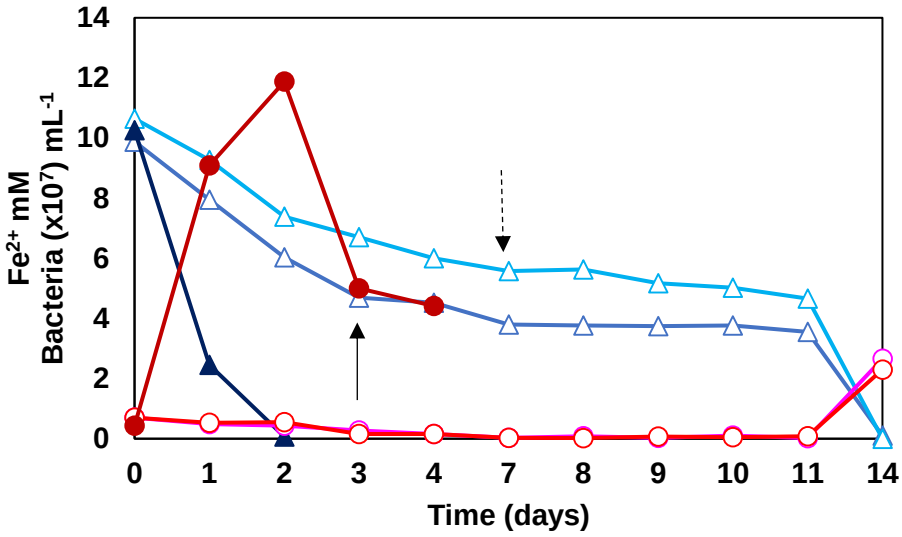

Figure 3.15. Test for the potential requirement of reduced sulfur compounds for growth of *"Ab. sulfuroxidans"* Y002^T. Cultures were grown in Fe liquid medium in the presence (closed symbols) and absence (open symbols) of 0.02% (w/v) yeast extract. Key: Fe^{2+} concentrations (▲, Δ, Δ) and cell numbers (●, o, o). Culture amended with 0.1 mM $K_2S_4O_6$ (Δ, o) at day 3 (solid arrow) and culture amended with 0.1 mM L-cysteine (Δ, o) at day 7 (broken arrow). At day 11 yeast extract (0.02%, w/v) was added to cultures previously without yeast extract.

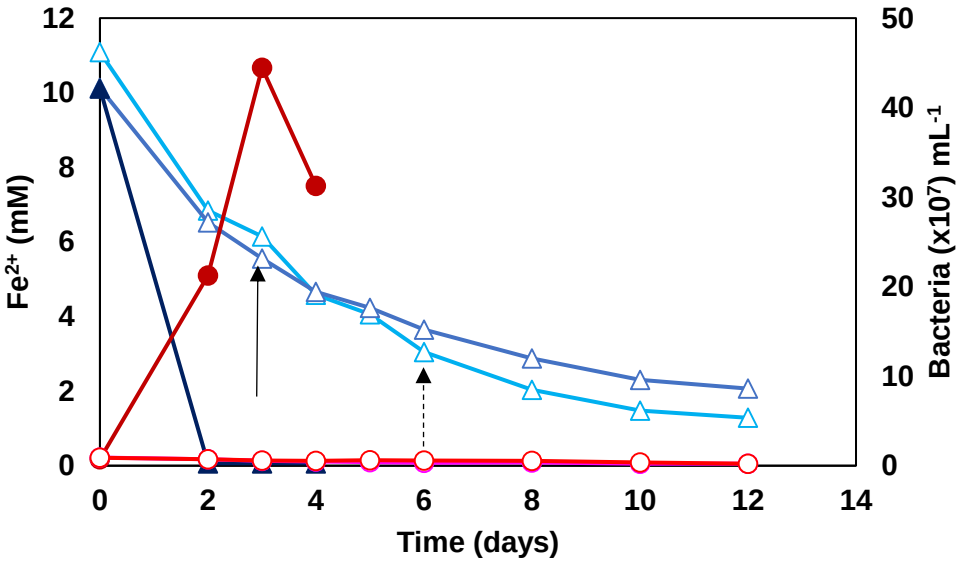

Figure 3.16. Test for the potential requirement of reduced sulfur compounds for growth of *"Ab. ferrooxidans"* SLC66^T. Cultures were grown in Fe liquid medium in the presence (closed symbols) and absence (open symbols) of 0.02% (w/v) yeast extract. Key: Fe^{2+} concentrations (▲, Δ, Δ) and cell numbers (●, o, o). Culture amended with 0.1 mM $K_2S_4O_6$ (Δ, o) at day 3 (solid arrow) and culture amended with 0.1 mM L-cysteine (Δ, o) at day 6 (broken arrow). At day 11 yeast extract (0.02%, w/v) was added to cultures previously without yeast extract.

3.4.3.3 Effect of different concentrations of yeast extract on the growth of "*Acidibacillus*" spp.

3.4.3.3.1 Methods

The effect of different concentrations (0, 0.005, 0.02 or 0.5%, w/v) of yeast extract on cell yields was examined in replicate flasks with Fe liquid medium (10 mM Fe^{2+}, section 2.2.4), adjusted to either pH 2.0 (for strains of *"Ab. ferrooxidans"* SLC66^T) or 1.8 (for strains of *"Ab. sulfuroxidans"* Y002^T). Cultures were incubated (at 30ºC for SLC66^T and 45ºC for Y002^T), shaken at 100 rpm, and residual ferrous iron and cells enumerated daily for up to 6 days.

3.4.3.3.2 Results

Cell numbers of both SLC66^T and Y002^T correlated (r = 0.97) with initial concentrations of yeast extract (Fig. 3.17), though those of SLC66^T were greater (for the same concentration of yeast extract) than those of Y002^T, due in part to more pronounced cell aggregation of the latter. In cultures of both SLC66^T and Y002^T containing 0.05% (w/v) of yeast extract, cell numbers were found to continue to increase beyond the point at which ferrous iron had been fully oxidized. However, at smaller concentrations (0.005% w/v), cell yields of both strains increased in parallel with the amount of ferrous iron oxidized.

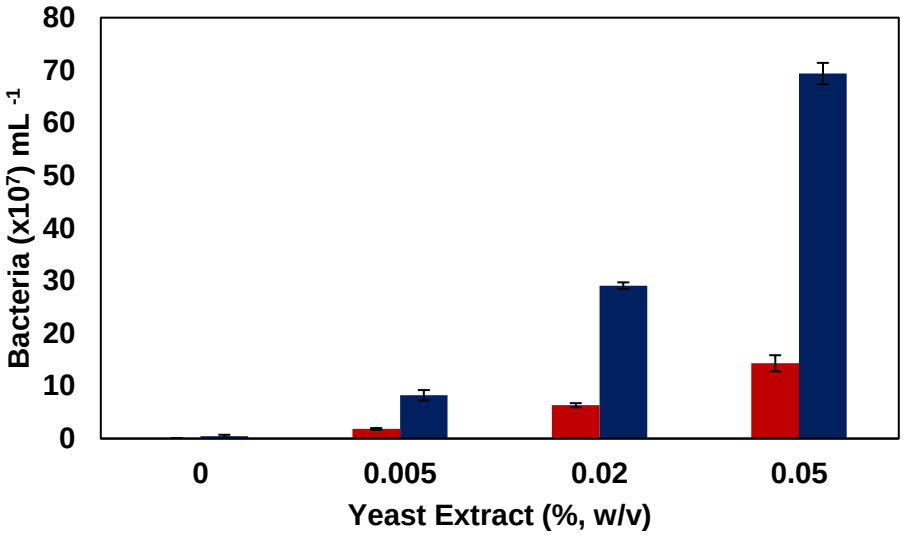

Figure 3.17. Effect of yeast extract concentrations on cell numbers of *"Ab. ferrooxidans"* SLC66^T (■) and *"Ab. sulfuroxidans"* Y002^T (■). Cultures were grown in liquid media containing 10 mM ferrous iron and different concentrations of yeast extract. Cells were enumerated after 2 days in cultures of Y002^T, and 4 days in cultures of SLC66^T. Bars indicate mean values and the error bars data range (n = 2).

3.4.3.4 Comparative growth using other complex or defined small molecular weight organic compounds

3.4.3.4.1 Methods

Growth was assessed in 1 mM Fe^{2+} liquid medium (section 2.2.4) supplemented with either casein hydrolysate or tryptone (both at 0.02%, w/v), or defined small molecular weight organic compounds. With the latter, liquid media also contained 0.005% (w/v) yeast extract. The latter were: (i) monosaccharides (glucose, fructose, and maltose, all at 5 mM); (ii) alcohols (15 mM ethanol, 10 mM glycerol and 5 mM mannitol); (iii) organic acids (citric acid and lysine, both at 5 mM); (iv) benzyl alcohol (5 mM). Tests contained different concentrations of defined organic compounds in order to provide similar amounts of organic carbon. In addition, comparative growth yields of all isolates (in triplicate cultures) were performed using liquid medium containing ABS (section 2.2.1) and TE (section 2.2.2) pH 2.0 (Group IA and IB) or pH 1.8 (Group II) supplemented with: (i) 1 mM ferrous iron; (ii) 20 mM ferrous iron; (iii) 1 mM ferrous iron/5 mM glucose; (iv) 20 mM ferrous iron/5 mM glucose; (iv) 1 mM ferrous iron/0.005% yeast extract. Biomass yields were determined from regular counts of bacteria in liquid media over 3 - 5 day incubation period.

To compare growth of "*Acidibacillus*" spp. to that of a known heterotrophic acidophile in a glucose-containing medium, the mesophilic acidophile *Acidiphilium cryptum* SJH and "*Acidibacillus"* type strains ($SLC66^T$ and $Y002^T$) were cultivated on 0.5 mM Fe^{2+} medium (section 2.2.4) supplemented with 0.005% (w/v) yeast extract and glucose (0 or 5 mM). Concentrations of glucose and cells numbers were determined for up to 7 days.

3.4.3.4.2 Results

As shown previously (sections 3.4.4.1 and 3.4.4.2) for the type strains $SLC66^T$ and $Y002^T$, all other isolates identified as strains of "*Acidibacillus*" required provision of organic carbon for growth, and yeast extract appeared to be superior to all others tested for this purpose (Fig. 3.18). Addition of some complex and defined organic compounds to ferrous iron/yeast extract liquid media resulted in increased cell numbers of all of the "*Acidibacillus*" strains, though this was limited in scale (Appendix 3A).

Figure 3.18 compares cell numbers of all eight isolates grown in organic carbon-free and organic carbon-amended liquid media. While there were some differences displayed between the "*Acidibacillus*" strains, there were also some general trends, including that greater amounts of ferrous iron did not generally result in enhanced cell yields in organic carbon-free media, though they did in most cases where glucose was also present. Cell numbers of all of the strains were also much greater in 1 mM ferrous iron medium containing 0.005% yeast extract than in those containing 5 mM glucose, even though the amount of organic carbon present in

the former (~ 25 mg/L assuming that yeast extract, like glucose, contains 40% C), was much less than in the latter (360 mg/L).

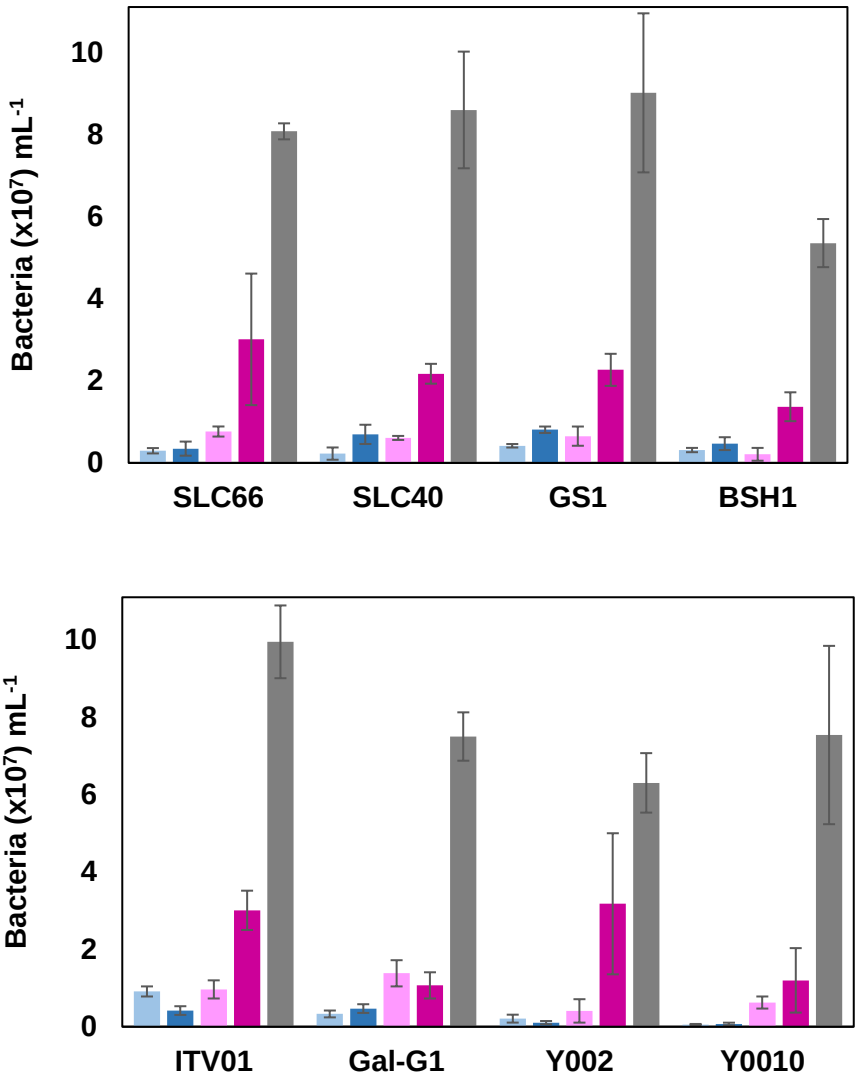

Figure 3.18. Cell counts of strains of "*Acidibacillus*" spp. grown in different liquid media; Key: (■) 1 mM Fe^{2+}; (■) 20 mM Fe^{2+}; (■) 1 mM Fe^{2+}/5mM glucose;(■) 20 mM Fe^{2+}/5 mM glucose; (■) 1 mM Fe^{2+}/0.005% yeast extract. Bars indicate mean values and error bars standard deviations (n = 3).

Comparative data for the mesophilic acidophiles SLC66^T and *A. cryptum* SJH (Fig. 3.19) show that numbers of the former were far fewer than those of *A. cryptum* SJH, when grown in identical glucose-containing liquid medium. Also, while all of the glucose provided was utilized in the *A. cryptum* SJH cultures within 3 days, only ~12% of the glucose in cultures of SLC66^T was metabolized. In the case of Y002^T, numbers were >50% greater in cultures containing glucose than in glucose-free controls, but only ~6% of the available glucose was utilized (Fig. 3.20). In the case of strain Y002^T, cell numbers were also significantly greater (by ~55% on

day 3 and ~500% on day 7) in ferrous iron/yeast extract cultures that contained glucose compared to those that did not, though again the amount of glucose consumed was relatively small (5.5% of that provided; concentrations, compensated for evaporative water loss, falling from 4.90 +/- 0.02 mM on day 0 to 4.62 +/- 0.06 on day 7).

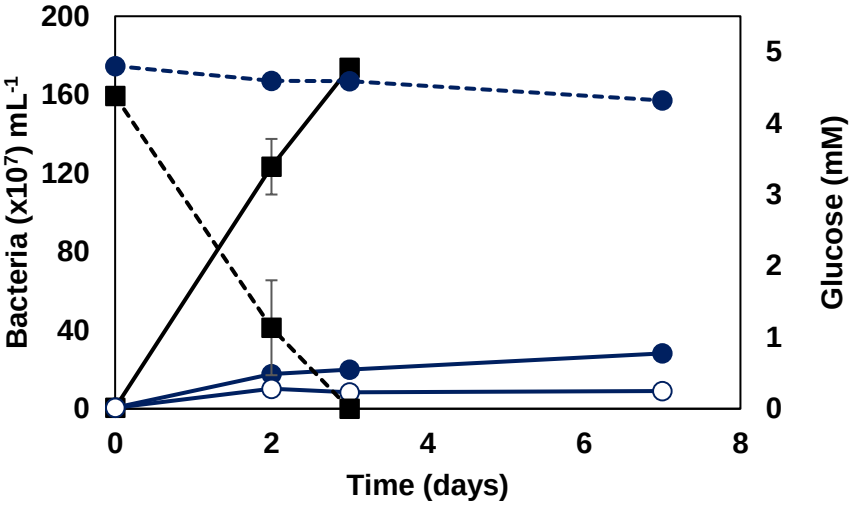

Figure 3.19. Comparison of changes in cell numbers (solid lines) and glucose concentrations (broken lines) in cultures of *"Ab. ferrooxidans"* SLC66^T and *A. cryptum* SJH. Key: cell numbers and glucose concentrations in cultures of *"Ab. ferrooxidans"* SLC66^T grown with (●) or without (o) 5 mM glucose; cell numbers and glucose concentrations in cultures of *A. cryptum* SJH grown with 5 mM glucose (■). Data points indicate mean values and error bars data range (n = 2).

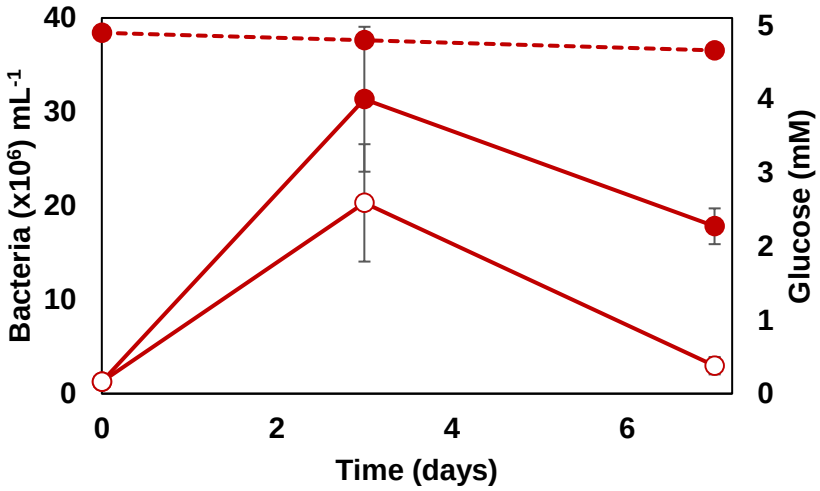

Figure 3.20. Comparison of changes in cell numbers (solid lines) and glucose concentrations (broken lines) in cultures of *"Ab. sulfuroxidans"* Y002^T. Key: cell numbers and glucose concentrations with (●) or without (o) 5 mM glucose. Data points indicate mean values and error bars data ranges (n = 2).

3.5 Chemotaxonomic analysis

3.5.1 Methods

The two proposed type strains of "*Acidibacillus"* (SLC66^T and Y002^T) were grown in FeYE liquid medium (10 mM Fe^{2+}, section 2.2.4, pH 2.0 and pH 1.8, respectively) and biomass were harvested and freeze-dried. Analysis of cellular fatty acids, polar acids, respiratory quinones and peptidoglycan structure were carried out by the *Deutsche Sammlung von Mikrooganismen und Zellkulturen* (DSMZ, Braunschweig, Germany).

3.5.2 Results

Frequently found within acidophilic gram-positive bacteria, the peptidoglycan structure of both isolates SLC66^T and Y002^T contained *meso*-diaminopimelic acid type A1γ (Johnson *et al.,* 2009*;* Kim *et al.,* 2014). The composition of respiratory quinones of both strains SLC66^T and Y002^T was exclusively MK-7(100%). The prevalence of MK7 in the respiratory quinones system is a feature found also in the species of *Alicyclobacillus* and *Bacillus* spp. (Mochida *et al.,* 2002) The two strains had also similar cellular fatty acid composition, having anteiso-$C_{15:0}$ as the most predominant type and anteiso-$C_{17:0}$ as the second. Comparison of the fatty acid composition of *"Acidibacillus"* and some other acidophilic *Firmicutes* is shown in Table 3.4. Strains SLC66^T and Y002^T contained significantly more anteiso-$C_{15:0}$ fatty acids than the three validated species listed, while all species, except *Alb. acidoterrestris* (which contained ω-alicyclic fatty acids) contain considerable proportion of anteiso-$C_{17:0}$. The polar acids distribution in the both type strains is shown in Table 3.5. Strains SLC66^T and Y002^T showed similar polar acids composition, except for the absence of phospholipid in the former.

Table 3.5. Polar acids detected in "*Acidibacillus"* spp. grown in FeYE liquid medium.

Polar acids	SLC66^T	Y002^T
Aminolipid	-	-
Glycolipid	+	+
Phospholipid	-	+
Phosphatidylglycerol	+	+
Diphosphatidylglycerol	+	+
Phosphatidylethanolamine	+	+

Table 3.4. Cellular fatty acids composition of *"Acidibacillus"* spp. and other species of acidophilic *Firmicutes* (average values of relative percentages).

	Composition (%)				
Fatty acid	**SLC66^T**	**Y002^T**	***Alb. pomorum*[a]**	***Alb. acidoterrestris*[a]**	***Sb. sibiricus*[bc]**
$C_{14:0}$			0.5	0-1.2	0.2
$C_{15:0}$	0.1	0.1	0.5	0-0.7	0.3
$C_{16:0}$	0.4	0.7	1.4	1.7-9.7	6.4
$C_{17:0}$	N.A	N.A	N.A	N.A	0.2
$C_{18:0}$	N.A	N.A	1.3	N.A	0.6
iso-$C_{14:0}$	0.6	1.9	N.A	N.A	N.A
iso-$C_{15:0}$	1.1	1.5	19.9	0.5 – 0.8	7.6
iso-$C_{16:0}$	6.7	14	18.3	0 – 0.5	10.3
iso-$C_{17:0}$	0.9	2.6	13.3	1.2 - 2	3.5
iso-$C_{18:0}$	N.A	0.5	N.A	N.A	0.6
iso-$C_{19:0}$	N.A	N.A	N.A	N.A	0.8
anteiso-$C_{15:0}$	62.9	40.2	9.9	0.5 – 1.1	11.3
anteiso-$C_{17:0}$	26.3	35.1	34.2	3.2 – 6.5	36.6
$C_{16:0}$ 2OH	N.A	N.A	N.A	N.A	0.3
iso-$C_{16:0}$ 2OH	N.A	N.A	N.A	N.A	0.8
$C_{16:1}$	N.A	N.A	N.A	N.A	0.4
$C_{16:1}$ ω7c alcohol	N.A	0.3	N.A	N.A	N.A
$C_{17:1}$ ω6c	0.5	2	N.A	N.A	N.A
$C_{17:0}$ 2OH	0.4	0.2	N.A	N.A	5.2
$C_{19:0}$ CYCLO ω8c	N.A	0.3	N.A	N.A	N.A
$C_{17:0}$ ω-Cyclohexane	N.A	N.A	N.A	62.6 - 68.4	N.A
$C_{19:0}$ ω-Cyclohexane	N.A	N.A	N.A	18.9 - 23.9	N.A
iso-$C_{17:1}$ /anteiso-$C_{17:1}$	N.A	0.64[d]	N.A	N.A	N.A

[a] Reported by Goto *et al.* (2003) [b] Reported by Melamud *et al.* (2003); [c] Unidentified fatty acids correspond to 14.9%; [d] Summed feature corresponded to 0.64% of the total composition; N.A, not applicable.

3.6 Discussion

The bacteria described in this report were isolated from geothermal and mine-impacted sites from different parts of the world. The fact that other closely related acidophiles have also been isolated (Appendix 3B) and clones (Appendix 3C) identified from sites all over the world suggests that "*Acidibacillus*" spp. are very widely distributed in extremely acidic environments. The first reported strains ("SLC series") were all described as obligately heterotrophic, mesophilic iron-oxidizing acidophiles, and were noted to be only distantly phylogenetically related to other *Firmicutes* (Johnson *et al.,* 2001). Six other phylogenetically-related isolates that have since then been added to the *Acidophile Culture Collection* at Bangor University since then were studied alongside two of the original "SLC series" strains in the present study. While the eight strains shared a number of physiological traits, there were also some significant differences. For instance, cells of *"Ab. sulfuroxidans"* Y002^T were larger (3 - 4 µm long and ~0.5 µm wide) than *"Ab. ferrooxidans"* SLC66^T (1.5 – 1.8 µm long, ~0.4 µm wide), and individual cells of Y002^T declined after few days of incubation (~2 days) due to cells aggregation (Fig. 3.21), a trait much less apparent in cultures of SLC66^T.

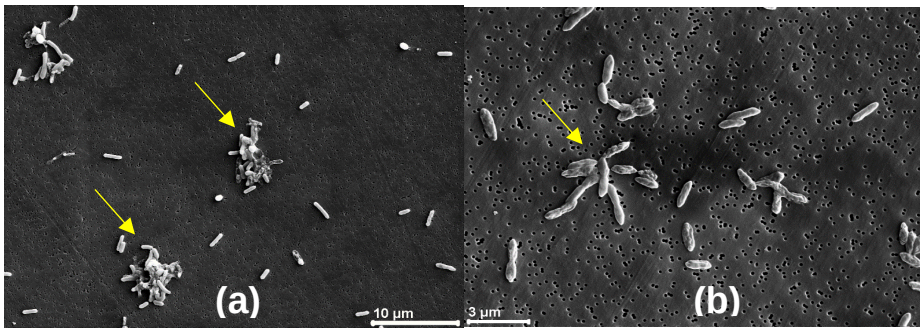

Figure 3.21. Scanning electron micrographs of cells of (a) *"Ab. sulfuroxidans"* Y002^T and (b) *"Ab. ferrooxidans"* SLC66^T (Holanda *et al.,* 2016). The arrows indicate aggregation of cells, which is typical feature of this species. Active cultures of strains SLC66^T and Y002^T were fixed in 2.5% glutaraldehyde followed by progressive ethanol dehydration. Fixed cultures were filtered through 0.2 µM pore size Nuclepore filters, and the immobilized bacteria critical point-dried and gold-coated, and were visualized using a Zeiss Sigma VP scanning electron microscope.

Comparison of 16S rRNA gene sequences clearly separated the eight strains studied, at the genus level, from currently classified acidophilic *Firmicutes*. While they formed a distinct clade, the fact that two of the isolates (Y002^T and Y0010) shared only 94% gene similarity with the other six confirmed that the isolates comprised two distinct species. Subsequent laboratory tests showed that these phylogenetic relationships were reflected in some marked differences in some key physiological characteristics, with the larger group being mesophilic iron-oxidizers

(*"Ab. ferrooxidans"*) and the smaller group moderately thermophilic iron- and sulfur-oxidizers that were more tolerant of extreme acidity (*"Ab. sulfuroxidans"*). Interestingly, phylogenetic analysis separated two strains of the larger group (strains ITV01 and Gal-G1) from the other four strains, even though the six strains shared ~99% gene similarity, and this was also reflected in some minor differences in their physiologies. For example, strains ITV01 and Gal-G1 grew at 40ºC, while the other four strains of *"Ab. ferrooxidans"* did not, both grew at pH 1.75 while only one Group IA strain (BSH1) grew at this pH value, and strains ITV01 and Gal-G1 were also the least effective of all eight strains at reducing ferric iron.

Following this study, two further isolates, S^0AB and Huett2, were identified as closely-related to "*Acidibacillus*" spp (Appendix 3B). From the phylogenetic analysis and the similarity of 16S rRNA genes shared between these isolates and species of "*Acidibacillus",* it is possible to infer that strain S^0AB represents a novel species of "*Acidibacillus"* and isolate Huett2 a strain of "*Ab. ferrooxidans*".

The three major physiological traits shared by all of the isolates studied were: (i) optimum growth at extremely low (< 3) pH, (ii) the ability to catalyse the dissimilatory oxidation of ferrous iron, and (iii) a requirement of organic carbon for growth. All of the isolates also catalysed the dissimilatory reduction of ferric iron under anoxic conditions though, as noted, this was limited in the case of the two Group IB strains, and it was not ascertained whether the bacteria could grow by ferric iron respiration. Ferrous iron is a widely used electron donor among acidophilic prokaryotes, due to it often being present in large concentrations in low pH environments, and also chemically stable at pH < 3 (Johnson and Aguilera, 2016). The ability to oxidize ferrous iron does not necessarily imply that microorganisms are able to conserve the energy from this reaction. However, the observation that cell numbers of both $SLC66^T$ and $Y002^T$ increased in parallel with the amount of iron oxidized (in organic-lean media) strongly suggests that this is the case with "*Acidibacillus*" spp. The specific rates of ferrous iron oxidation recorded for "*Acidibacillus*" spp. were much lower than those reported for other iron-oxidizing acidophiles (Table 3.6).

Table 3.6. Specific rates of ferrous iron oxidation by "*Acidibacillus*" type strains and by other iron-oxidizing acidophiles reported previously (Johnson *et al.,* 2012).

Isolates/species	Ferrous iron oxidised (mg min^{-1} mg $protein^{-1}$)
Y002^T	48.5 ± 1.3
SLC66^T	36.1 ± 3.4
***Acidithiobacillus* spp.**	192 - 484
***Leptospirillum* spp.**	426 - 484
***Sulfobacillus* spp.**	236 - 449

The two *"Ab. sulfuroxidans"* strains (Y002^T and Y0010) also catalysed the dissimilatory oxidation of both zero-valent sulfur and tetrathionate, and it was assumed (though not confirmed) that they also conserved the energy from these reactions. The ability to oxidize both ferrous iron and sulfur is not uncommon among chemolitho-autotrophic and chemolitho-heterotrophic acidophiles, and has been reported for some *Acidithiobacillus* spp. (*At. ferrooxidans, At. ferridurans, At. ferrivorans* and *At. ferriphilus*; Falagán and Johnson, 2016), *Acidihalobacter prosperus* (Cárdenas *et al.,* 2015), *Acidiferribacter thiooxydans* (Hallberg *et al.,* 2011) and "*Acidithiomicrobium*" (Norris *et al.,* 2011). Among the acidophilic *Firmicutes*, all classified *Sulfobacillus* spp. (*Sb. thermosulfidooxidans, Sb. acidophilus, Sb. thermotolerans, Sb. benefaciens,* and *Sb. sibiricus*) (Johnson and Aguilera, 2016), as well as *Alicyclobacillus tolerans* and *Alb. aeris* (Guo *et al.,* 2009) can oxidize both ferrous iron and reduced sulfur. Other species of acidophilic bacteria (e.g. *Leptospirillum ferrooxidans, Ferrimicrobium acidiphilum, Acidimicrobium ferrooxidans, Acidithrix ferrooxidans* and "*Ferrovum myxofaciens*") catalyse the dissimilatory oxidation of ferrous iron but not sulfur (Johnson and Aguilera, 2015) as was the case with the six strains of *"Ab. ferrooxidans"*.

Yeast extract acted as both an energy and carbon source for these bacteria, as evidenced by: (i) growth continuing in cultures well after all of the ferrous iron had been depleted, (ii) growth yields correlating with concentrations of yeast extract provided (in cultures containing relatively little ferrous iron) and (iii) active growth in yeast extract/ferric iron media. "*Acidibacillus*" spp. can therefore be classified as facultative chemolitho-heterotrophs (i.e. they can obtain energy from both inorganic and organic electron donors but require an organic carbon source). It was noted that cell yields of *"Ab. ferrooxidans"* SLC66^T tended to be greater than those of *"Ab. sulfuroxidans"* Y002^T in liquid media that contained the same concentrations of yeast extract. This was thought to be due, at least in part, to strain SLC66^T being able to utilize a wider range

of organic compounds present in this complex material, as both carbon and energy sources, than strain Y002^T. Cell yields of both SLC66^T and Y002^T were significantly greater when glucose was added to ferrous iron/yeast medium, suggesting that it was metabolized to some extent. In contrast to those of the heterotrophic acidophile, *Acidiphilium* SJH, only small amounts (5.5 - 6%) of the available glucose was utilized in these cultures, suggesting that this compound served as a carbon source, but not an energy source, for "*Acidibacillus*" spp., and that growth was ultimately limited by the energy source available (ferrous iron, and that fraction of yeast extract that could be broken down to generate ATP) in these cultures. Glucose also acted as a carbon source for "*Acidibacillus*", but was far less effective than yeast extract. For most strains, growth yields in glucose-containing media was limited by the availability of ferrous iron, suggesting again that the later served as the sole or main energy source, and glucose as the carbon source for these bacteria.

The tolerance of "*Acidibacillus*" spp. to some transition metals, aluminium and sodium chloride reported by Holanda *et al.* (2016) is shown in Table 3.7. In general, strains belonging to *"Ab. ferrooxidans"* had greater tolerance of most of the metals tested than the two *"Ab. sulfuroxidans"* isolates. The proposed type stain of *"Ab. ferrooxidans"* (SLC66^T) had a lower MIC for copper than the other strains belonging to the same species. Strain BSH1 displayed less tolerance of copper, but had by far the highest tolerance threshold for cobalt compared to the other strains of this species. Strains of *"Ab. sulfuroxidans"* were far more sensitive to copper and cobalt than the two *"Ab. ferrooxidans"* strains. None of the isolates were halotolerant, though both strains of *"Ab. sulfuroxidans"* were able to grow in liquid media containing higher concentrations of sodium chloride than the six strains of *"Ab. ferrooxidans"* tested.

Data from the preliminary annotation of the genomes of the strains SLC66^T (Ñancucheo *et al*, 2016), ITV01 (Dall'Agnol *et al.*, 2016) and Y002^T (I. Ñancucheo, personal communication) showed that they had G+C contents of 52%, 50% and 46%, respectively. The genomes of strains SLC66^T and ITV01 contained genes with relatively low (34% and 36%, respectively) similarity to the *cbbL* gene (which encodes the large subunit of RuBisCO form IA, involved in CO_2 assimilation), but not the *cbbM* gene (which encodes the large subunit for type II RuBisCO). Tests with cultures not amended with yeast extract and tests under enhanced CO_2 atmosphere confirmed that none of the strains could grow in the absence of an organic form of carbon. No gene identified as being necessary for nitrogen fixation were identified in the three genomes, but BLAST searches revealed a low sequence identity (36%) for the gene encoding for rusticyanin (a protein involved in ferrous iron oxidation in the iron-oxidizing acidithiobacilli and some other acidophiles) in the genomes of the three "*Acidibacillus*" strains. Intriguingly, there are no reports to date that other iron-oxidizing *Firmicutes* have genes

encoding rusticyanin. Further annotation of the genomes of these bacteria will undoubtedly throw more light on the biochemical constraints that restrict glucose utilization by these novel acidophiles.

In theory, any acidophilic bacterium that catalyses the dissimilatory oxidation of ferrous iron should accelerate the oxidative dissolution of pyrite, as ferric iron is the primary oxidant of this mineral in acidic liquors (Vera *et al.,* 2013). This was the case with both "*Acidibacillus*" type species, though it was not immediately apparent for strain Y002^T. Autoclaving pyrite in the presence of yeast extract (as is common practice in the Bangor research laboratories, and has not previously proven problematic) generated some, currently unidentified, by-product that inhibited growth and iron oxidation by strain Y002^T, though not by strain SLC66^T or *Sb. thermosulfidooxidans*. Adding sterile yeast extract after heat-sterilization of pyrite eliminated this impediment, though pyrite leaching by Y002^T was far less effective, and appeared to require more yeast extract, than *Sb. thermosulfidooxidans*.

The major industrial use of iron- and sulfur-oxidizing acidophilic bacteria is in the commercial bio-processing of sulfide mineral ores to extract and recover base and precious metals ("biomining"; Johnson, 2014). Whether or not "*Acidibacillus*" spp. have a potential role in mineral bioleaching consortia has yet to be evaluated. Both species could, in theory, carry out two critical roles (those of regenerating ferric iron and removing potentially inhibitory organic carbon) and *"Ab. sulfuroxidans"* strains could also contribute to the process by generating sulfuric acid. Another important required characteristic – that of being able to tolerate highly elevated concentrations of transition and other metals – also appears to be adequate, as the data obtained showed that metal tolerance is similar to that of most of the iron-oxidizing *Acidithiobacillus* spp.. A more significant constraint, however, may be their tolerance to extreme acidity, as many biomining practices operate at pH values < 2, and often (in stirred tanks) at ~ pH 1.5. Mesophilic *"Ab. ferrooxidans"* may, however, play a more important role in the natural attenuation of acidic (pH >2) ferruginous mine waters by catalysing the oxidation of ferrous iron and thereby facilitating the hydrolysis and precipitation of ferric iron (Blowes *et al.,* 2014).

Table 3.7. Tolerance of strains of "*Acidibacillus*" to selected metals and chloride reported by Holanda *et al.,* 2016. The values (in mM) shown are minimum inhibitory concentrations (MICs) and those in parentheses are the highest concentrations of that metals/chloride where growth was observed.

Strain	Cu	Zn	Ni	Co	Al	Mn	Fe(II)	Mo	Cl
SLC66^T	300 (200)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	800 (600)	<0.05	100 (50)
SLC40	600 (400)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	600 (400)	<0.05	50 (25)
BSH1	400 (200)	1000 (800)	400 (200)	>600	1000 (800)	800 (600)	800 (600)	<0.05	100 (50)
GS1	800 (600)	800 (600)	400 (200)	300 (200)	600 (400)	800 (600)	600 (400)	<0.05	100 (50)
ITV01	600 (400)	1000 (800)	400 (200)	300 (200)	800 (600)	>800	800 (600)	<0.05	100 (50)
G1	800 (600)	600 (400)	400 (200)	300 (200)	800 (600)	600 (400)	800 (600)	<0.05	50 (25)
Y002^T	100 (50)	200 (100)	200 (100)	50 (30)	400(300)	800 (600)	600 (400)	<0.05	250 (100)
Y0010	300 (200)	200 (100)	300 (200)	150 (100)	600 (400)	400 (300)	600 (400)	<0.05	250 (100)

Chapter 4. Dissimilatory reduction of zero-valent sulfur by acidophilic *Firmicutes*

4.1 Introduction

Many studies have found evidence of bacterial sulfidogenesis at extremely low pH in natural and engineered ecosystems (Ñancucheo and Johnson, 2012; Kimura *et al*., 2006; Koschorreck, 2008 and section 1.1.2.5.1). However, there are only few reports describing acidophilic ZVS- and sulfate-reducing bacteria. *Desulfosporosinus acidiphilus* and *Desulfosporosinus acididurans* are moderately acidophilic sulfate-reducing bacteria that are able to grow at pH 3.5 and to reduce ZVS (Kimura *et al*., 2006; Alazard *et al.,* 2010; Sanchez-Andrea *et al.,* 2015) while the latter was reported to represent ~6% of the bacterial population in a bioreactor maintained at pH 2.4 (Ñancucheo and Johnson, 2012). Moreover, studies reported that strains of the most widely studied acidophilic bacteria, *Acidithiobacillus ferrooxidans*, were able to reduce ZVS at pH 2.5 (Jameson *et al.,* 2010) and at pH 1.3 (Ohmura *et al.,* 2002). However, in the study of Jameson and colleagues a non-inoculated control also generated H_2S, though at lower rates than that measured for *At. ferrooxidans*. More recently, a novel *Deltaproteobacteria, Desulfurella amilsii,* was shown to reduce ZVS at low pH (growth occurred at pH 3 – 7 with optimum at pH 6 - 6.5; Florentino *et al*., 2016a). This strain, an acid-tolerant ZVS-reducer, was obtained from a low pH enrichment containing ZVS and sediment from the Tinto river, a naturally acidic metal-rich environment. Currently, the only acidophiles that grow optimally at pH < 3 which have been shown unambiguously to catalyse the dissimilatory reduction of ZVS are archaea (e.g. *Acidianus* spp., *Stygiolobus azoricus*, *Sulfurisphaera ohwakuensis*).

This Chapter describes research related to the dissimilatory reduction of ZVS in acidic conditions by acidophilic bacteria. The first part describes the characteristics of a new isolate, *Firmicute* strain I2511, an acidophilic ZVS-respiring bacterium isolated from sediment from an acidic stream in an abandoned copper mine in UK. The second section describes tests carried out with strains of moderate acidophilic and acid-tolerant sulfate-reducing bacteria to determine whether they could also grow by sulfur respiration at low pH.

4.2 Isolation and characterisation of *Firmicute* I2511

4.2.1 Enrichment cultures

Black sediments with a discernible hydrogen sulfide odour were collected from an acidic stream draining an abandoned copper mine, Mynydd Parys, in Anglesey, North Wales (53° 22' 59.9988" N; 4° 20' 60" W; Fig. 4.1).

Figure 4.1. Location of Mynydd Parys Copper Mine in Anglesey, North Wales.

The sediments were used to enrich acidophilic sulfur-reducers in chloride salts medium (CBS, section 2.2.1) using different types of zero-valent sulfur and electron donors. A two-step enrichment was performed. In the first (enrichment A), 10 g of the black sediment was added to 100 mL Src liquid medium (0.01% w/v, yeast extract; section 2.2.4) containing 5 mM glycerol and 1%, w/v sterile microbially pre-wetted ZVS (section 2.2.3). This enrichment was placed into an Oxoid™ AnaeroJar™ under an anaerobic atmosphere (section 2.2.6) and incubated at 30ºC, shaken at 50 rpm. Hydrogen sulfide production was detected after 12 days of incubation, and four new cultures were set up (enrichments B to E) inoculated with 1 % (v/v) of enrichment A, using the same liquid medium and incubation conditions of this enrichment (Table 4.1).

Table 4.1. Description of enrichments for acidophilic ZVS-reducers using sediment from the abandoned Mynydd Parys copper mine.

Enrichment	Electron acceptor	Electron Donor	Inoculum	H_2S production
A	pre-wetted ZVS	5mM glycerol	black sediments	yes
B	pre-wetted ZVS	5mM glycerol	enrichment A	yes
C	pre-wetted ZVS	5mM mannitol	enrichment A	yes
D	biosulfur[a]	5mM glycerol	enrichment A	no
E	biosulfur[a]	5mM mannitol	enrichment A	no

[a]medium contained 1 % (w/v) of biosulfur prepared as described in section 2.2.3

Enrichments containing biosulfur as electron acceptor (D and E) did not produce H_2S after ~50 days of incubation. Enrichments containing microbially pre-wetted ZVS (A to C) produced H_2S continuously after being sub-cultured several times.

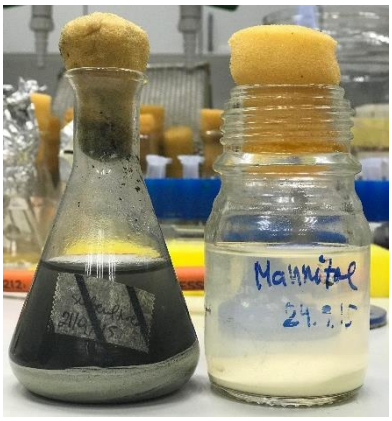

Figure 4.2. Enrichment A (left) inoculated with black sediment, containing microbially pre-wetted ZVS (1%, w/v) as electron acceptor and glycerol (5 mM) as electron donor; and enrichment C (right) inoculated with 1 % (v/v) of enrichment A and contained microbially pre-wetted ZVS (1%, w/v) as electron acceptor and mannitol (5 mM) as electron donor.

Biomass was collected from enrichments A to C and DNA extracted as described in section 2.6.1. Bacterial 16S rRNA genes from extracted DNA were amplified and digested with the restriction enzymes HaeIII, CfoI and AluI (sections 2.6.2 and 2.6.3). The bacterial communities of enrichments A, B and C were evaluated by T-RFLP analysis (Fig. 4.3 – 4.5). The bacterial population of enrichment A exhibited higher diversity than enrichments B and C. Enrichment A at 18 days of incubation displayed two dominant T-RFs, 202 nt and 297 nt in length, and low abundance (≤ 5% of relative abundance) T-RFs represented ~40 % of the bacterial population. At 36 days of incubation, the 297 nt T-RF could not be detected, the 215 nt T-RF

became more abundant (24% relative abundance) and two new T-RFs emerged: (i) a group of T-RFs (261 to 267 nt) that were represented by 266 nt and amounted to 4.5% relative abundance, and (ii) 300 nt T-RF with 6% relative abundance. However, by 59 days of incubation, the bacterial population had exhibited a shift, in which the 266 nt T-RF became the more predominant T-RF accounting for 45% of the summated T-RFs, a novel T-RF emerged (243 nt, 23% relative abundance) and the low abundance T-RFs represented only ~20 % of the bacterial population. The T-RFLP profile from enrichment B displayed low diversity compared to enrichment A, with T-RFs 266 nt and 220 nt comprising 94% of the population. At 47 days, 220 nt T-RF could not be detected, 266 nt T-RF represented 58% of the population and low abundance T-RFs accounted for ~30 %. The 243 nt T-RF also emerged in enrichment B with ~14% of relative abundance. Enrichment C was dominated by the 220 nt T-RF at 24 days of incubation, however the TRFLP profiles changed dramatically at 47 days, with two dominant T-RFs (138 nt and 209 nt) accounting for 99% of the summated T-RFs . Attempts to amplify archaeal genes from all enrichment cultures proved negative.

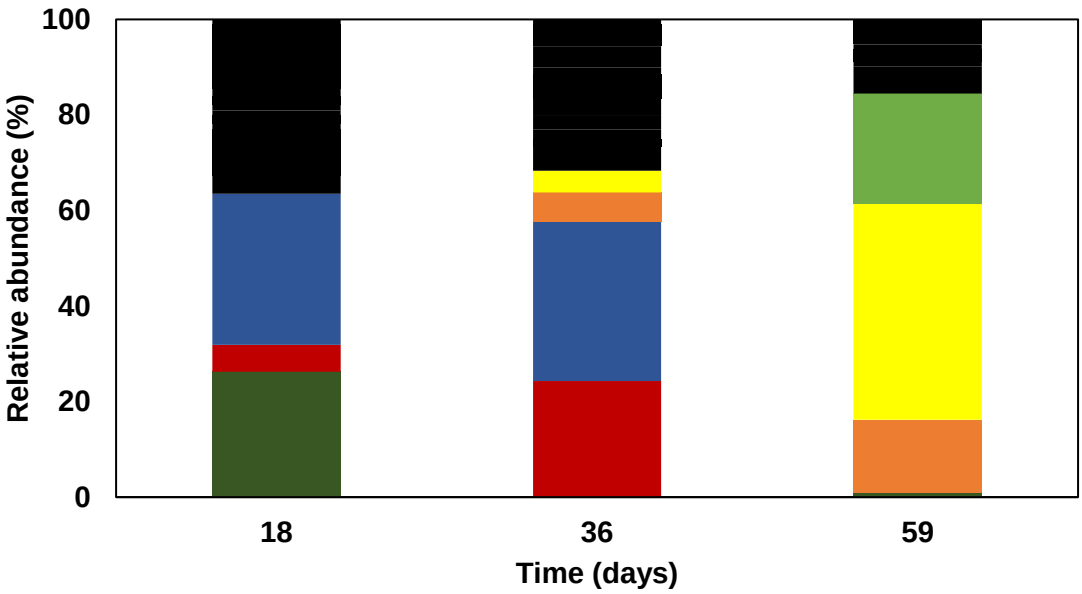

Figure 4.3. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts of enrichment A and digested with the restriction enzyme HaeIII. Key: (■) 266 nt T-RF (*Firmicute* strain I2511), (■) 243 nt T-RF, (■) 300 nt T-RF, (■) 202 nt T-RF, (■) 215 nt T-RF, (■) 297 nt T-RF (■) low abundance T-RFs (≤ 5% relative abundance).

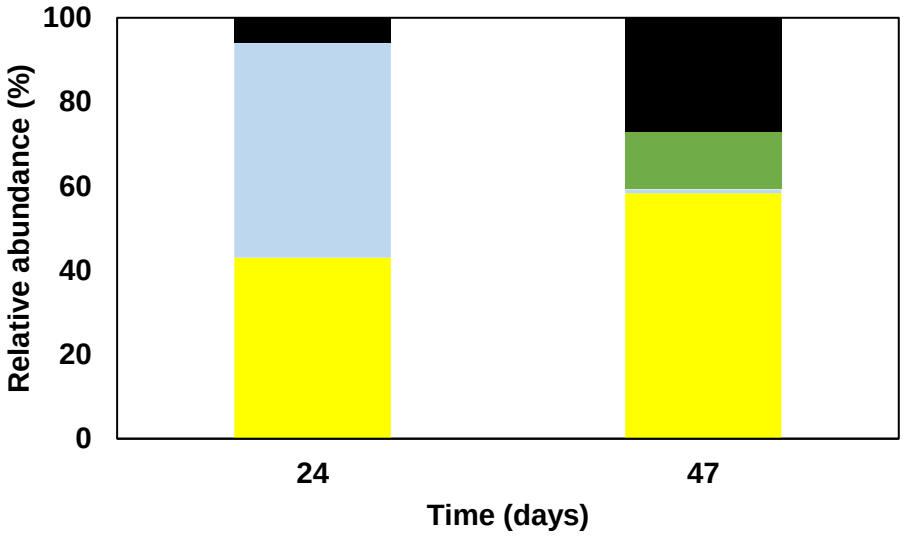

Figure 4.4. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts of enrichment B and digested with the restriction enzyme HaeIII. Key: (■) 266 nt T-RF (*Firmicute* strain I2511), (■) 220 nt T-RF, (■) 243 nt T-RF, (■) low abundance T-RFs (≤ 5% relative abundance).

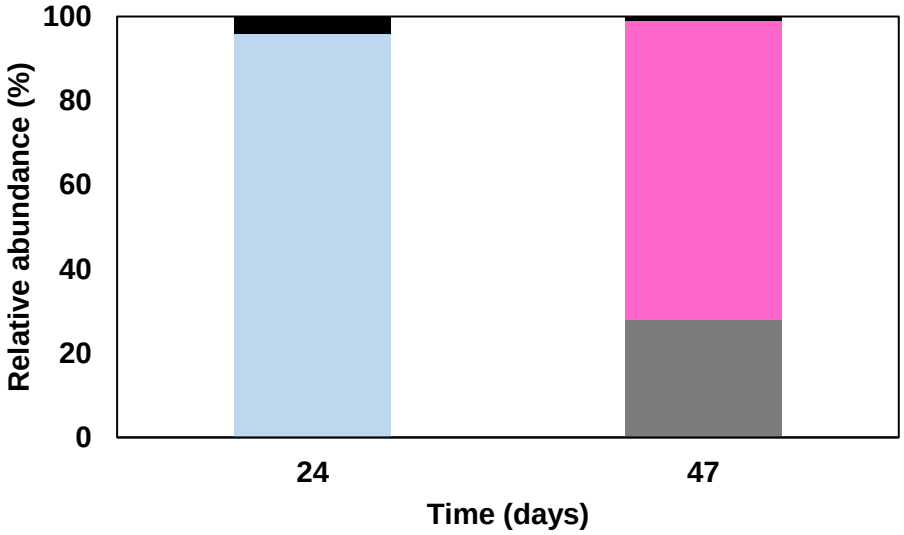

Figure 4.5. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts of enrichment C and digested with the restriction enzyme HaeIII. Key: (■) 220 nt T-RF, (■) 138 nt T-RF, (■) 209 nt T-RF (■) low abundance T-RFs (≤ 5% relative abundance).

4.2.2 Isolation, identification and cultivation of isolate I2511

4.2.2.1 Methods

Enrichments A to C were streaked onto aSRB plates (section 2.2.5), placed in sealed jars under anaerobic atmospheres (section 2.2.6) and incubated at 30ºC. Colonies were differentiated by their morphologies and purified by repeated re-streaking of single colonies onto fresh solid media. These were then transferred into universal bottles containing Src liquid medium (section 2.2.4) containing either 1 % (w/v) ZVS, 5 mM glycerol or 5 mM mannitol adjusted to pH 3.0 with hydrochloric acid, and incubated in sealed jars under anaerobic conditions at 30ºC.

Hydrogen sulfide production was determined by visualisation of CuS precipitates inside a "copper trap", which consisted of an open universal bottle containing 10 mL of ~ 15 mM $CuSO_4$ which was placed inside the sealed jar (Fig. 4.6).

The purity of liquid cultures was assessed by T-RFLP analysis (i.e. by displaying a single peak). Cells from liquid cultures were visualised using a Leitz Labolux phase contrast microscope (section 2.4.2).

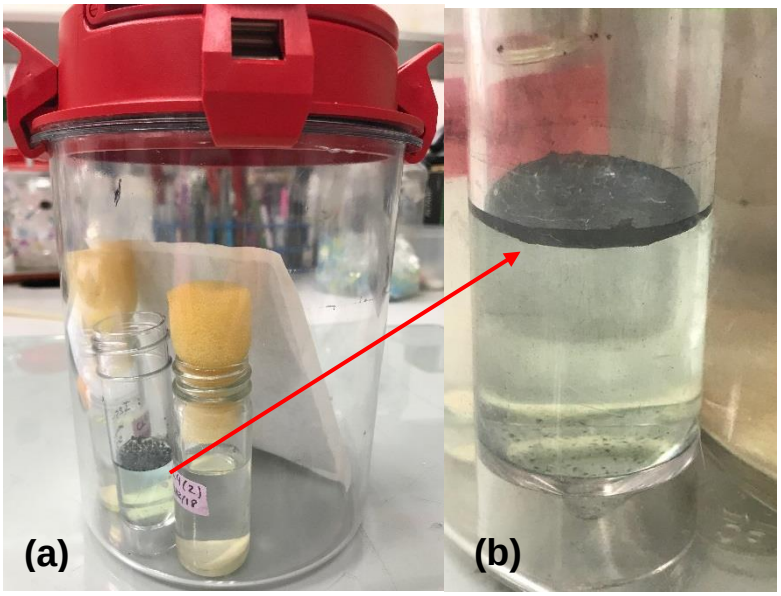

Figure 4.6. (a) Src cultures incubated anaerobically in a sealed jar, (b) universal bottle containing solution of $CuSO_4$ and CuS precipitation (black layer).

4.2.2.2 Results

Colonies that grew on aSRB plates inoculated with enrichment cultures (A, B and C) were not encrusted with ZnS, a characteristic that readily differentiate sulfate-reducers from anaerobes that do not reduce sulfate (Ñancucheo *et al.,* 2016). Generation of H_2S causes deposition of ZnS on the aSRB colonies, which can develop hard white/silver surface coats with prolonged incubation.

Of four liquid cultures obtained from the selected single colonies, only one culture (isolate I2511), which originated from enrichment B, generated H_2S. T-RFLP analysis confirmed purity of the culture. The restriction enzyme HaeIII generated terminal fragments with different sizes, ranging from 261 to 267 nt (Fig. 4.7), though this was not the case for the restriction enzymes Cfo1 and Alu1 where only single T-RFs were observed (Fig. 4.8 and 4.9). *In silico* digestion of partial and full length of 16S rRNA gene carried out with GeneDoc (version 2.7) indicated that digestion with HaeIII would generate T-RFs of 266 nt, 310 nt and 332 nt in length, but that multiple T-RFs would also be generated with CfoI (63 nt and 241 nt) and AluI (207 nt and 247 nt).

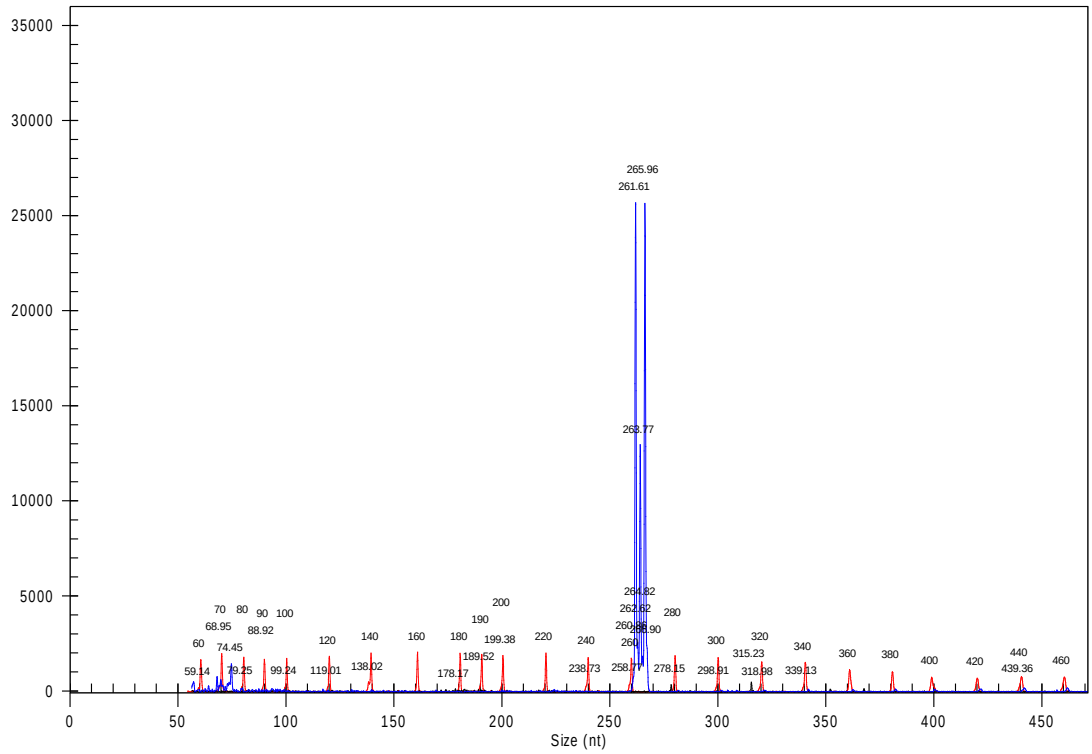

Figure 4.7. T-RFLP profile of isolate I2511 with 16S rRNA gene digested by restriction enzyme HaeIII. Profile shows several peaks between ~261 to 267 nt.

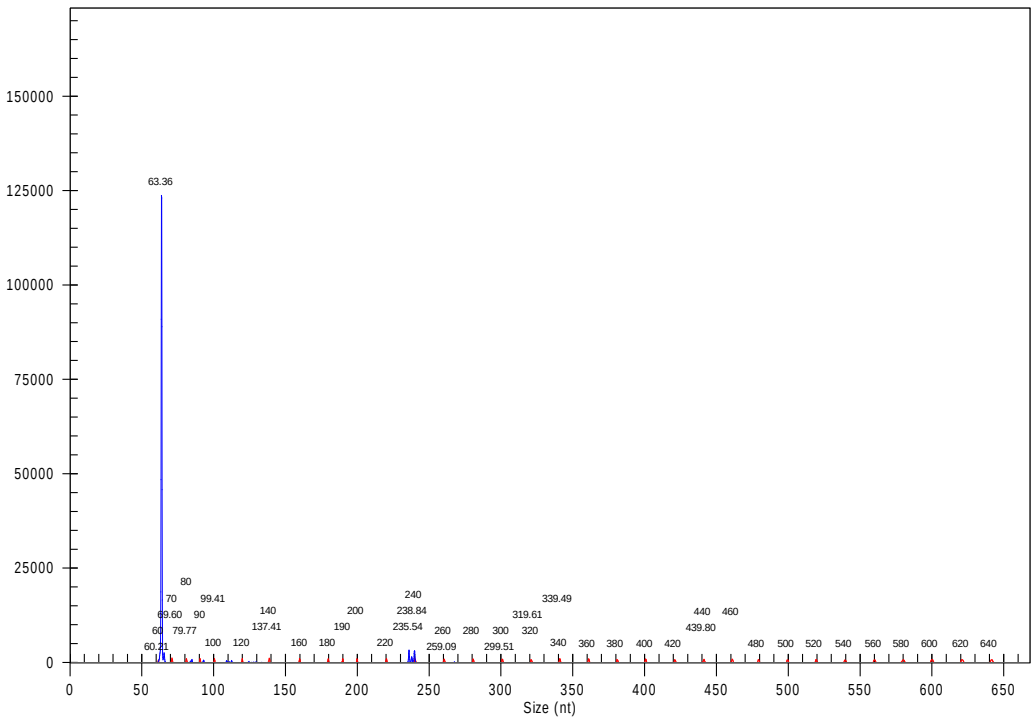

Figure 4.8. T-RFLP profile of isolate I2511 with 16S rRNA gene digested by restriction enzyme CfoI. Profile show single peak at ~63 nt.

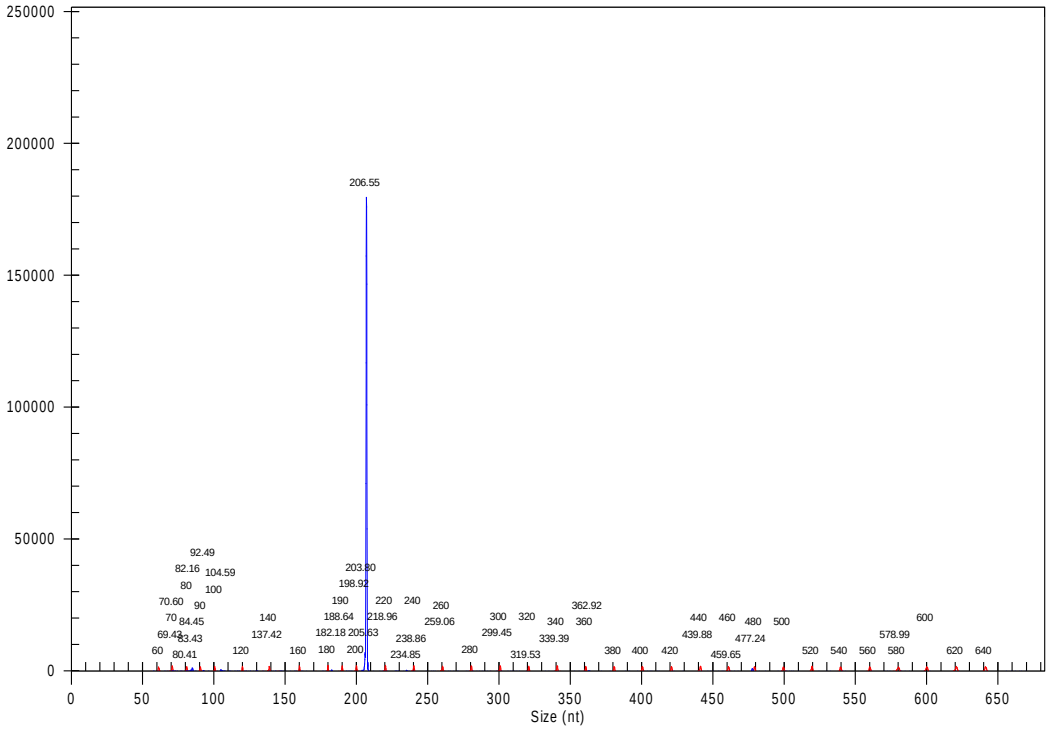

Figure 4.9. T-RFLP profile of isolate I2511 with 16S rRNA gene digested by restriction enzyme AluI. Profile show single peak at ~207 nt.

Cells of strain I2511 were motile rods (3 - 5 µm long and ~0.4 µm wide) that formed oval endospores located at the cell termini (Fig 4.10). Although strain I2511 was obtained from colonies on aSRB plates (which produced very small and scarce colonies on this solid medium), several attempts to cultivate this isolate in aSRB liquid medium (section 2.2.4) in the absence of ZVS, did not result in increased cell numbers or H_2S production, and it was therefore, inferred that the isolate was not able to catalyse the dissimilatory reduction of sulfate. For this reason, the liquid medium selected to cultivate I2511 was Sr liquid medium that contained sulfate salts, rather than the chloride-based basal salts that was used in the enrichment cultures (section 2.2.1).

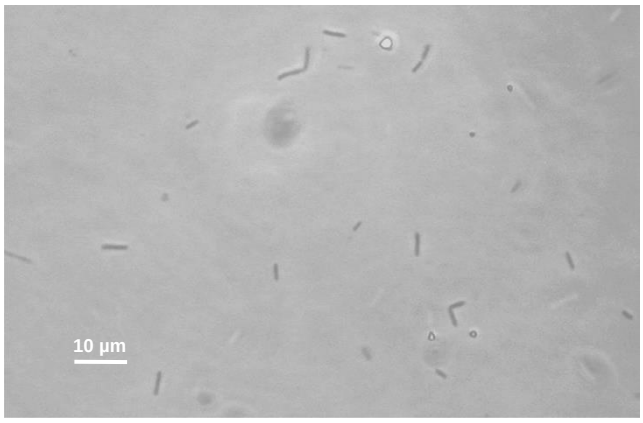

Figure 4.10. Phase contrast micrograph of cells of isolate I2511.

4.2.3 Phylogenetic analysis

4.2.3.1 Methods

The 16S rRNA gene of strain I2511 was amplified (section 2.6.2) using the primers 27F (5′–3′ AGAGTT TGATCM TGGCTCAG) and 1387R (5′–3′ GGGCGGWGTGTACAAGGC) and sequenced by Macrogen Inc., South Korea. The resulting partial gene sequence was analysed (section 2.6.4) and deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

ContEst16S (https://www.ezbiocloud.net/tools/contest16s, Lee *et al.,* 2017) was used to obtain the full length of the 16S rRNA gene sequence from the draft genome sequence (section 4.2.9). The full length 16S rRNA gene sequence was searched against the database of the EzBioCloud web server, which is a quality-controlled database of 16S rRNA gene sequences (https://www.ezbiocloud.net/identify, Yoon *et al*., 2017). The 16S rRNA gene sequences of closely-related species were retrieved from EZBioCloud and aligned with the full length 16S rRNA gene sequence of strain I2511 using Muscle (Edgar, 2004) *via* MEGA7 software (Kumar *et al.,* 2016). A phylogenetic tree using the full length 16S rRNA gene sequence was constructed by a neighbour-joining method (Saitou and Nei, 1987) with the

Kimura 2-parameter method (Kimura, 1980) using MEGA7 software, which provided higher bootstrap support for tree branches. Tree topology reliabilities were confirmed by bootstrap analysis using 1000 replicates.

4.2.3.2 Results

The partial 16S rRNA gene sequence of isolate I2511 obtained by amplification and sequencing was 1,388 bp long and deposited on GenBank (Appendix 4C; accession number KY576736). The full length 16S rRNA gene sequence of isolate I2511 obtained from its draft genome (1,549 bp, GenBank accession number MH686031; Appendix 4B) was aligned with the partial sequence (BLAST analysis, https://blast.ncbi.nlm.nih.gov/Blast.cgi), showed >99% similarity to that of the amplified gene, therefore representing the full length 16S rRNA gene of isolate I2511.

Analysis of the full length 16S rRNA gene sequence from isolate I2511 showed that it aligned within the phylum *Firmicutes* (order *Bacillales*, family *Alicyclobacillaceae)* and the closest validated species were *Alicyclobacillus contaminans* 3-A191^{T} and *Alicyclobacillus tolerans* K1^{T}, which shared 92.9% and 93.1% sequence similarity, respectively. Isolate IR2 (Ñancucheo and Johnson, 2012) and that represented by clone G13 (Winch *et al.,* 2009) were more closely related bacteria, both sharing 96% identity of their 16S rRNA genes. The phylogenetic relationship of I2511 with the validated species of the genus *Alicyclobacillus* and closely related bacteria is shown in Figure 4.11. Isolate I2511 was placed in a separate clade from the *Alicyclobacillus* species, and clustered with isolate IR2 and clone G13 in a separate branch.

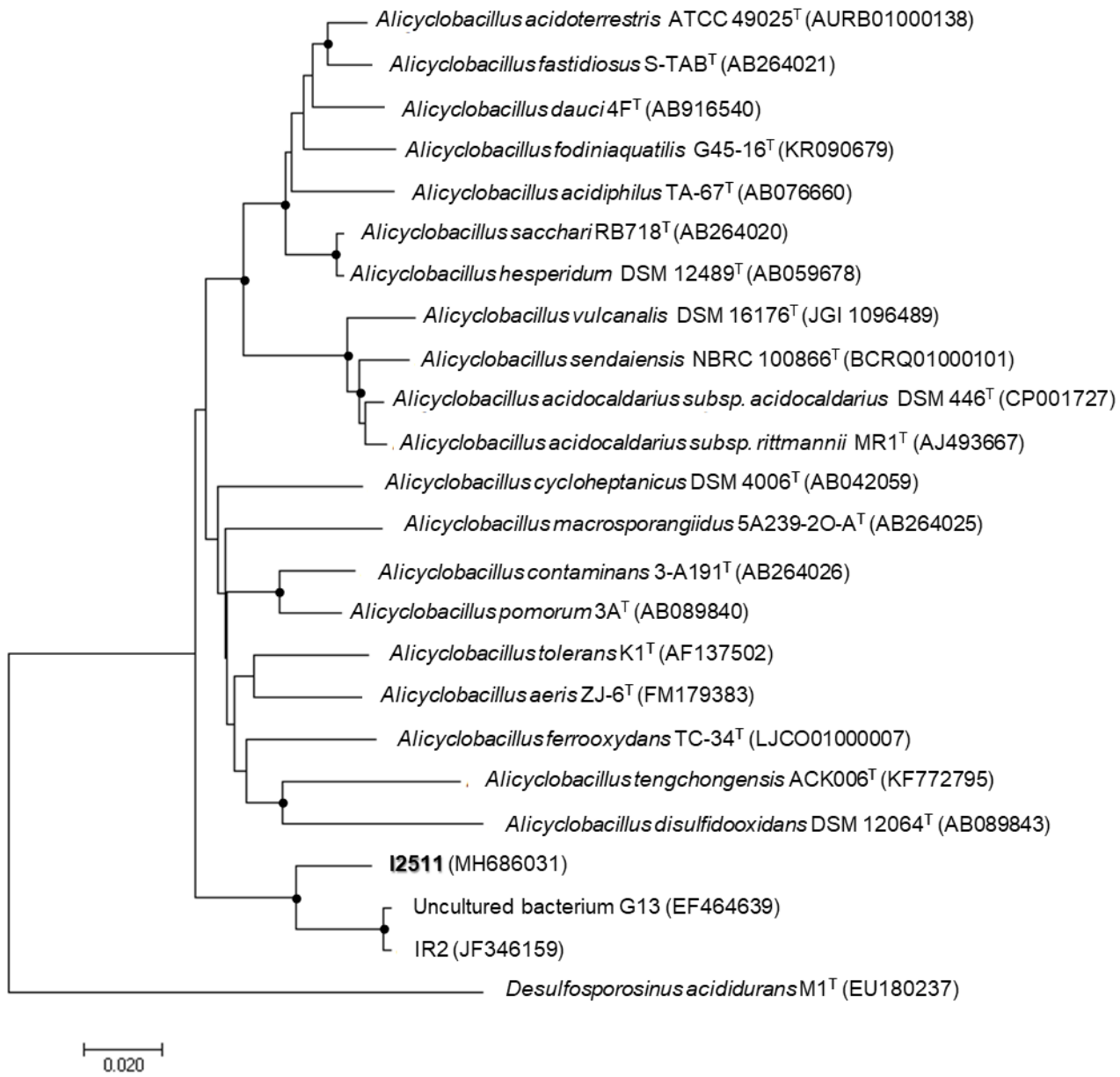

Figure 4.11. Rooted neighbour-joining tree based on 16S rRNA gene sequences showing the relationship of isolate I2511 to species of the genus *Alicyclobacillus* and other bacteria. The support in bootstrap analysis (1000 replicates) with values ≥70% are indicated by "●". The bar represents 0.02 substitutions per site. The 16S rRNA gene sequence of *Desulfosporosinus acididurans* M1^T (EU180237) was used as the outgroup.

4.2.4 Effects of pH and temperature on the growth of isolate I2511

4.2.4.1 Methods

Growth rates and optimum pH values and temperature for growth of isolate I2511 were determined by growing the bacterium in a pH- and temperature-controlled bioreactor (FerMac 310/60 unit, Electrolab Biotech, UK) fitted with a 2.2 L glass vessel. The liquid medium used was a modified Sr medium (containing 1%, w/v ZVS, 5 mM glycerol, 0.005%, w/v yeast extract, ABS and TE; section 2.2.4). The bioreactor was stirred at 150 rpm and a continuous stream of oxygen-free nitrogen (OFN~ 200 mL min^{-1}) was used to maintain anaerobic conditions and to deliver H_2S generated inside the bioreactor vessel to an off-line glass vessel that contained 500 mL of $CuSO_4$ (20 - 50 mM). Growth rates were determined at pH between 2.8 and 4.5, cultures maintained at 30 ºC, and at temperatures between 23 °C and 35 °C, at pH 3.7. Samples from the bioreactor vessel and the off-line vessel were removed regularly to determine concentrations of glycerol, acetic acid and copper. Growth rates were routinely determined from semi-logarithmic plots of glycerol oxidised against time. Changes in of-line copper concentrations were used to determine rates of H_2S production.

Additional tests were carried out in replicate universal bottles with the modified Sr liquid medium to assess the minimum pH (carried out at 30ºC) and maximum temperature (at pH 3.7) at which isolate I2511 could grow. Cultures were incubated anaerobically in sealed jars (section 2.2.6) together with a “copper trap”, shaken at 50 rpm. Planktonic bacterial cells were counted (section 2.4.2) after 11 and 18 days of incubation to evaluate growth.

4.2.4.2 Results

Production of hydrogen sulfide was correlated with glycerol oxidation (r = 0.93) and with planktonic cell numbers (r =0.91) (Fig. 4.12). Acetic acid production was detected during growth and concentrations increased with time. During growth experiments in the bioreactor, the ratio of acetic acid produced: glycerol oxidised was ~0.4 (Fig 4.13). Isolate I2511 had an optimum growth at pH ~3.7 (when incubated at 30ºC) with a culture doubling time of ~ 62 h (Fig. 4.14). The effect of temperature on culture doubling times of isolate I2511 maintained at pH 3.7 is shown in Figure 4.16; the lowest culture doubling time determined here was 54 h (at 35ºC). Isolate I2511 did not grow or generate H_2S in the bioreactor at 38°C or above (at pH 3.7). Due to time constraints growth at temperatures between 35°C and 38°C was not assessed. In addition, no growth or H_2S production was observed in bioreactor cultures maintained at pH 2.5 and 30ºC.

In contrast to results from the bioreactor culture, tests carried out in universal bottles suggested that the minimum pH for growth was pH 1.8 (Fig. 4.15); planktonic cell numbers

increased from ~ 5 x10^5 cells mL^{-1} at day zero to 2 ± 1.4 x10^7 cells mL^{-1} at day 18 with no measurable changes in culture pH by the end of the experiment.

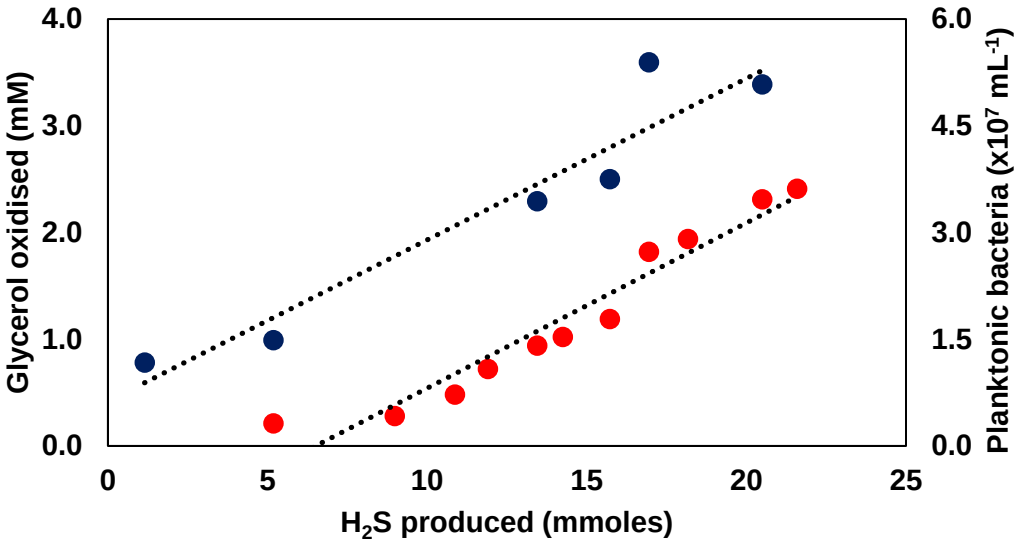

Figure 4.12. Relationship between hydrogen sulfide production and glycerol oxidation (●; *r* = 0.93) and hydrogen sulfide production and planktonic cell numbers (●; *r* = 0.91) when isolate I2511 was grown in a bioreactor in modified Sr medium containing 1% (w/v) ZVS, 5 mM glycerol and 0.005% (w/v) yeast extract at pH 3.2, and 30°C.

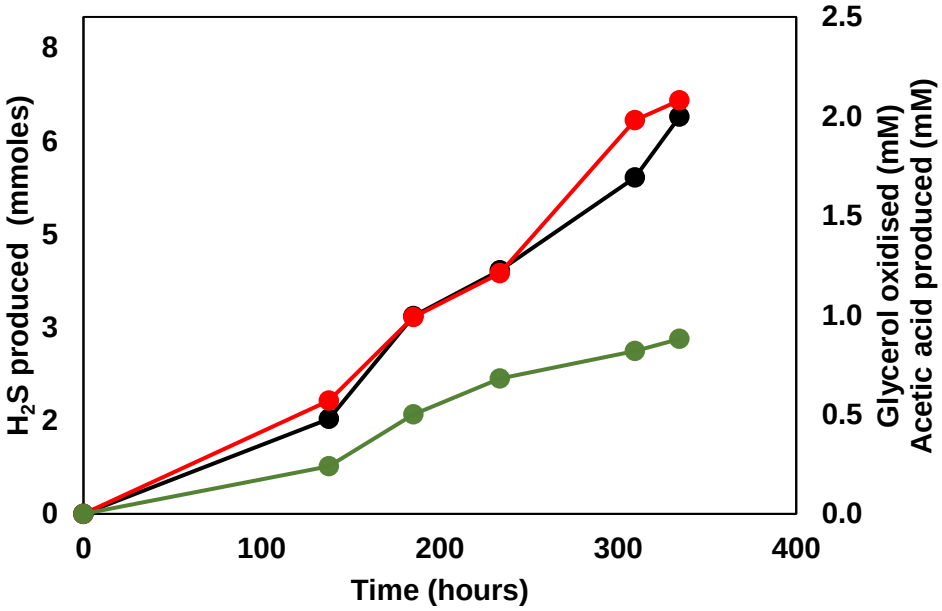

Figure 4.13. Cumulated amounts of hydrogen sulfide produced (●), glycerol oxidised (●) and acetic acid produced (●) of isolate I2511 grown in a bioreactor in modified Sr medium containing 1% (w/v) ZVS, 5 mM glycerol and 0.005% (w/v) yeast extract at pH 2.8 and 30°C.

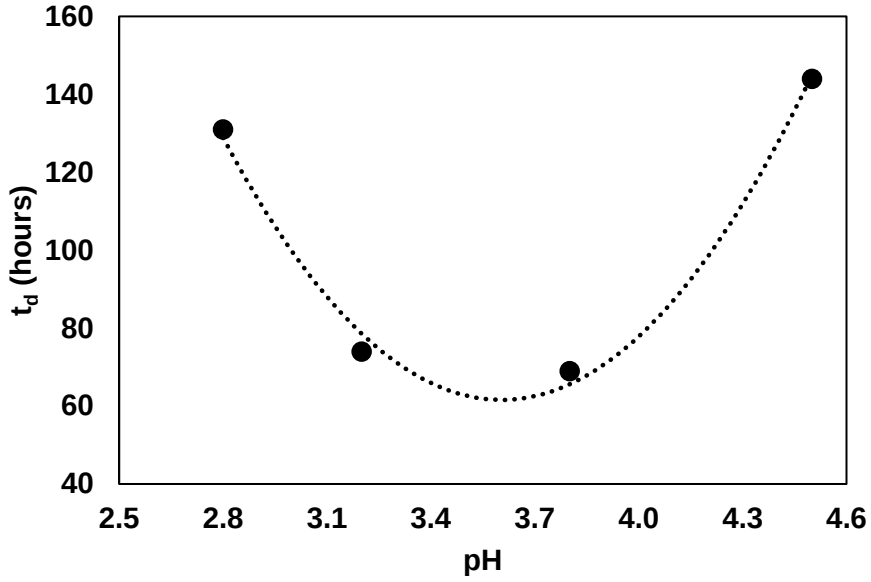

Figure 4.14. Effect of pH on the culture doubling times (t_d) in hours at fixed temperature (30°C) of isolate I2511 grown in a bioreactor in in modified Sr medium containing 1% (w/v) ZVS, 5 mM glycerol and 0.005% (w/v) yeast extract. Polynomial regression applied for curve fitting.

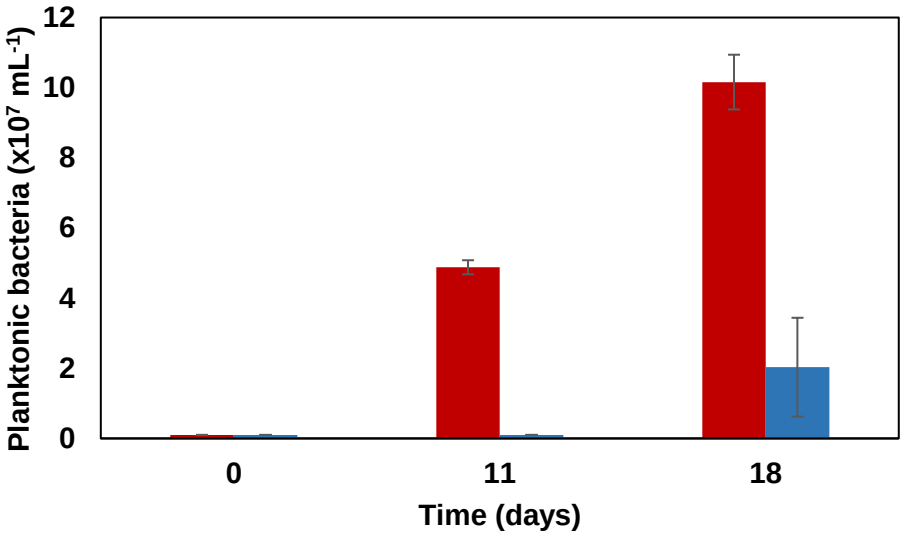

Figure 4.15. Growth of isolate I2511 at pH 1.8 in universal bottles. Cultures were grown in replicate bottles in modified Sr medium containing 1% (w/v) ZVS, 5 mM glycerol and 0.005% (w/v) yeast extract at 30°C. Key: (■) pH 3.0 (as a positive control) and (■) pH 1.8. Bars indicate mean values and the error bars data ranges (n=2).

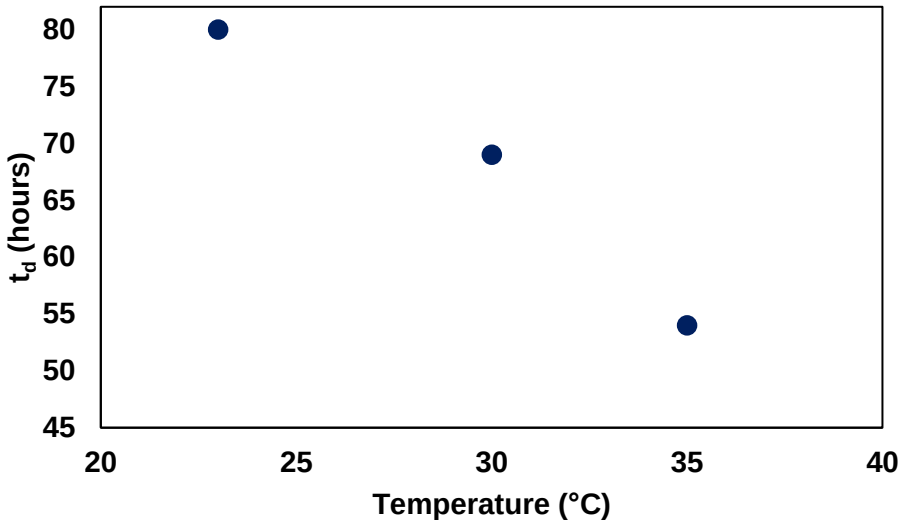

Figure 4.16. Effect of temperature on the culture doubling times (t_d) in hours at fixed pH (3.7) of isolate I2511 grown in a bioreactor in modified Sr medium containing 1% (w/v) ZVS, 5 mM glycerol and 0.005% (w/v) yeast extract.

4.2.5 Growth under aerobic and micro-aerobic conditions

4.2.5.1 Methods

Growth under aerobic conditions was assessed in liquid and on solid media and for micro-aerobic conditions only on solid media. The solid media used were: YE3o, FeSo and aSRB (all overlay solid media; section 2.2.5). Liquid media selected to assess aerobic growth were: (a) Sr medium containing ZVS (1%, w/v) 5 mM glycerol and yeast extract (0.01%, w/v), pH 3.0 and b) FeYE medium, which contained 10 mM Fe^{2+} and yeast extract (0.02%, w/v), pH 2.0. Solid and liquid media were inoculated with culture grown in modified Sr medium containing 1% (w/v) ZVS, 5 mM glycerol and 0.005% (w/v) yeast extract pH 3.5, anaerobically. Inoculum for tests in liquid media contained ~ 3 x10^5 cells mL^{-1}. To evaluate growth, planktonic bacterial cells were counted and pH measured after 10 and 20 days.

4.2.5.1 Results

In all solid medium tests (both aerobic and micro-aerobic conditions) no colonies were detected after ~45 days of incubation. In tests with liquid medium no increase in planktonic cell numbers or changes in pH was detected. These results indicated that isolate I2511 is a strict anaerobe. However, exposure of ~ 3 h to oxygen (e.g. manipulating cultures in atmospheric air) did not result in loss of cells viability.

4.2.6 Dissimilatory redox transformations of inorganic electron acceptors by isolate I2511

4.2.6.1 Dissimilatory reduction of zero-valent sulfur on solid medium

4.2.6.1.1 Methods

To assess reduction of ZVS on solid media, two different types of ZVS were used: colloidal and microbially pre-wetted. Overlay colloidal sulfur (Sco) plates were developed based on solid medium described elsewhere (Florentino *et al.,* 2015) and overlay pre-wetted ZVS (S^0o) plates were developed jointly with Ivan Ñancucheo (*Facultad de Ingeniería y Tecnología*, Universidad San Sebastian, Chile). Isolate I2511 was grown in Src medium 1% (w/v) ZVS, 5 mM glycerol, 0.01% (w/v) yeast extract, chloride basal salts (section 2.2.1) and adjusted to pH 3.0 with hydrochloric acid. The culture was streaked onto Sco and S^0o plates and placed in sealed jars in anaerobic conditions jointly with a "copper trap" to indicate production of H_2S.

4.2.6.1.2 Results

Growth of isolate I2511 in Sco plates was not detected after ~60 days of incubation. Conversely, S^0o plates exhibited consistent growth and generation of H_2S (confirmed by visualisation of CuS precipitates in the universal bottles) after repeatedly re-streaking. Colonies on S^0o plates were very small (<1 mm diameter) and white. ZVS particles disappeared where colonies grew, indicating they were completely reduced to H_2S (Figure 4.17).

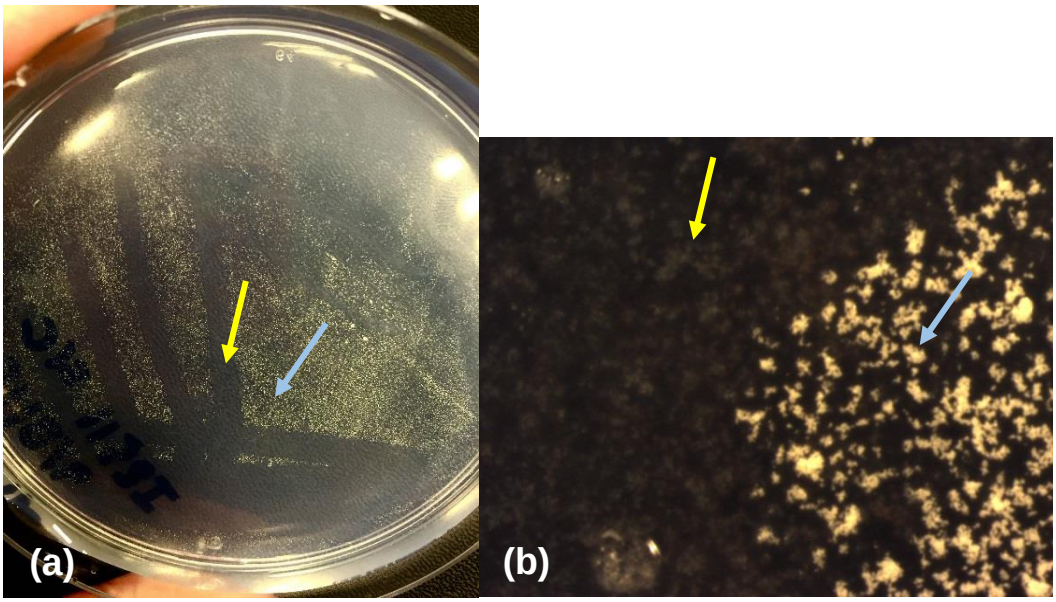

Figure 4.17. (a) Image of S^0o plate inoculated (streak plate method) with liquid culture of strain I2511; (b) Detailed view of I2511 colonies grown on an S^0o plate. Yellow arrow indicates where ZVS particles were replaced by white colonies and blue arrow indicate ZVS particles with apparently no grown colonies.

4.2.6.2 Dissimilatory reduction of ferric iron

4.2.6.2.1 Methods

Dissimilatory reduction of ferric iron was tested by growing strain I2511 in liquid medium containing glycerol and yeast extract and (mostly) solid phase ferric iron, incubated under anaerobic conditions. A liquid medium was prepared as follows: ferric iron was added from a sterile stock solution of ~ 0.7 M of $Fe_2(SO_4)_3$ pH 1.8 to ABS and TE (sections 2.2.1 and 2.2.2, respectively), glycerol and yeast extract added (to give final concentrations of 5 mM and 0.005% (w/v), and the pH was adjusted to 2.6 with sterile 1 M NaOH, causing partial precipitation of the ferric iron. Replicate cultures were inoculated with an active culture of strain I2511, grown previously in the presence of ferric iron. Replicate cultures were set up, together with a non-inoculated control. Samples were withdrawn after 17, 25 and 30 days and concentrations of ferrous iron determined using the Ferrozine assay (section 2.3.1.2).

Reduction of soluble ferric citrate was also assessed. Replicates cultures with medium containing ~ 7 mM tribasic ferric citrate, 5 mM glycerol, 0.005% (w/v) yeast extract, and ABS, pH 3 were inoculated with cells harvested from the solid phase ferric iron cultures (above). Sterile porous glass beads (Poraver Dennert GmbH, Germany) were added to another set of replicate cultures containing ferric citrate medium in order to assess whether isolate I2511 required attachment to a solid surface to grow effectively in the absence of a solid substrate (which was usually ZVS). A negative control was set up to assess abiotic reduction of ferric citrate in glycerol/yeast extract containing acidic medium.

4.2.6.2.2 Results

Ferric iron (mostly present in solid phase) was reduced to ferrous iron in cultures of isolate I2511, as shown in Figure 4.18. Concentrations of Fe^{2+} and the pH of inoculated cultures increased considerably after 17 days of incubation, though only marginal increase in both parameters were detected from day 17 to day 25. Additional yeast extract (0.005%, v/v) added at day 25, did not result in further iron reduction over the next 5 days. Non-inoculated cultures exhibited only minor changes in pH and Fe^{2+} concentrations throughout the time course of the experiment. Concentrations of glycerol and acetic acid measured after 25 days of experiment in one of the replicate cultures showed changes in glycerol and acetic acid concentrations (0.54 mM of glycerol was oxidised and 0.51 mM of acetic acid was produced).

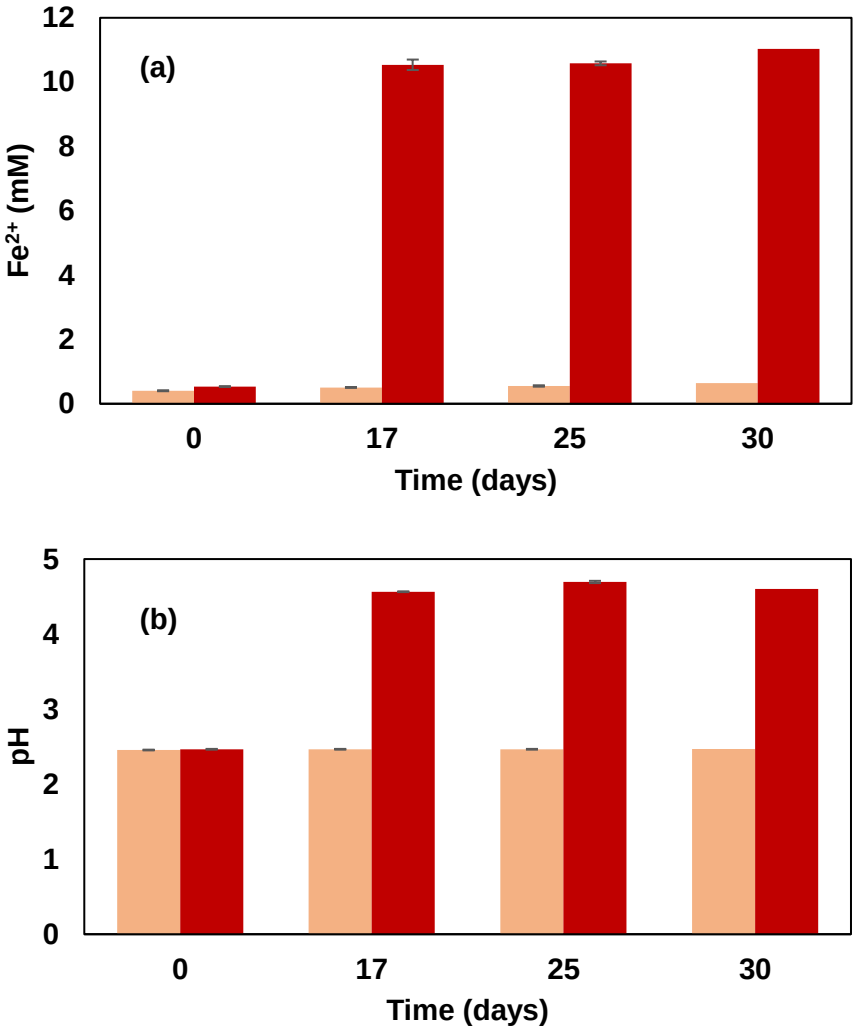

Figure 4.18. Reductive dissolution of solid phase ferric iron by isolate I2511: (a) ferrous iron concentrations (mM) and (b) pH, after 17, 25 and 30 days of incubation at 30°C in medium containing ~15 mM Fe^{3+}, 5 mM glycerol, 0.005% (w/v) yeast extract, ABS and TE (pH 2.6). Key: (■) cultures of isolate I2511; (■) non-inoculated cultures. Bars show mean values and error bars indicate data ranges (n=2).

In contrast, isolate I2511 did not appear to be able to reduce soluble ferric citrate. Although after 7 days of incubation there was more ferrous iron in inoculated cultures (especially those that contained Poraver beads) than in the sterile controls, by day 13 days differences were not significant (Fig 4.19 and 4.20). The pH of in cultures and non-inoculated bottles increased during incubation, reaching (at day 13) mean values of pH 4.22 for non-inoculated bottles and inoculated cultures without beads, and pH 4.72 for inoculated cultures containing beads. There were no changes in glycerol concentration in inoculated cultures (with and without Poraver beads) after 13 days of incubation.

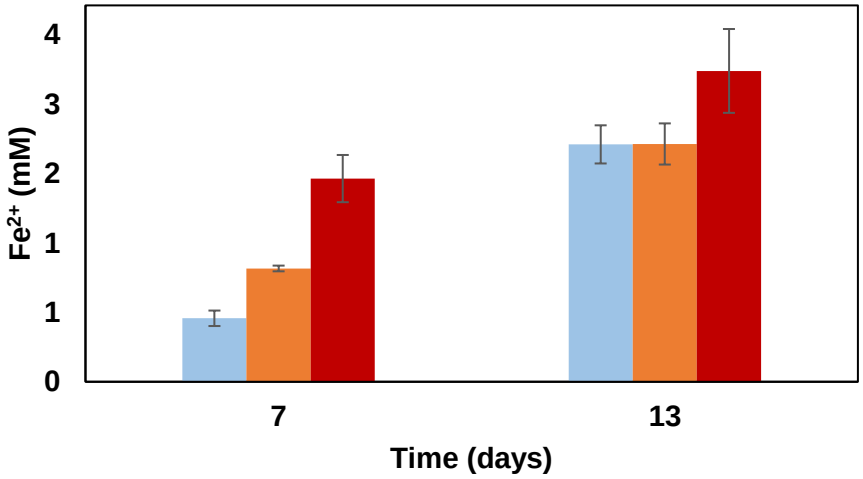

Figure 4.19. Results of tests of the ability of isolate I2511 to reduce soluble ferric citrate. Ferrous iron (mM) after 7 and 13 days of anaerobic incubation, 30°C, in medium containing ~ 7 mM ferric citrate, 5 mM glycerol, 0.005% (w/v) yeast extract and ABS pH 3.0. Key: (■) non-inoculated cultures containing Poraver beads; (■) inoculated cultures without beads; (■) inoculated cultures containing beads. Bars show mean values and error bars indicate data ranges (n=2).

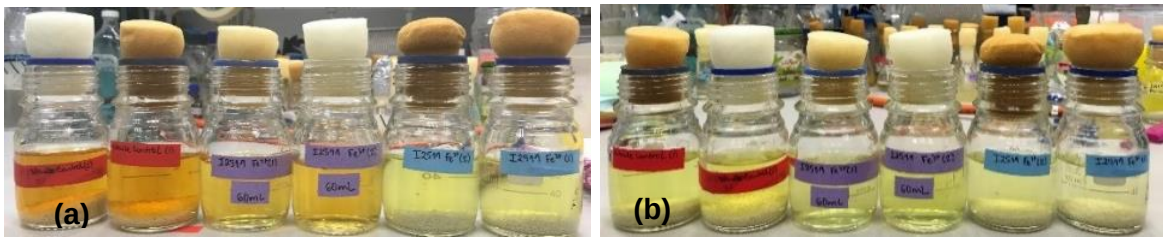

Figure 4.20. Image of I2511 cultures containing ferric citrate (initial pH 3.0): (a) after 7 days and (b) 13 days incubation. Bottles labelled with red labels are non-inoculated cultures containing Poraver beads; purple labels are inoculated cultures without beads and light blue labels are inoculated cultures containing beads.

4.2.6.3 Dissimilatory reduction of other electron acceptors

4.2.6.3.1 Methods

Dissimilatory reduction of tetrathionate, thiosulfate and nitrate were assessed in cultures incubated anaerobically on solid and in liquid media. Overlay aSRB plates were supplemented with either 5 mM potassium tetrathionate, 10 mM sodium thiosulfate or 10 mM sodium nitrate and non-supplemented overlay aSRB plate was used as a negative control. To evaluate

growth in liquid media, replicate cultures in 20 mL universal bottles containing aSRB medium were supplemented with the electron acceptors listed above (and at the same concentrations). Replicate cultures in Sr medium and standard aSRB medium were incubated in parallel to act as a positive and negative control, respectively. Growth on solid medium was evaluated by detection of colonies and in liquid medium by enumerating planktonic cells.

Dissimilatory reduction of manganese (IV) was assessed by growing strain I2511 in replicate 50 mL flasks containing 0.5% (w/v) manganese (IV) oxide (Sigma-Aldrich, USA), 5 mM glycerol, 0.005% (w/v) yeast extract, ABS and TE, at initial pH 2.5. Non-inoculated flasks were incubated in parallel. Tests were incubated anaerobically in sealed jars at 30°C.Samples were withdrawn after 18 and 60 days and concentrations of manganese (II) were determined (section 2.3.1.4).

Dissimilatory reduction of cysteine was assessed in replicate 20 mL universal bottles using a liquid medium containing 0.02% (w/v) L-cysteine hydrochloride monohydrate, 5 mM glycerol, 0.005% (w/v) yeast extract, ABS and TE, initial pH 3.0.

4.2.6.3.2 Results

Figure 4.21 shows planktonic cell numbers in and pH values of cultures containing tetrathionate, thiosulfate and nitrate after 0, 12, 20 and 32 days incubation. Cell counts in tetrathionate and thiosulfate cultures were similar to the negative control (standard aSRB medium); cell counts in nitrate-amended cultures exhibited a small increase with time but were far fewer than those in the positive control cultures (in Sr medium). Although concentrations of nitrate decreased from 10 mM to 5.46 ± 0.03 mM, and 4.37 ± 0.02 mM nitrite was present in cultures after 32 days, there were no corresponding changes in glycerol concentrations, while 3 mM glycerol was consumed in the positive control (with ZVS).The pH of all cultures (except tetrathionate) increased with time, with those in the nitrate-containing medium being greater than the others. Cell numbers did not increase and pH did not change in cultures amended with cysteine or with manganese (IV) oxide. While in the latter, non-inoculated controls and cultures exhibited similar increases in concentrations of Mn (II) (0.08 µmoles). No colonies were visible on any of the solid media prepared with potential alternative electron acceptors.

A second experiment was set up to confirm growth of isolate I2511 by nitrate respiration. Replicate cultures in 50 mL flasks were set up in similar conditions to the first experiment (same medium, pH and incubation) using the first test as inoculum (~ 1 x10^6 cells mL^{-1}). After 35 days of incubation there was no increase in cell numbers or measurable changes in pH.

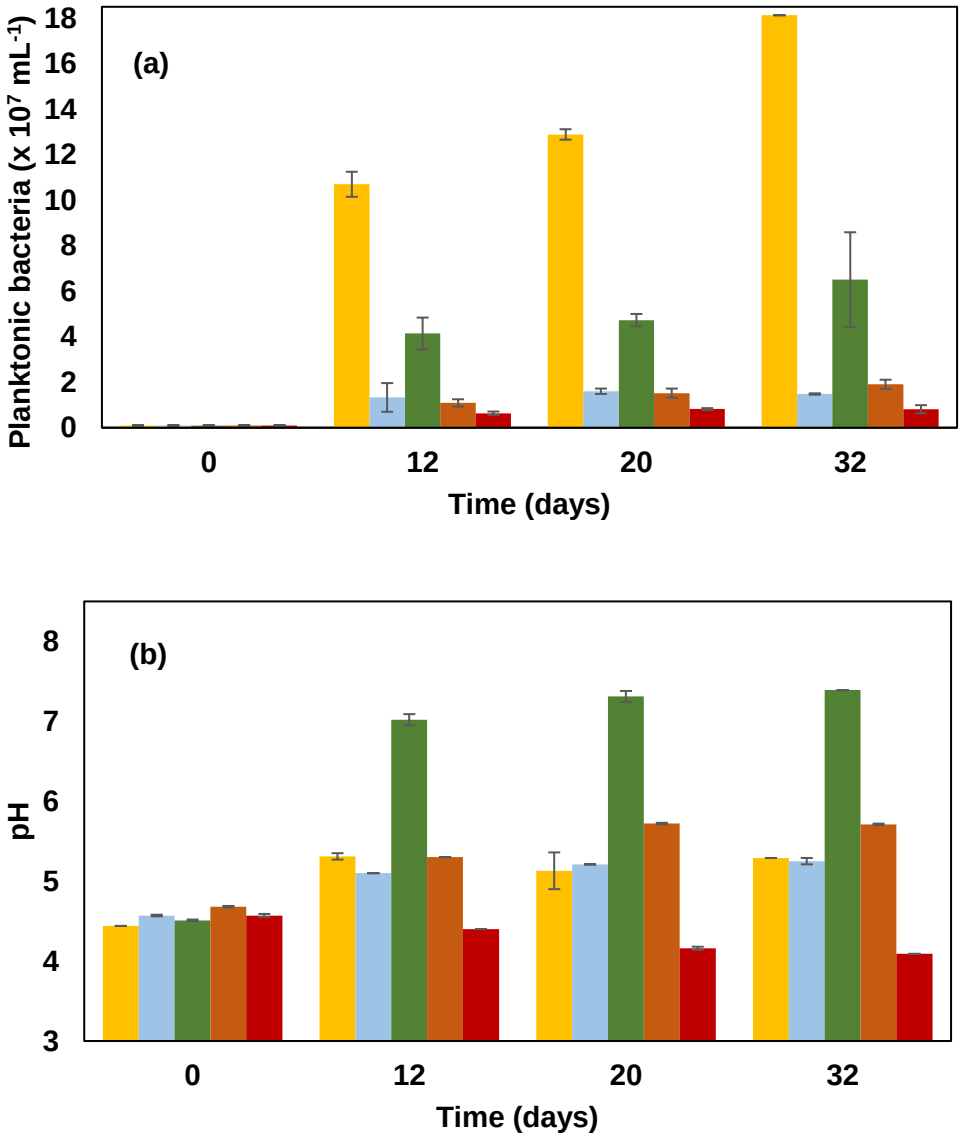

Figure 4.21. Results of tests for the ability of isolate I2511 to grow by dissimilatory reduction of various alternative electron acceptors. (a) Planktonic cell numbers and (b) pH of I2511 cultures after 0, 12, 20 and 32 days of anaerobic incubation at 30°C in liquid medium amended with different potential electron acceptors. Key: (■) Sr medium containing 1% (w/v) ZVS, 5 mM glycerol, 0.005% w/v, yeast extract pH 3.5; (■) standard aSRB medium; (■) aSRB medium amended with 10 mM sodium nitrate; (■) aSRB medium amended with 10 mM sodium thiosulfate; (■) aSRB medium amended with 5 mM tetrathionate. Bars show mean values and error bars indicate data ranges (n = 2).

4.2.7 Carbon metabolism

4.2.7.1 Growth of isolate I2511 on yeast extract and peptone.

4.2.7.1.1 Methods

The requirement of yeast extract for growth of isolate I2511 in media containing defined organic compounds was tested for using replicate cultures in Sr liquid medium (pH 3.0) containing 5 mM glycerol, with or without yeast extract (added at 0.01% (w/v)). Planktonic cells were enumerated after 11 and 18 days of anaerobic incubation at 30°C and agitation at 50 rpm

The effect of different concentrations (0.005 or 0.05%, w/v) of yeast extract or peptone on cell yields was tested in replicate cultures modified Sr liquid medium containing 1% (w/v) ZVS, ABS and TE pH 3.0. Two other culture variants were set up at the same time: (a) a liquid medium containing 0.05% (w/v) yeast extract or peptone but no ZVS; (b) Sr medium containing both 5 mM glycerol and 0.005% (w/v) yeast extract. Planktonic cells were enumerated after 18 days of anaerobic incubation at 30°C and agitation at 50 rpm.

4.2.7.1.2 Results

Isolate I2511 did not grow in medium where glycerol was the only carbon source and electron donor provided (Fig 4.22). However, the isolate grew in medium that contained glycerol and yeast extract. The isolate coupled oxidation unidentified organic materials in yeast extract to the reduction of both ZVS (Fig. 4.23) and ferric iron (Fig 4.18). Yeast extract and peptone acted both as electron donor and carbon source for isolate I2511 (Fig. 4.23) and because isolate I2511 grew solely in peptone, this substrate substituted yeast extract as growth factor. The isolate was not able to grow cells by fermentation of yeast extract or peptone, and culture pH increased only in cultures with ZVS and 0.05% (w/v) yeast extract or peptone. Acetic acid was produced (~ 0.3 mM) in both replicates of cultures containing ZVS and 0.05% (w/v) yeast extract or peptone.

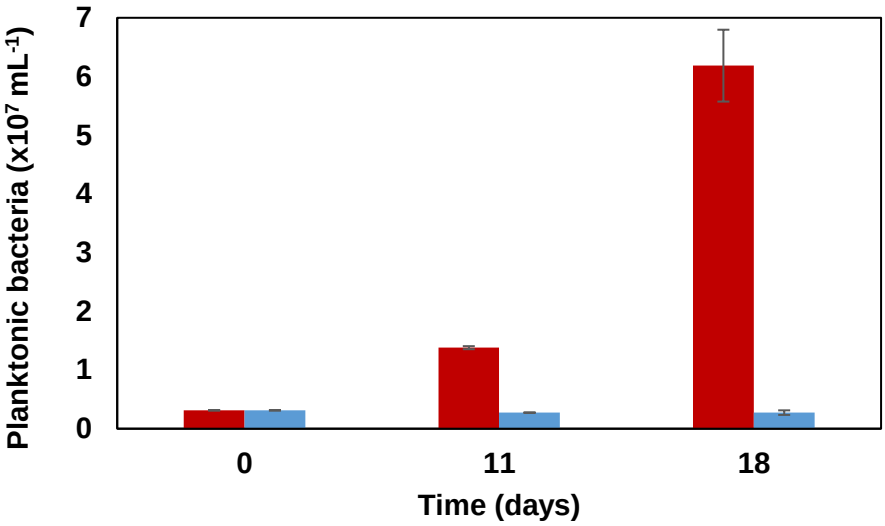

Figure 4.22. Growth of isolate I2511 in modified Sr medium (containing 5 mM glycerol) with (■) and without (■) 0.01% (w/v) yeast extract. Bars indicate mean values and the error bars data range (n=2).

4.2.7.2 Use of defined small molecular weight organic compounds by isolate I2511.

4.2.7.2.1 Methods

To assess the ability of isolate I2511 to use defined small molecular weight organic compounds, replicate cultures were set up in Sr medium containing 1% (w/v) ZVS, 0.01% (w/v) yeast extract pH 3.0 supplemented with the following compounds:

(i) monosaccharides: glucose and fructose (both at 5 mM);

(ii) alcohols: 1,3-propanediol and glycerol (both at 5 mM), ethanol and methanol (all at 10 mM);

(iii) organic acids: lactic acid (pK_a = 3.86) and citric acid (pK_a = 3.13, 4.76 and 6.40) (both at 5 mM).

Control cultures containing ZVS and 0.01% (w/v) yeast extract were set up in parallel.

The effect of different concentrations (1, 5 and 10 mM) of pyruvate (sodium pyruvate, pK_a = 2.5) on cell yields was examined in replicate cultures in Sr medium containing 0.01% (w/v) yeast extract (pH 3.0). The following controls were used: (a) 1 mM pyruvate without ZVS, and (b) Sr medium without pyruvate.

Growth by fermentation was assessed in replicate cultures supplemented with glucose, glycerol, L-malate (pK_a = 3.4 and 5.2) or fumarate (pK_a = 3 and 4.4) (all at 5 mM) in the absence of ZVS. The media contained 0.01% (w/v) yeast extract, ABS and TE (pH 4.0).

In all tests, growth was assessed from counts of planktonic cells after incubation for 12 - 18 days at 30°C and agitation at 50 rpm.

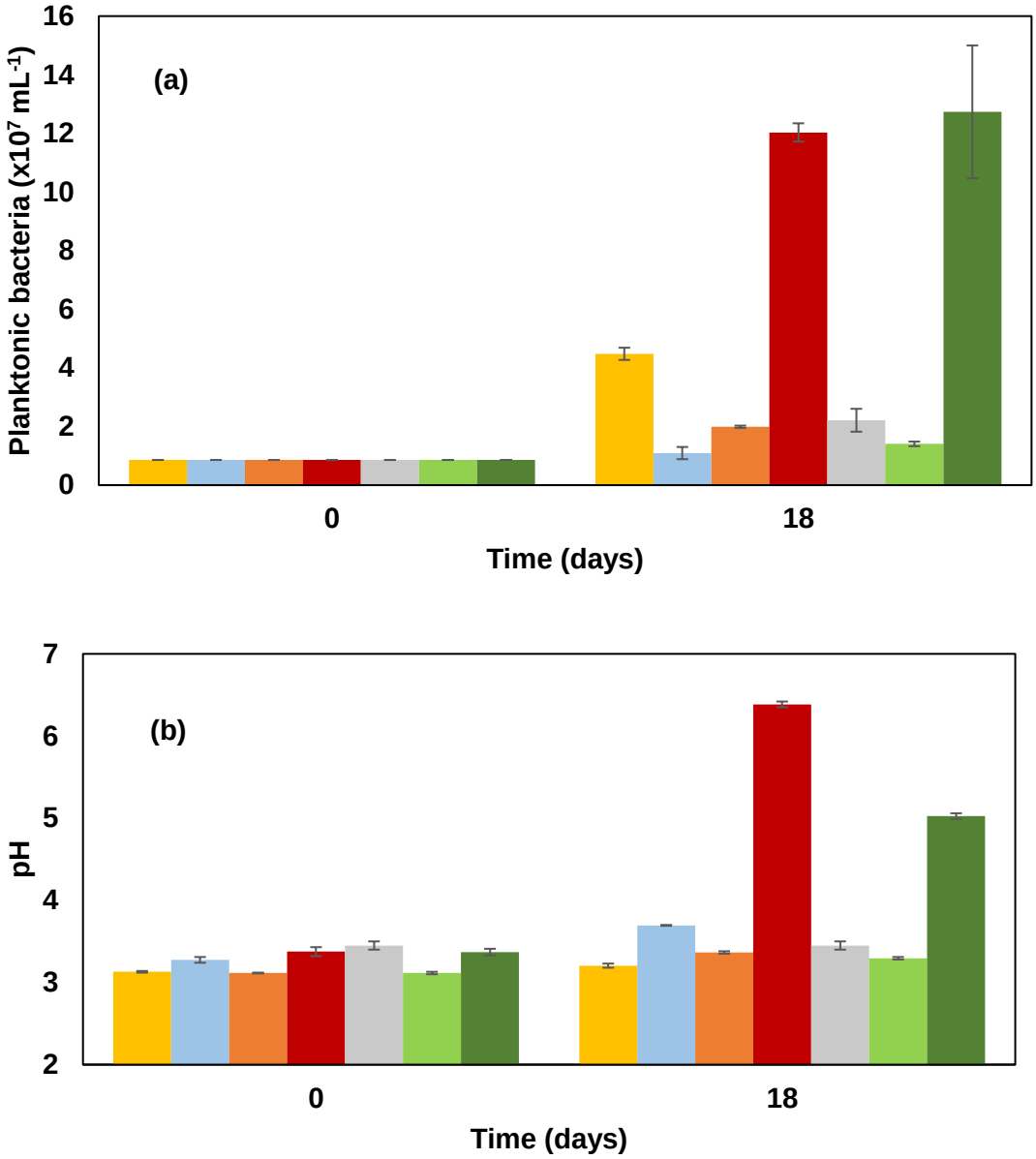

Figure 4.23. Effect of different concentrations of yeast extract and peptone on growth of isolate I2511: (a) planktonic cell numbers and (b) pH. Key: (■) Sr medium containing 5 mM glycerol and 0.005% (w/v) yeast extract; (■) ZVS-free medium containing 0.05% (w/v) yeast extract; (■) modified Sr medium containing 0.005% (w/v) yeast extract; (■) modified Sr medium containing 0.05% (w/v) yeast extract; (■) ZVS-free medium containing 0.05% (w/v) peptone; (■) modified Sr medium containing 0.005% (w/v) peptone and (■) modified Sr medium containing 0.05% (w/v) peptone. Bars indicate mean values and error bars data ranges (n=2).

4.2.7.2.2 Results

Isolate I2511 grew on a range of organic compounds (Fig. 4.24), coupled to the dissimilatory reduction of ZVS, as production of hydrogen sulfide was detected in the “copper trap” incubated jointly. Biomass yields of cultures increased when more pyruvate was provided (Fig. 4.25). Culture pH also increased during incubation in cultures supplemented with 5 mM or 10 mM pyruvate (Fig. 4.25). Growth was not detected in cultures with 1 mM pyruvate in the absence of ZVS. Moreover, no growth was detected *via* fermentation in cultures with any of the substrates tested.

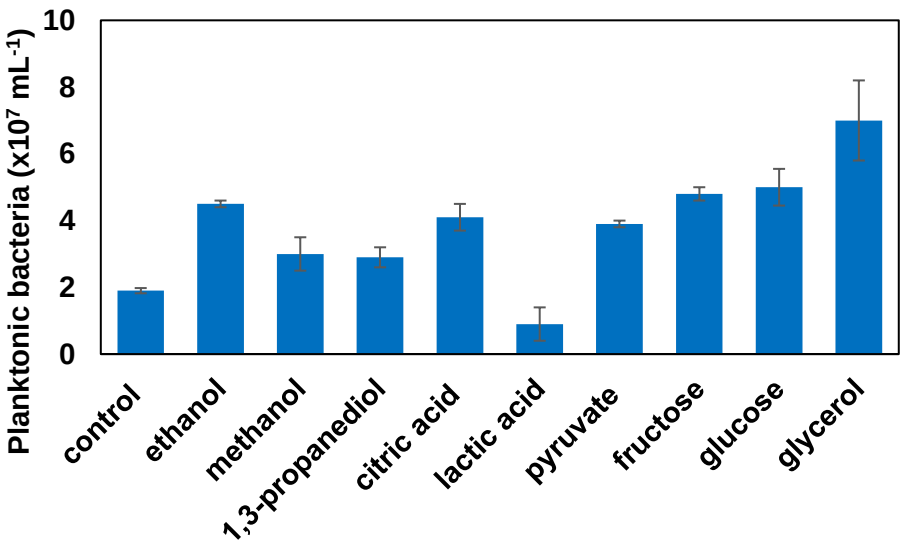

Figure 4.24. Growth of isolate I2511 on defined small molecular weight organic compounds. Planktonic cell numbers were counted after 17 days of anaerobic incubation in Sr medium containing 1% (w/v) ZVS, 0.01% (w/v) yeast extract, ABS and TE, pH 3, at 30°C. Control cultures contained ZVS, 0.01% (w/v) yeast extract, ABS and TE pH 3. Bars indicate mean values and error bars data ranges (n=2).

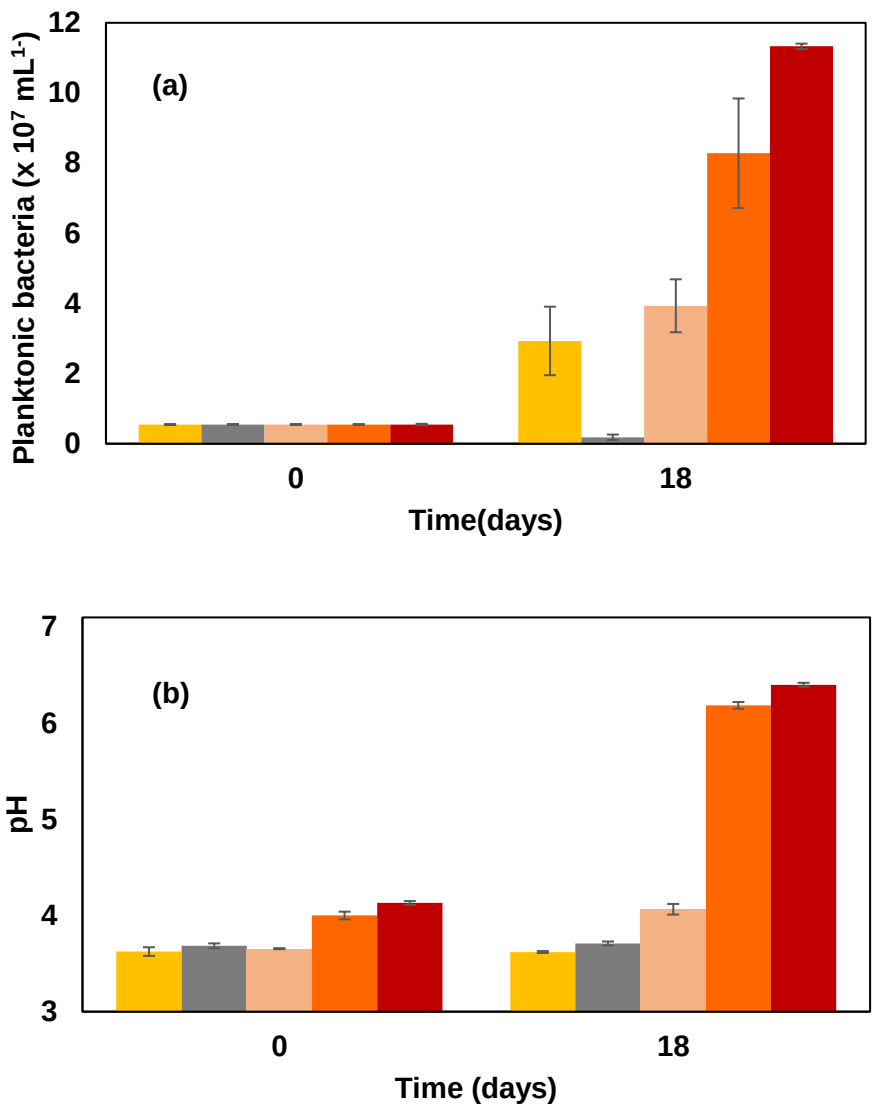

Figure 4.25. Effect of different concentration of pyruvate on the growth of isolate I2511: (a) planktonic cell numbers and (b) pH at day 0 and after 18 days of anaerobic incubation at 30 °C. Key: (■) Sr medium containing 0.01% (w/v) yeast extract (pyruvate-free); (■) ZVS-free medium containing 1 mM pyruvate; (■) Sr medium containing 1 mM pyruvate; (■) Sr medium containing 5 mM pyruvate; (■) Sr medium containing 10 mM pyruvate. Bars indicate mean values and error bars data ranges (n=2).

4.2.8 Tolerance of isolate I2511 to copper, sodium chloride and acetic acid

4.2.8.1 Methods

Copper sulfate was added (at 0.1, 1.0, 5.0 and 10 mM) to replicate cultures of I2511 in Sr medium containing 5 mM glycerol and 0.01% (w/v) yeast extract (pH 2.5). Cultures were incubated anaerobically at 30ºC and shaken at 50 rpm. Control cultures without Cu^{2+} were set

up in parallel. Growth was assessed by enumerating planktonic bacterial cells and from formation of CuS (as a result of production of H_2S). A similar approach was used to determine the tolerance of I2511 to sodium chloride (50, 100 and 500 mM). To assess tolerance of I2511 to acetic acid, replicate cultures containing 0.5, 1.0 and 3.0 mM acetic acid were set up in Sr medium containing 5 mM glycerol and 0.005% (w/v) yeast extract at pH 2.5. Control cultures without acetic acid were set up at both pH 2.5 and 3.0.

4.2.8.2 Results

Growth of isolate I2511 in the presence of Cu^{2+} and NaCl was inhibited at the lowest concentrations of both that were tested (i.e. 50 mM NaCl and 0.1 mM Cu^{2+}). Figure 4.26 shows cell counts of cultures containing different concentrations of acetic acid. After 10 days of incubation, cell counts of all cultures supplemented with acetic acid were lower than in the control culture at same pH. However, after 15 days incubation, cultures containing 0.5 and 1.0 mM acetic acid had similar planktonic cell counts to the control cultures at same pH. No growth was detected in cultures containing 3.0 mM acetic acid. At the end of the experiment (at day 15) culture pH for test (including those that showed no growth) and control cultures were similar at ~ pH 2.6

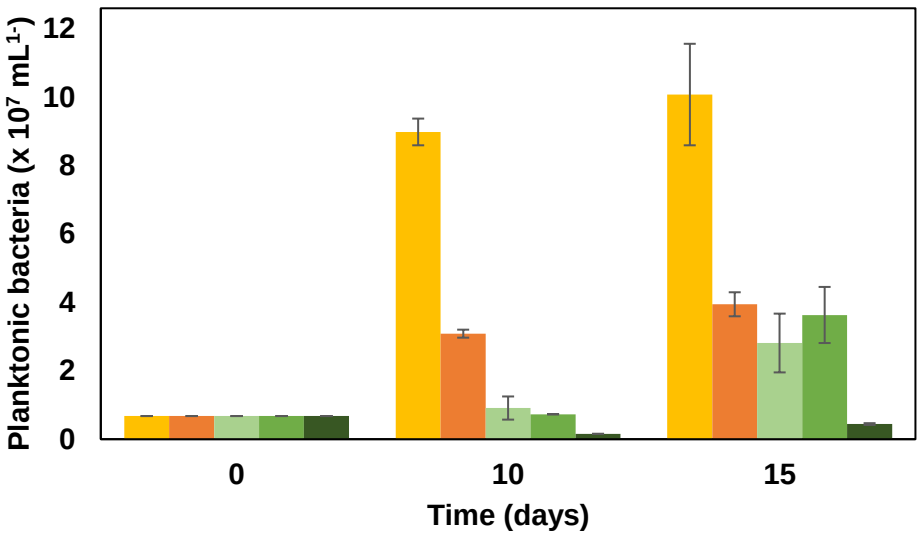

Figure 4.26. Growth of isolate I2511 in Sr medium containing 5 mM glycerol and 0.005% (w/v) yeast extract, and different concentrations of acetic acid. Key: (■) acetic acid-free medium, pH 3.0; (■) acetic acid-free medium, pH 2.5; (■) Sr medium containing 0.5 mM acetic acid, pH 2.5; (■) Sr medium containing 1.0 mM acetic acid, pH 2.5; (■) Sr medium containing 3.0 mM acetic acid, pH 2.5. Bars indicate mean values and error bars indicate data ranges (n=2).

4.2.9 Genome Analysis

4.2.9.1 Methods

Genomic DNA of isolate I2511 was extracted from a liquid culture (Sr medium supplemented with 5 mM glycerol, 0.01% w/v, yeast extract pH 3.5) using a CTAB method (section 2.6.1). Whole-genome sequencing was performed by MicrobesNG (http://www.microbesng.uk, Birmingham, UK) using an Illumina HiSeq 2500 sequencer with 2x250 bp paired-end reads. MicrobesNG provided a draft genome sequence with bioinformatics analysis that consisted of mapping the reads using BWA-MEM (http://bio-bwa.sourceforge.net/) against the closest available reference genome. *De novo* genome assemblies were generated using SPAdes (version 3.9.1; http://cab.spbu.ru/software/spades/). The draft genome sequence has been deposited at DDBJ/ENA/GenBank under the accession number QXHL00000000. Automated annotation was performed by RAST tool kit (Brettin *et al.,* 2015). Protein-coding sequences (CDSs) and RNA genes were predicted by PATRIC using *Comprehensive Genome Analysis Service* (Wattam *et al.,* 2017). Genome completeness and contamination was assessed by CheckM software (Parks *et al.,* 2014).

For comparative genomic analysis, the genome sequences of the five closest relatives (with available whole genome sequence) of strain I2511 were retrieved from NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/). The species and the accession numbers of the sequences were: *Alb. contaminans*^{T} (NZAUCA00000000.1), *Alb. hesperidum*^{T} (NZ_FNOJ00000000.1), *Alb. acidiphilus*^{T} (NZ_BCQI00000000.1), *Alb. sendaiensis*^{T} (NZ_BCRQ00000000.1), *Alb. acidoterrestris*^{T} (NZ_AURB00000000.1). The average amino acid identity (AAI) values between the genomes were calculated using the online tool AAI-matrix calculator (http://enve-omics.ce.gatech.edu/g-matrix/; Rodriguez-R and Konstantinidis, 2016), considering only the reciprocal best hits (two-way AAI). The percentage of conserved proteins (POCP) in the genomes were determined by “BLAST” analysis of CDSs against a database of peptide sequences (UniProt Trembl and Swissprot databases combined, http://www.uniprot.org/downloads) using the sequence aligner software DIAMOND (https://github.com/bbuchfink/diamond). The conserved proteins from a query genome were defined as a BLAST match with the Expect value (E) less than 1 e-5, a sequence identity of more than 40%, and an alignable region of the query protein sequence (query coverage) of more than 50%.

4.2.9.2 Results

The draft genome of strain I2511 produced 185,106 paired-end reads with a total length of 3,482,793 bp, assembled into 242 contigs with 19-fold coverage and 96% completeness. The automated annotation of the genome predicted 3,803 protein-coding sequences (CDS) with

72 repeat regions, one copy each ribosomal RNA genes (16S rRNA, 23S rRNA and 5S rRNA) and 56 tRNA genes. From the annotation, the genome encoded 1,881 hypothetical proteins and 1,922 proteins with function assignments. Table 4.2 compares the genomic information of the isolate I2511 with the five closest-related *Alicyclobacillus* spp.

Table 4.2. General genomic information of strain I2511 and the five closest-related *Alicyclobacillus* spp.

Strain	Genome size (Mb)	Contigs	N_{50} contig length (bp)[a]	Genes	G+C (mol%)
I2511	3.48	242	53,710	3,803	50.2
Alb.contaminans^T (DSM 17975)	3.27	111	64,314	3,304	58.2
Alb. hesperidum^T (DSM 12489)	2.86	55	147,474	2,840	53.9
Alb. acidiphilus^T (NBRC 100859)	3.86	165	57,905	3,768	54.9
Alb. sendaiensis^T (NBRC 100866)	2.79	153	38,367	2,789	62.7
Alb. acidoterrestris^T (ATCC 49025)	4.06	207	44,524	4,240	52.2

[a] 50% of the genome contain contigs with length larger than or equal the N_{50} contig size.

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The average amino acid identity (AAI) obtained between strain I2511 and *Alb. contaminans*^{T} was 58 ± 15% from 1,885 proteins (AAI ≥ 65 is the cut-off value for species of the same genus, Konstantinidis *et al.,* 2017). The AAI values obtained by the comparison of the genome of strain I2511 and other species of *Alicyclobacillus* are shown at Table 4.3. In terms of POCP values, 50% of the protein-coding sequences of strain I2511 matched those of the genus *Alicyclobacillus* (the cut-off value for species of same genus is POCP > 50%). Table 4.4 show the POCP values of five closest-related *Alicyclobacillus* spp.

Table 4.3. Pairwise comparison of the average amino acid identity (AAI) between genomes of the isolate I2511 and *Alicyclobacillus* spp.

Pairwise comparison with strain I2511	AAI[a] (%)
Alb. contaminans	58
Alb. hesperidum	54
Alb. acidiphilus	53
Alb. sendaiensis	53
Alb. acidoterrestris	54

[a]standard deviation for all AAI values was ± 15%

Table 4.4. Percentage of conserved proteins (POCP) of the genomes of isolate I2511 and *Alicyclobacillus* spp.

Strain	POCP (%)
I2511	50
Alb. contaminans^T	66
Alb. hesperidum^T	99
Alb. acidiphilus^T	71
Alb. sendaiensis^T	95
Alb. acidoterrestris^T	71

4.3 Reduction of zero-valent sulfur by acidophilic and acid-tolerant sulfate-reducing bacteria (aSRB)

Three strains of aSRB, *Peptococcaceae* CEB3, *Desulfosporosinus acididurans* strain M1^T and "*Desulfobacillus acidavidus"* CL4, were cultivated in Src medium in order to determine whether they could grow by dissimilatory reduction of zero-valent sulfur (ZVS).

4.3.1 Methods

To ascertain whether the three aSRB strains could catalyse the dissimilatory reduction of ZVS, cultures were initially sub-cultured in liquid media in which sulfate salts were gradually replaced by chloride salts and the pH (usually 4.0) was adjusted with hydrochloric rather than with sulfuric acid. Cultures of each isolate were incubated in a separate sealed jar under anaerobic atmospheres (section 2.2.6) together with a "copper trap" (open universal bottle containing 10 mL of ~ 15 mM $CuSO_4$), and incubated at 30ºC. After confirmation that cultures were generating H_2S (by formation of CuS in the "copper trap"), cells were harvested, re-suspended (in Src medium) and 50 µL aliquots used to inoculate modified Src liquid medium (~ 0.6 x10^7 cells mL^{-1}), which contained 5 mM glycerol, 0.005% (w/v) yeast extract, 0.35 mM $ZnCl_2$, pH 5.0 (adjusted with HCl). ZVS-free control cultures were also placed in a separate jar and incubated in parallel. Bacterial growth was evaluated by enumerating planktonic cells and generation of H_2S was assessed by changes in the concentrations of soluble zinc in the cultures.

A second experiment was carried out with strains M1^T, CL4 and isolate I2511 to evaluate consumption of glycerol coupled to the dissimilatory reduction of ZVS. Replicate cultures containing modified Src medium, as above but containing 1 mM $ZnCl_2$, were incubated in separate sealed jars under anaerobic atmospheres, as described previously.

4.3.2 Results

Strains $M1^T$, CEB3 and CL4 shown to be metabolically active, as indicated by generation of H_2S, as assessed by visualization of CuS precipitates in the “copper trap”, and also to grow in sulfate-free media. Results from the adaptation period are shown in Figure 4.27. Cell numbers of cultures of strains CEB3 and $M1^T$ containing ZVS were greater than in ZVS-free controls, although cell numbers also increased in the latter. For strain CL4, cell counts also increased in cultures with and without ZVS, and numbers were very similar in both (Fig. 4.27). The final pH of cultures of CEB3 both with and without ZVS were similar and slightly higher the initial pH (4.0). However, pH of ZVS-free controls of both $M1^T$ and CL4 increased from 4 to ~ 6 and 5.5, respectively, and were higher than cultures containing ZVS (pH ~ 4.5).

Following adaption of the cultures to sulfate-free liquid medium, fresh cultures were incubated for 13 days (Figures 4.28). In general, planktonic cell numbers of all cultures containing ZVS were higher than ZVS-free controls though planktonic cells in cultures of isolate CEB3 were far fewer in number than those of isolates $M1^T$ and CL4. Generation of H_2S was confirmed by formation of CuS precipitates in the “copper trap” and by the decrease of Zn^{2+}concentrations in all three aSRB cultures. Concentrations of Zn^{2+} in all cultures containing ZVS were below the detection limit (<0.01 mg L^{-1}), while in the ZVS-free control cultures, Zn^{2+} concentrations were 0.33 - 0.36 mM. The pH of the cultures containing ZVS of all isolates were below 3.5 (Fig. 4.28), due to the precipitation of ZnS which is a proton-generating reaction ($Zn^{2+} + H_2S \rightarrow ZnS + 2\ H^+$). In contrast, the pH of the ZVS-free controls of strains $M1^T$, CEB3 and CL4 decreased only slightly from 5 to ~ 4.5.

In the second experiment, generation of hydrogen sulfide by isolates $M1^T$, CL4 and I2511 was also detected by formation of CuS in the “copper traps”. The pH of cultures of strains $M1^T$, CL4 and I2511 decreased from 5.0 to ~2.8 (Fig. 4.29). Oxidation of glycerol varied between strains, with strain $M1^T$ displaying higher values (also higher deviation) than strains CL4 and I2511. Generation of acetic acid paralleled glycerol oxidation. Precipitation of Zn^{2+} was similar for strains $M1^T$ and CL4 (~0.6 mM), while less zinc precipitated in cultures of I2511 (~0.4 mM).

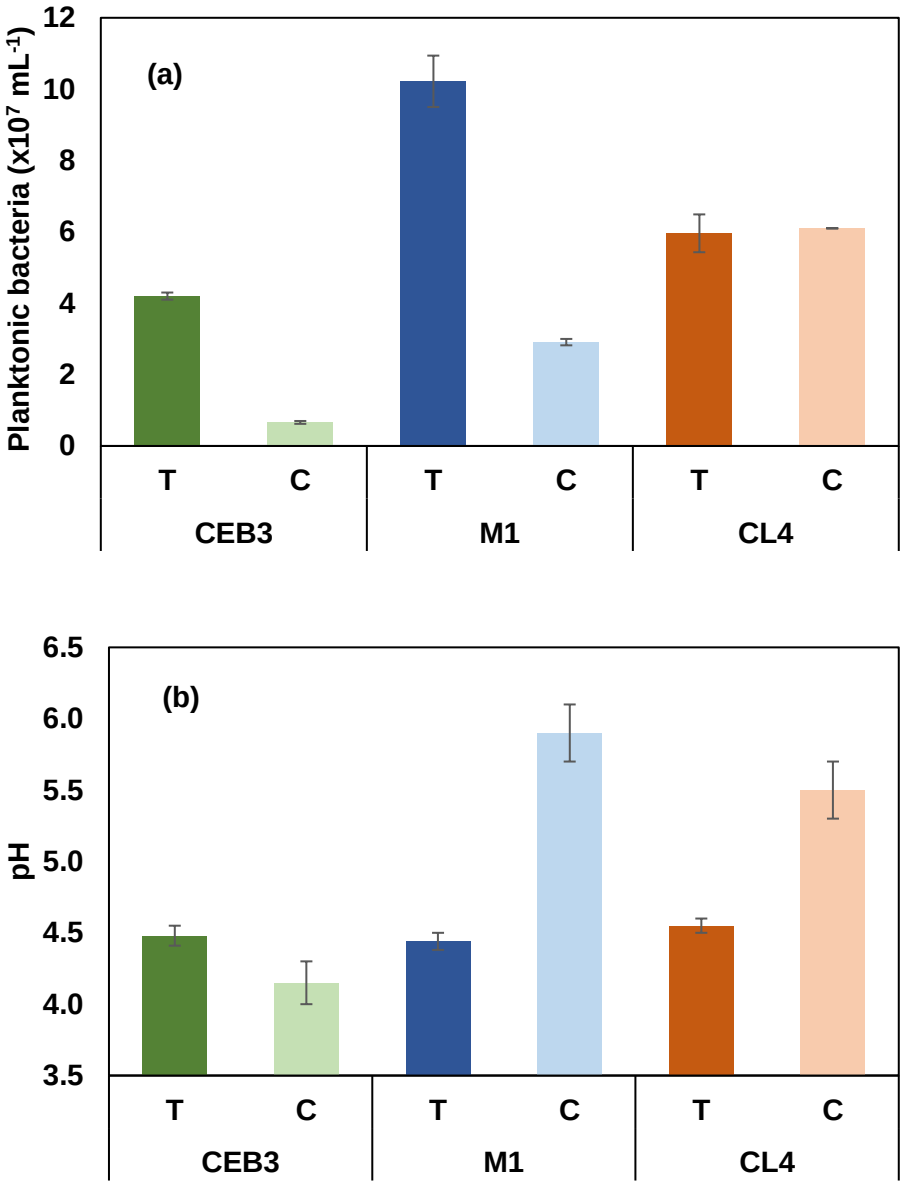

Figure 4.27. Dissimilatory reduction of ZVS by aSRB isolates *Peptococcaceae* CEB3, *D. acididurans* T (strain M1) and "*Ds. acidavidus"* CL4: (a) planktonic cell numbers and (b) pH in Src medium containing 0.005% (w/v) yeast extract and 5 mM glycerol pH 4.0. "T" refers to cultures containing ZVS and "C" refers to ZVS-free control cultures. Bars indicate mean values and the error bars data ranges (n=2).

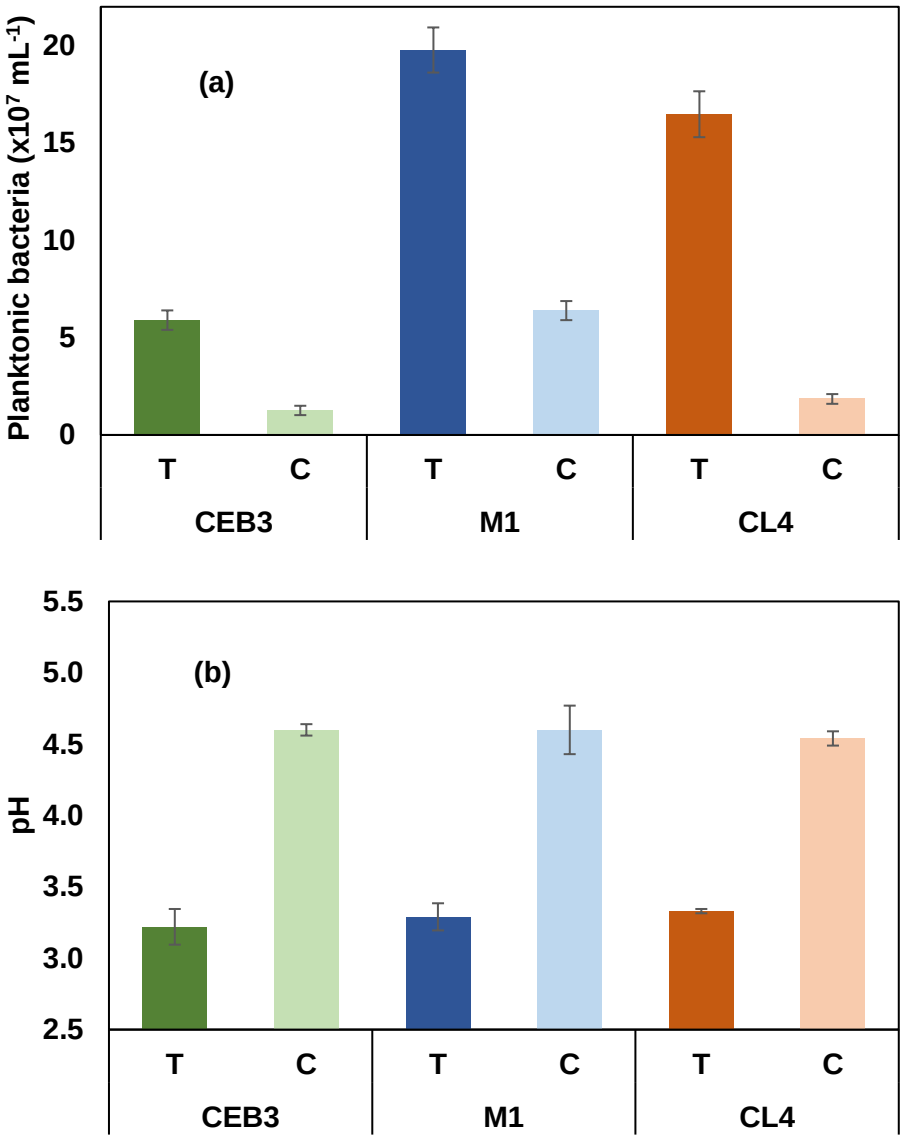

Figure 4.28. Dissimilatory reduction of ZVS by aSRB isolates *Peptococcaceae* CEB3, *D. acididurans*^{T} (strain M1) and "*Ds. acidavidus"* CL4: (a) planktonic bacterial cell numbers and (b) pH of cultures in modified Src medium containing 5 mM glycerol, 0.005% (w/v) yeast extract and 0.35 mM $ZnCl_2$ pH 5.0. "T" refers to cultures containing ZVS as electron acceptor and "C" refers to ZVS-free control cultures. Bars indicate mean values and the error bars data ranges (n=2).

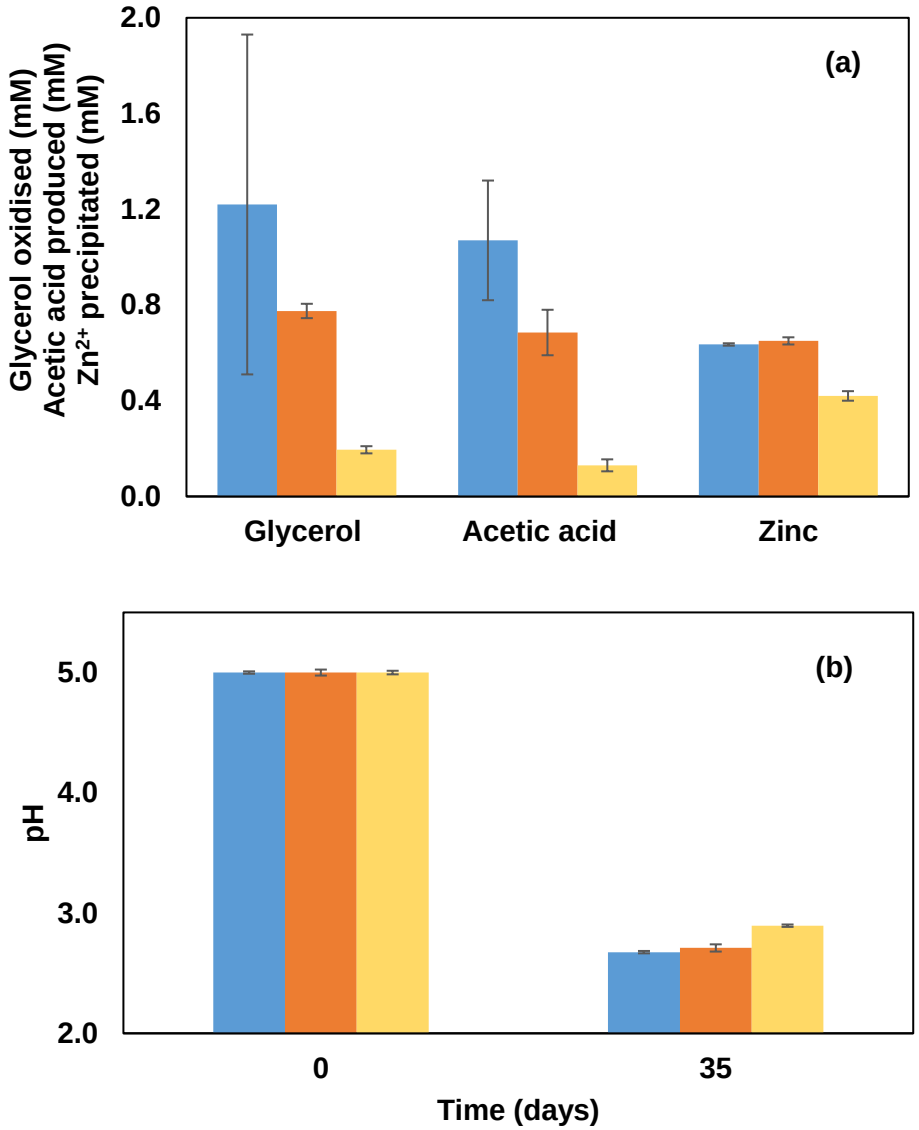

Figure 4.29. Dissimilatory reduction of ZVS by isolates *D. acididurans*^{T}, "*Ds. acidavidus"* CL4 and isolate I2511: (a) glycerol oxidised, acetic acid produced and Zn^{2+} precipitated after 35 days of incubation, and (b) pH, at the start and after 35 days of incubation in Src medium containing 5 mM glycerol, 0.005% (w/v) yeast extract and 1.0 mM $ZnCl_2$ pH 5.0. Key: *D. acididurans*^{T} (■), "*Ds. acidavidus"* CL4 (■) and *Fimicute* I2511 (■). Bars indicate mean values and the error bars data ranges (n=2).

4.4 Discussion

This chapter describes the dissimilatory reduction of ZVS at low pH by acidophilic isolates of the phylum *Firmicutes.* Several experiments were performed to assess the characteristics of a novel acidophilic sulfur-reducing bacterium, isolate I2511, which was able to reduce ZVS at very low pH. This chapter also confirmed that three strains of aSRB *Firmicutes*, *Peptococcaceae* CEB3, *Desulfosporosinus acididurans*^{T} and "*Desulfobacillus acidavidus"* CL4, were all able to reduce ZVS at low pH

The enrichments performed with mine water sediments to isolate acidophilic sulfur-reducers produced only one isolate, strain I2511 that grew by ZVS respiration, generating H_2S. T-RFLP analysis of enrichment cultures showed that the bacterial population changed greatly with time in composition and in relative abundance during incubation. Bacterial diversity decreased with time in enrichment A with *Firmicute* I2511 becoming increasingly dominant while in contrast the population diversity increased with time in enrichments B and C. Some factors might have influenced the low diversity of enrichments: (i) not every prokaryote can utilise solid electron acceptors, (ii) low pH conditions and (iii) the use of chloride salts in the medium composition (chloride ions can be inhibitory at very low pH; Falagán and Johnson, 2018).
The composition of the bacterial population of enrichments A, B and C were quite distinct during incubation. These differences were reflected in cell morphologies seen under light and electron microscopy, in which most of the cells in enrichment C were larger than those in enrichments A and B (Fig. 4.30).

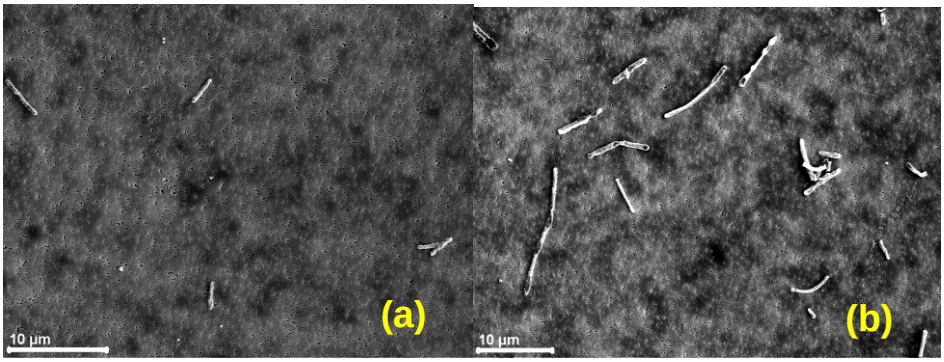

Figure 4.30. Scanning electron micrographs of cultures used to enrich acidophilic sulfur-reducers: (a) enrichment A; (b) enrichment C. Images were provided by Ivan Ñancucheo (*Facultad de Ingeniería y Tecnología*, Universidad San Sebastian, Chile).

Following the inoculation of aSRB plates using enrichments A, B and C, none of the colonies that grew deposited zinc sulfide (i.e. none appeared to grow by sulfate reduction). Intriguingly, although isolate I2511 was obtained from aSRB plates inoculated with enrichment B, this

strain was also shown subsequently not be capable of dissimilatory sulfate reduction, and that it could use only two electron acceptors of those tested - ZVS and ferric iron. aSRB plates contain 100 µM Fe^{2+} and the solid medium pH is > 4 (the pH of liquid components before combining with the agarose suspensions is 4, and addition of this gelling agent increase the final pH of the medium). In the presence of oxygen and at pH values >3.5, ferrous iron oxidises to ferric iron abiotically (Johnson *et al.,* 2012). Since overlay plates can be stored aerobically at 4°C for up to several weeks before being used, it is possible that some, at least, of the Fe^{2+}oxidised to Fe^{3+} during this time. In that case, the growth of isolate I2511 on aSRB plates could be explained by cells growing by ferric iron respiration. As stated above, the total concentration of iron in aSRB solid medium is only 100 µM, which meant that very little of the potential electron donor (ferric iron) would have present, which was reflected by the sizes of the colonies of isolate I2511, which were very small.

Digestion of the 16S rRNA gene of isolate I2511 by the restriction enzyme HaeIII generated several terminal fragments, with lengths ranging from 261 nt to 267 nt, with the 262 nt and 266 nt fragments being most predominant. The 266 nt T-RF was obtained by *in silico* digestion of the 16S rRNA gene obtained from the genome, but not the 262 nt T-RF. Several factors can affect the ability of restriction enzymes to cut DNA at these specific sites (Osborn *et al.,* 2000; Qiu *et al*., 2001). For instance, variations in the gene sequence such as point mutation (e.g. the secondary structure of 16S rRNA gene may generate mutations/deletions during PCR amplification) can alter restriction sites, generating fragments of different sizes (Qiu *et al*., 2001). In addition, many restriction enzymes tend to have a preferential restriction site. In contrast, *in silico* digestion performed with CfoI and AluI indicated several possible terminal fragment sizes, but both of these enzymes cut the 16S rRNA gene amplified from DNA extracted from isolate I2511 at only one restriction site.

In order to overcome the problem of poor growth on aSRB plates of isolate I2511, a different solid medium ($S^0\underline{o}$ medium) was developed in which sterile microbially pre-wetted ZVS was added to the surface of a gelled overlay medium. This plate formulation proved to be an effective way to grow I2511 on solid media, possibly because it allowed direct contact of the bacterial cells with ZVS particles. To confirm whether this solid medium was selective for ZVS-reducing microorganisms, $S^0\underline{o}$ plates were inoculated with enrichments A, B and C. Colonies were analysed by T-RFLP and all of them were identified as isolate I2511.

Growth rates of isolate I2511 confirmed that this *Firmicute* is an acidophile with an optimum growth pH of ~3.7, Tests carried out in universal bottles suggested it was able to grow at pH values as low as 1.8, which was very different to results obtained in a bioreactor where it was

unable to grow at pH 2.5. There are two possible reasons for this apparent discrepancy: (i) carryover of more acetic acid in the bioreactor (generally ~ 80% of the bioreactor was drained and replaced when setting up a new growth test); (ii) greater mixing in the bioreactor, so eliminating the potential (as in universal bottles) to develop pH gradients and niches. Interestingly, in Chapter 5, in the experiments with the hybrid sulfidogenic bioreactor at pH 2.0 (sections 5.3.3) isolate I2511 was shown to be the dominant member of the bacterial population (present at ~50% relative abundance).

When isolate I2511 was grown in liquid medium to which ferric sulfate had been added ~ 10 mM ferrous iron was generated. The initial pH of this culture was 2.5, which meant that most of the added ferric iron would have hydrolysed, forming solid phase amorphous and poorly crystalline ferric iron minerals, such as schwertmannite (Hedrich and Johnson, 2012). Reduction came to a halt at day 17, by which time the culture pH had increased to ~ 4.6. The increase in pH is consistent with that reported for the reductive dissolution of ferric iron minerals, such as goethite (Hallberg *et al.,* 2011). Acidophilic iron-reducing bacteria are thought to use soluble ferric iron, and not solid phase ferric minerals, as electron acceptors (Hallberg *et al.,* 2011). By doing so, they cause the equilibrium between solid phase and soluble ferric iron to shift, inducing more dissolution of the solid phase (Eq 4.1):

$$Fe(III)_{solid} \leftrightarrow Fe(III)_{soluble} \rightarrow Fe(II) \qquad \text{Eq 4.1}$$

The equilibrium between solid/mineral-phase ferric iron and soluble ferric iron is both pH- and mineral-dependent, with solubilisation being favoured by low pH and less crystalline minerals (e.g. goethite is more readily dissolved than is haematite). As the pH increased in these cultures, so the abiotic dissolution of solid phase ferric iron would have slowed down, thereby limiting the ability of I2511 to continue to grow by ferric iron respiration. One way to test this hypothesis would be to grow the isolate at a fixed pH (~ 2.5) in a bioreactor, where growth would be expected to continue until either the electron donor(s) or acceptor (ferric iron) are depleted.

Interestingly, although it appeared initially that isolate I2511 could also use soluble ferric citrate as an electron acceptor, this was not confirmed with protracted incubation of cultures. It appears therefore that complexed ferric iron was not used, and that chemical reduction of soluble ferric citrate was probable reason for the observed increases in ferrous iron seen in both inoculated and control cultures.

Bonch-Osmolovskaya *et al.* (1990), when describing *Desulfurella acetivorans*., a genus of sulfur-respiring bacteria, defined the term “true sulfur reducers” for organisms that use ZVS

respiration as the only or the preferable catabolic reaction. Considering that the most effective way to grow isolate I2511 was by reduction of ZVS, is possible to infer that this isolate is a "true sulfur reducer".

Isolate I2511 was able to utilise several small molecular weight organic compounds such as glycerol, ethanol, methanol, glucose, fructose, 1,3-propanediol, pyruvate and citric acid as electron donors, and to couple these to the reduction of ZVS. However, growth in cultures containing these defined organic compounds did not occur unless a complex organic material (yeast extract in this experiment) was also added. Isolate I2511 could grow using either yeast extract or peptone as the sole organic material present, where presumably some of the components of both acted as electron donors as well as carbon sources.

Planktonic cell numbers of cultures containing 0.05% (w/v) yeast extract (200 mg C L^{-1}, assuming that yeast extract, like glucose, contains 40% of carbon) were ~3 times greater than in cultures containing 5 mM glycerol (180 mg C L^{-1}) and 0.005% (w/v) yeast extract (20 mg C L^{-1}, even though the total amount of organic carbon was the same in both media. Therefore, isolate I2511 grew more effectively when provided complex organic sources, which is a characteristic exhibited by other acidophilic bacteria (e.g. "*Acidibacillus"* spp.) and some neutrophilic ZVS-reducing archaea (e.g. *Pyrobaculum islandicum*, *Thermoproteus tenax;* Rabus *et al*., 2006). The preference for complex rather than defined organic substrates these and other prokaryotes may be attributed to the former acting as both carbon and energy sources for those organisms, while the latter act as either electron donor or carbon source. This was the case of "*Acidibacillus"* spp. described in Chapter 3, in which glucose (and other defined organic compounds) was thought to be utilised as carbon source but not as an electron donor.

Various small molecular weight organic acids were used or detected in these investigations. These have different pK_a values, and would have been present in both dissociated (anionic) and non-dissociated (uncharged) forms in the acidic media used. The terms used (e.g. acetic acid and pyruvate) reflect the major forms that these acids would have occurred in at the pH of the media used.

The ratios of acetic acid generated to glycerol consumed by *Desulfosporosinus acididurans*^{T}, "*Desulfobacillus acidavidus"* CL4 and isolate I2511 were ~ 0.9, 0.9 and 0.7 (mean values) respectively, in universal bottle cultures containing very small amounts (0.005% w/v) of yeast extract, and 5 mM glycerol. During growth experiments of isolate I2511 in bioreactor, the mean ratio of the acetic acid produced and glycerol consumed was ~0.4. In cultures of isolate I2511

containing 0.05% (w/v) of either yeast extract or peptone as the sole organic material acetic acid was produced at final concentrations of ~0.3 mM. Concentrations of 0.05% (w/v) of both of these complex organic materials is equivalent to ~ 200 mg L^{-1} (or about 17 mM) of carbon. The stoichiometry of yeast extract/peptone metabolised (assuming most of it was) to acetic acid produced is therefore much less than in the case of glycerol. Generation of acetic acid as a by-product during growth by isolate I2511 indicates that this isolate is an incomplete oxidiser, as is *D. acididurans*^{T}, "*Ds. acidavidus"* and many other neutrophilic sulfidogens (e.g. *Sulfospirillum* spp.*, Wolinella* spp.*, Shewanella* spp.). The lower ratio for acetic acid produced:glycerol oxidised in cultures isolate of I2511 when cultivated in the bioreactor can be attributed to loss during the experiments. Acetic acid is a volatile compound therefore has high vapour pressure. The stream of OFN and intense agitation (150 rpm) during growth in the bioreactor may had shifted the equilibrium between the liquid and vapour phase of acetic acid, causing an increase in the evaporation rates.

At pH 2.5, the presence of 0.5 and 1.0 mM acetic acid increased the lag phase and decreased the growth rate of I2511 cultures and growth was completely inhibited by 3 mM acetic acid. The pK_a of acetic acid is 4.76, which means that at pH 2.5, acetic acid is present predominantly in the undissociated form, which is lipophilic and can diffuse into the cells lowering intracellular pH, causing cell death. This result indicates that a co-culture of isolate I2511 and the acidophilic acetate-consumer, *Ac. aromatica*^{T} which can metabolise acetic acid at low pH (Jones *et al.,* 2013) could be beneficial to the former. In contrast, isolate I2511 was able to grow in media containing 10 mM pyruvate at pH 3.0, where only 24% of the acid would have been present initially in its non-dissociated form, and even less as the pH increased as sodium pyruvate was metabolised.

Isolate I2511 was highly intolerant of salt (sodium chloride) and copper ions. Previous reports showed that some species of moderately acidophilic sulfate- and sulfur-reducing bacteria have relatively low tolerance to metals and/or sodium chloride. For instance, *D. acididurans*^{T} was completely inhibited by 1 mM copper but tolerated up to 300 mM NaCl (Sanchez *et al.,* 2015); *Desulfurella amilsii*^{T} was inhibited by 0.5 mM copper (Florentino *et al.,* 2015); mixed cultures of acidophilic aSRB grew up to 0.2 mM copper (Hedrich and Johnson, 2014) but were inhibited by 100 mM NaCl (A.L. Santos and D.B. Johnson, Bangor University, unpublished data).

The establishment of a "copper trap" in tests performed in sealed jars provided an effective way to confirm generation of H_2S, which is an indicator of bacterial growth, without opening the anaerobic jars. In addition, the removal of part of the H_2S produced by precipitating CuS in the "copper trap" was also beneficial since this gas is toxic in high concentrations. The

medium devised for sulfate-reducing bacteria (aSRB medium, section 2.2.4) contains $ZnSO_4$, which has two important functions. Firstly, it is used to counterbalance the consumption of protons as a consequence of the dissimilatory reduction of sulfate at pH < 4.5 (Eq 4.2):

$$4\ C_3H_8O_3 + 7\ SO_4^{2-} + 14\ H^+ \rightarrow 7\ H_2S + 12\ CO_2 + 16\ H_2O \qquad \text{Eq. 4.2}$$

This is achieved by the acidity generated when zinc is precipitated as ZnS (Eq. 4.3):

$$7\ Zn^{2+} + 7\ H_2S \rightarrow 7\ ZnS + 14\ H^+ \qquad \text{Eq. 4.3}$$

The second function is (like the "copper trap") to act as an indicator of H_2S production: ZnS precipitates have a white/silver colour and form a "crust" on the sides of shake flasks, which are readily observed. However, in the case of dissimilatory sulfur-reduction, which is a pH neutral reaction at pH < 4.5 (Eq 4.4), the presence of soluble zinc in the medium would decrease the pH.

$$C_3H_8O_3 + 7\ S^0 + 6\ H_2O \rightarrow 3\ CO_2 + 7\ H_2S \qquad \text{Eq. 4.4}$$

Phylogenetic analysis of the 16S rRNA gene of strain I2511 showed that the isolate clustered with strain IR2 and clone G13 in a separate clade from that represented by the closest validated *Alicyclobacillus* species. Strain IR2 was isolated by Ñancucheo and Johnson (2012) from a sulfate-reducing bioreactor inoculated with an enrichment containing biomass collected from a stream draining an abandoned copper mine. Strain IR2 was isolated from the bioreactor (maintained at pH 2.2) on aSRB plates and identified as a non-sulfidogenic facultative anaerobe. Clone G13 was obtained from enrichment cultures used to isolate sulfate-reducing bacteria that were inoculated with mine tailings containing high concentrations of methylmercury (Winch *et al.,* 2009). The origins of isolate IR2 and clone G13 (H_2S-generating mining-related environments) were therefore similar to that of strain I2511.

Some characteristics of strain I2511 (i.e. a mesophilic obligate anaerobe, which catalyses the dissimilatory reduction of both ZVS and ferric iron and has relatively low tolerance to copper and sodium chloride) are distinct from its close relatives *Alb. contaminans*^{T} and *Alb. tolerans*^{T}. For example, *Alb. contaminans*^{T} is a moderately thermophilic strict aerobe, and tolerates up to ~350 mM NaCl. *Alb. tolerans*^{T} is a moderately thermophilic facultative anaerobe and grows as a heterotroph and also autotrophically using Fe^{2+} and ZVS as electron donors (Appendix 4A). The acidophilic genera of the family *Alicyclobacillaceae* (i.e. *Alicyclobacillus, Sulfobacillus* and "*Acidibacillus"*) share several characteristics. Many species are strict aerobes, others are

facultative anaerobes and use either molecular oxygen or ferric iron as electron acceptors, many species are thermo-tolerant or moderate thermophilic, several are obligate heterotrophs, some are facultative autotrophs, some species can use ferrous iron or reduced sulfur as electron donors, and display elevated tolerance to transition metals. However, none of the species described to date can grow anaerobically by sulfur respiration, which clearly delineates isolate I2511 (which is a strict anaerobe) from other species of the family *Alicyclobacillaceae.*

Several authors have discussed the criteria required for circumscribing prokaryotic genera (Yarza *et al.,* 2008; Tindall *et al.,* 2010; Rodriguez-R and Konstantinidis, 2014; Qin *et al.,* 2014; Yarza *et al.,* 2014; Aliyu *et al*., 2016; Konstantinidis *et al.,* 2017). As general guidelines, the authors suggested the following conditions should be considered when confirming a novel genus: (i) 16S rRNA gene similarity shared with the closest related genus should be <94%; (ii) clear separation of a monophyletic group; (iii) collection of unique phenotypic characteristics distinct from those of other related genera; (iv) compliance with thresholds defined by overall genome relatedness indices (OGRI), e.g. AAI and POCP. In relation to these guidelines, strain I2511 shared 93% identity of its 16S rRNA gene with *Alb. contaminans*^{T}, formed a separate clade in the phylogenetic analysis and exhibited very distinct physiology from *Alicyclobacillus* spp. In terms of OGRI, the AAI between I2511 and *Alb. contaminans*^{T} was 58 ± 15%, overlapping with the cut-off value for species in the same genus (AAI > 65). The pairwise comparison of strain I2511 with other *Alicyclobacillus* species also showed overlapping of AAI values. From Table 4.4 (section 4.2.9.2) is clear that the genome of isolate I2511 exhibited far less relatedness to the genus *Alicyclobacillus*, in terms of conserved proteins (POCP = 50%), than the other species where POCP ≥ 66% (these species shared with isolate I2511 92-93% similarity of their 16S rRNA genes). Nonetheless, the POCP obtained for isolate I2511 is very close to the cut-off for species of the same genus (POCP >50 %). The use of POCP as genomic index for prokaryotic genus delimitation has been described by Qin *et al.* (2014) and applied in several studies (Nicholson *et al.,* 2018; Salvettia *et al.,* 2018; Ishii *et al.,* 2017; Aliyu *et al*., 2016; Sachse *et al.,* 2015). The POCP index was devised based on the concept that the proteins encoded in a genome define the morphological, physiological, and biochemical traits of a prokaryotic strain, and that a prokaryotic genus could be described as a group of species that share more than half of their proteins. Both OGRI values measured (AAI and POCP) were not able to resolve the taxonomy of the *Firmicute* I2511. However, the percentage of conserved proteins was effective in underlining the distinct physiology of isolate I2511 compared to *Alicyclobacillus* spp.. While further analysis are needed to confirm the taxonomy of the isolate I2511, the overall results

obtained in this study suggests that strain I2511 is a representative of a novel genus within the phylum *Firmicutes*.

Dissimilatory reduction of ZVS in acidic conditions was assessed in three strains of aSRB: *Peptococcaceae* CEB3, *Desulfosporosinus acididurans* M1^T and "*Desulfobacillus acidavidus"* CL4. All strains tested could grow by sulfur respiration at low pH, as indicated by CuS in the "copper trap", decrease in Zn^{2+} concentrations in test cultures and higher cell numbers in test cultures compared to ZVS-free controls. A previous report had shown that *D. acididurans*^{T} was able to reduce ZVS (Sanchez-Andrea *et al.,* 2015), though this was tested at pH 5.5. As described later in this thesis (Chapter 5), *D. acididurans*^{T} represented ~10% of the ZVS-attached bacterial communities of the T-RLFP profile at both pH 3 and 4 in a "hybrid" sulfidogenic bioreactor operated as a ZVS-only reducing bioreactor. *Peptococcaceae* CEB3 represented 6% of the population at pH 3 and was not detected in the bioreactor at pH 4.

Cell numbers of both *D. acididurans*^{T} and "*Ds. acidavidus"* CL4 increased (as did pH) in ZVS-free control cultures in sulfate-free Src medium, though this was not the case for *Peptococcaceae* CEB3. This could imply that these strains were able to grow by fermentation of yeast extract or glycerol, as well as by sulfate/ZVS respiration. However, it is also possible that this result was due to the carryover of small amounts of ZVS from inoculum, and confirmation of the ability of these bacteria to grow by fermentation requires further tests. Sanchez-Andrea *et al.* (2015) reported that *D. acididurans*^{T} was able to grow by fermentation in sulfate-free media containing yeast extract, pyruvate or fumarate, but not glycerol. Consumption of glycerol and production of acetic acid differed between *D. acididurans*^{T}, "*Ds. acidavidus"* CL4 and isolate I2511, with the latter exhibited the lowest values. This result reflects physiological differences between isolate I2511 and *D. acididurans*^{T}, "*Ds. acidavidus"* CL4. Isolate I2511 has much slower growth rates than the two aSRB (e.g. culture doubling times at optimum conditions of *D. acididurans*^{T} is ~10-fold lower than I2511). Another factor was the starting pH of the medium (5.0) which was well above the optimum value for strain I2511 (pH 3.7), whereas that of *D. acididurans*^{T} is pH 5.5 and that of "*Ds. acidavidus"* CL4 has not yet been determined.

Results of research described in this Chapter showed clearly that dissimilatory reduction of ZVS at low pH (< 3) could be mediated by several species of sulfidogenic bacteria of the phylum *Firmicutes*. Application of this metabolic potential is the theme of later Chapters (5 and 6)

Chapter 5. Development of a novel “hybrid” low pH sulfidogenic bioreactor.

5.1 Introduction

Increasing worldwide demand for metals and depleting reserves of high-grade primary ores drives the development of technologies that recycle metals from waste material and metal-impacted effluents. Sulfidogenic bioreactors are engineered systems that present an alternative approach to recover metals from and remediate metal-contaminated waters. These systems are referred as “active biological treatments” and harness the abilities of microorganisms to catalyse redox transformations of sulfur compounds. Oxidised sulfur compounds (and zero-valent sulfur; ZVS) are used by some prokaryotes as terminal electron acceptors, generating H_2S. This can react with chalcophilic metals, such as copper and zinc, precipitating them as sulfide phases and, in situations where pH is controlled, can facilitate selective removal of metals from contaminated waters.

Current industrial-scale sulfidogenic systems use neutrophilic species of sulfate- (or ZVS) reducing bacteria that need to be protected from direct contact with elevated concentrations of hydronium ions (H_3O^+; often quoted as proton (H^+) acidity) present in acidic waste waters, such as acid mine drainage. When applied to the treatment of acidic effluents, acidophilic and acid-tolerant species have several advantages over their neutrophilic counterparts (Santos and Johnson, 2016)

A number of reports have described the application of sulfidogenic bioreactors that operate at low pH (<5) (Ňancucheo and Johnson, 2012; 2014; Hedrich and Johnson, 2014; Santos and Johnson, 2016). These have utilised microbial consortia that include novel species of sulfate-reducing acidophiles which use sulfate as terminal electron acceptor in their anaerobic metabolisms. However, even though sulfate (in which sulfur occurs in its most oxidised state; +6) is usually present in elevated concentrations in mine waters, the number of known species of prokaryotes that catalyse the dissimilatory reduction of this oxy-anion in acidic conditions is relatively low (Dopson and Johnson, 2012).

Zero-valent sulfur is a relatively abundant element on Earth’s surface and a by-product of industrial processes (e.g. desulfurization processes in the oil and gas industries). Sulfate and ZVS may be used as electron acceptors by sulfidogenic bacteria, coupled to the oxidation of organic (e.g. glycerol) or inorganic (e.g. hydrogen) electron donors. In thermodynamic terms, ZVS is a superior electron acceptor due to having no requirement for ATP-consuming activation as a first step in its reduction (Rabus *et al.,* 2006) and because the redox potential

of the S^0/H_2S couple (-270 mV) is more positive than that of the SO_4^{2-}/H_2S couple (-303 mV). In theory, smaller amounts of electron donor are required to generate H_2S from ZVS (a 2-electron reduction) than from sulfate (an 8-electron reduction). Nevertheless, being a solid, ZVS is inherently less accessible to sulfidogenic bacteria and is rarely found in significant amounts in mine drainage waters. Therefore, when used in bioreactors treating meal-contaminated waters, sulfur needs to be added from an external source.

While sulfate reduction is a net proton-consuming reaction at low pH (Eq. 5.1), ZVS reduction is a pH-neutral reaction (Eq. 5.2). In situations where chalcophilic metals (Zn, Cu etc.) combine with the H_2S formed by sulfidogenesis, proton acidity is generated, causing the ZVS reduction process to be net acid-producing (Eq. 5.3). Generation of protons in this manner can be neutralised by the natural alkalinity of circum-neutral pH metal contaminated waters.

$$4\ C_3H_8O_3 + 7\ SO_4^{2-} + 14\ H^+ \rightarrow 12\ CO_2 + 7\ H_2S + 16\ H_2O \qquad \text{Eq. 5.1}$$

$$C_3H_8O_3 + 7\ S^0 + 6\ H_2O \rightarrow 3\ CO_2 + 7\ H_2S \qquad \text{Eq. 5.2}$$

$$C_3H_8O_3 + 7\ S^0 + 7\ Zn^{2+} + 3\ H_2O \rightarrow 3\ CO_2 + 7\ ZnS + 14\ H^+ \qquad \text{Eq. 5.3}$$

This chapter describes a new variant of low pH sulfidogenic bioreactors, referred to as a “hybrid” sulfidogenic bioreactor (HSB), which was designed to target the removal of chalcophilic metals from circum-neutral pH metal-contaminated liquors (Chapter 6). In this novel approach, both sulfate and ZVS were available as potential electron acceptors, and glycerol was provided as the electron donor. The HSB housed a microbial consortium that included novel species of acidophilic sulfidogens, including that were able to utilise both sulfate and ZVS.

5.2 Design of a novel “hybrid” low pH sulfidogenic bioreactor

5.2.1 Microbial community

Novel species of acid-tolerant and acidophilic sulfate-reducing bacteria (aSRB) isolated from mine sites have been used previously as members of consortia of low pH sulfidogenic bioreactors (Ňancucheo and Johnson, 2012, 2014; Santos and Johnson, 2016). They have the duel advantages of considerably greater tolerance to both acidity and dissolved metals than neutrophilic species.

The “hybrid” sulfidogenic bioreactor (HSB) was populated with microorganisms immobilized on porous glass beads, comprising species of aSRB and non-sulfidogenic acidophilic bacteria (Ňancucheo and Johnson, 2012). The HSB was also inoculated with a mixed culture of acidophilic ZVS-reducing bacteria and non-sulfidogenic acidophilic bacteria, obtained from sediments of an abandoned copper mine in North Wales, United Kingdom, previously enriched

with sulfate-free medium containing ZVS, glycerol and chloride basal salts, as described in Chapter 4 (section 4.2.1; enrichment B).

5.2.2 Operation of the “hybrid” sulfidogenic bioreactor

A novel low pH sulfidogenic upflow biofilm reactor was set up and operated as described by Ňancucheo and Johnson (2012). A FerMac 310/60 unit (Electrolab., UK) was used to control pH, temperature (maintained at 30°C during the entire test period) and agitation (50 rpm) of the 2.2 L (working volume) reactor vessel (Fig. 5.1). Mostly, the bioreactor was operated in a continuous flow mode, with a constant flow rate of 455 mL h^{-1}, corresponding to a hydraulic residence time (HRT) of approximately 5.0 h (HRT = V/F, where V is the volume of the bioreactor and F is the flow rate). A continuous stream of oxygen-free nitrogen (OFN ~200 mL min^{-1}) was used to maintain a slight positive pressure within the reactor vessel and to remove hydrogen sulfide (H_2S) produced inside the bioreactor vessel and deliver it to an off-line vessel. The influent liquor used in most experiments contained acidophile basal salts (ABS, section 2.2.1) supplemented with glycerol (1 - 2 mM) as principal electron donor, yeast extract (0.01%) to provide necessary growth factors (and a possible additional electron donor), varying concentrations of zinc sulfate, and a standard pH of 6.5. Zinc was selected to assess in-line metal precipitation due to its lower toxicity (compared to many other transition metals) to aSRB (e.g. Sánchez-Andrea *et al.,* 2015) and due to the fact that it is found in elevated concentrations in many metal-contaminated circum-neutral pH waste-waters.

When required, the bioreactor pH was controlled using automated addition of sterile acid and alkaline solutions. In experiments with the HSB that did not have pH control, the bioreactor pH was determined by a combination of factors related to conditions specific to particular experiments (e.g. pH and alkalinity of the influent liquor used, relative rates of sulfate and sulfur reduction, and concentrations of precipitated metals).

Porous glass beads (Poraver Dennert GmbH, Germany) were used as support material for the microbial consortium. These 300g were mixed with 100 g of sterile microbially pre-wetted ZVS (section 2.2.3) previously washed with ABS pH 4.0.

Figure 5.1. Photographic image of the “hybrid” sulfidogenic bioreactor (HSB).

Two different approaches were used to precipitate metals: in-line and off-line (Fig.5.2). In-line precipitation involved feeding the HSB with zinc-containing influent liquors (with added organic materials) and precipitating zinc as ZnS inside the reactor vessel. Off-line precipitation involved precipitating copper in a separate attached glass vessel containing 300 mL of 20 – 50 mM copper sulfate, so that the microbial community had no direct contact with the copper-containing liquor. With the latter, H_2S produced in the bioreactor was delivered in the OFN gas-stream to the separate vessel where it reacted with copper, producing insoluble copper sulfide (Fig. 5.2).

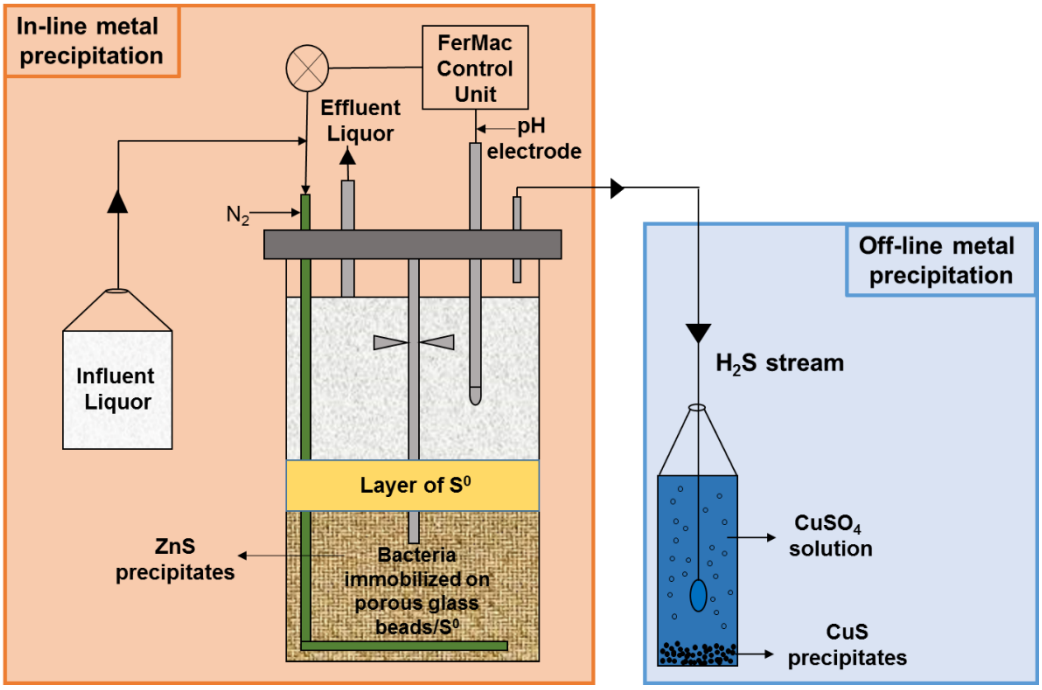

Figure 5.2. Schematic representation of the “hybrid” sulfidogenic bioreactor.

Analytical methods: The pH of the liquid phase above the ZVS layer in the HSB was measured continuously with an in-place glass electrode (Fig. 5.2) and that of the effluent liquor was measured regularly using a Accumet 50 pH meter couple to a pHase combination glass electrode (section 2.3.1.1). Concentrations of glycerol, sulfate and zinc (in both influent and effluent liquors) and of acetic acid (effluent liquors only) were determined by ion chromatography (section 2.3.1.6). Concentrations of copper in the off-line vessel were measured by colorimetry (section 2.3.1.3).

Microbiological analysis: Cells present in effluent liquors were enumerated using a Thoma counting chamber (section 2.4.2). Terminal restriction enzyme fragment length polymorphism (T-RFLP) was used to obtain semi-quantitative data on planktonic (upper liquid phase) and sulfur-attached bacterial communities (section 2.6.3). Bacterial 16S rRNA genes of the HSB were amplified and digested by restriction enzymes (HaeIII, CfoI or AluI), though only results obtained using enzyme HaeIII are described below.

Calculations: Data from these experiments are listed in Appendix 5A – 5U. Various calculations were made from the raw analytical data obtained:

(i) *Glycerol consumption (Δ glycerol):* the difference in glycerol concentrations in the influent and effluent liquors (mM).

(ii) *Complete oxidation of glycerol (%):* this is the percentage of glycerol that had been oxidised completely to CO_2, and was evaluated as

{Δ glycerol (mM) – effluent acetic acid (mM)} / Δ glycerol (mM) x 100.

(iii) *Zinc precipitation (Δ zinc):* the difference in zinc concentrations in the influent and effluent liquors (mM).

(iv) *Net sulfate reduction (Δ SO_4^{2-}):* the difference in sulfate concentrations in the influent and effluent liquors (mM). “Net” sulfate reduction was quoted since there was the possibility of some oxidation of reduced S occurring due to the fact that influent liquors were not de-aerated.

(v) *Rates of net sulfate reduction*: Δ SO_4^{2-} x F (flow rate; L h^{-1}) to give µmoles reduced h^{-1}, and divided by the working volume of the bioreactor (V; 2.2 L) to give µmoles reduced L^{-1} h^{-1}.

(vi) *Rates of H_2S production*. These were calculated by combining the rates of ZnS formation inside the bioreactor and those of CuS formation in the external vessel, and assumed that there was no leakage of H_2S gas from the system. Rates of ZnS formation (µmoles L^{-1} h^{-1}) were calculated as {Δ zinc x F} / V, while those of CuS formation were evaluated from changes in soluble copper concentrations in the off-line vessel during the same period. The amount of copper precipitated (mmoles)

was then calculated by multiplying this number by the volume of copper sulfate solution present in the off-line vessel (300 mL) and rates of CuS precipitation by dividing the latter number by the time period concerned (e.g. 10 h). Finally, by dividing the last number by the volume of the bioreactor (2.2 L), rates of CuS formation as µmoles L^{-1} h^{-1} were obtained. Since the stoichiometry H_2S to both ZnS and CuS precipitated is 1:1, the combined rates of ZnS and CuS formation gave total (Σ) values of H_2S production, as (µmoles L^{-1} h^{-1}).

(vii) *Relative % of sulfate and ZVS used as electron acceptors*: Since it was possible to monitor changes in sulfate concentrations (presumed to be entirely due to dissimilatory sulfate reduction) but not those of ZVS in the bioreactor vessel, the amounts of sulfate removed relative to those of H_2S generated was used to assess the amount of the latter formed from sulfate reduction, and that from reduction of ZVS was inferred from the differences.

H_2S produced from reduction of sulfate (%) =

net SO_4^{2-} reduced (µmoles L^{-1} h^{-1}) / ΣH_2S produced (µmoles L^{-1} h^{-1}) x 100

H_2S produced from reduction of ZVS (%) =

100 - H_2S produced from reduction of sulfate (%)

5.3 Commissioning and preliminary tests with the novel "hybrid" low pH sulfidogenic bioreactor

Preliminary experiments, referred to as the "priming phase", were conducted to evaluate the use of the HSB to produce H_2S in acidic conditions, and to remove metals efficiently from circum-neutral pH metal-rich liquors, in continuous flow mode. Following this, further tests were carried out to assess production of H_2S under more defined conditions.

5.3.1 Priming phase

5.3.1.1 Methods

Five experiments, each lasting for up to 8 days, were initially set up to evaluate in-line metal precipitation and generation of H_2S by the HSB *via* combined sulfur and sulfate reduction. These were performed in sequence, with a "resting period" of few days between each experiment during which time samples were not collected and analysed, though the HSB was maintained in continuous flow mode. The compositions of influent liquors used in each experiment are shown in the Table 5.1. One day before the start of each experiment, approximately 50 g of sterile microbially pre-wetted ZVS (section 2.2.3) was added to the bioreactor. Between experiments 3 and 4, microbial biomass was allowed to accumulate within the HSB during 5 days of using medium containing 5 mM glycerol, 0.02% (w/v) yeast extract

and 0.5 mM $ZnSO_4$ pH 2.5. During this time, the bioreactor was operated in intermittent rather than in continuous flow mode (to minimise washout of bacteria) and the bioreactor was maintained at pH 4.0.

For all experiments carried out in the priming phase, the bioreactor pH was not controlled and was determined by a combination of factors, including: influent liquor pH, relative rates of sulfate reduction (a proton consuming reaction at pH < 7) and ZVS reduction (a pH neutral reaction at pH < 4.5) and precipitation of soluble zinc (a proton-generating reaction). Hydrogen sulfide produced in excess of that needed to precipitate zinc in-line, was removed in a continuous OFN gas stream, and used to precipitate soluble copper off-line in a separate vessel containing 300 mL of 50 mM copper sulfate (Fig. 5.2).

Table 5.1: Influent liquor composition used in the priming phase of operating the HSB.

Exp	Influent liquor
1, 2	1 mM glycerol, 1 mM $ZnSO_4$, 0.01% (w/v) yeast extract, acidophile basal salts, pH 6.5
3, 4	2 mM glycerol, 1 mM $ZnSO_4$, 0.01% (w/v) yeast extract, acidophile basal salts, pH 6.5
5	1 mM glycerol, 0.8 mM $ZnSO_4$, 0.01% (w/v) yeast extract, acidophile basal salts, pH 6.5

5.3.1.2 Results

Data from the priming phase experiments are shown in Figures 5.3 – 5.11, and Appendices 5A – 5F. The priming phase lasted for approximately 50 days and the results confirmed the potential of the HSB for generating H_2S and for removing zinc from the influent liquors. However, consistent highly efficient removal of zinc occurred only during experiment 5 (Fig.5.3)

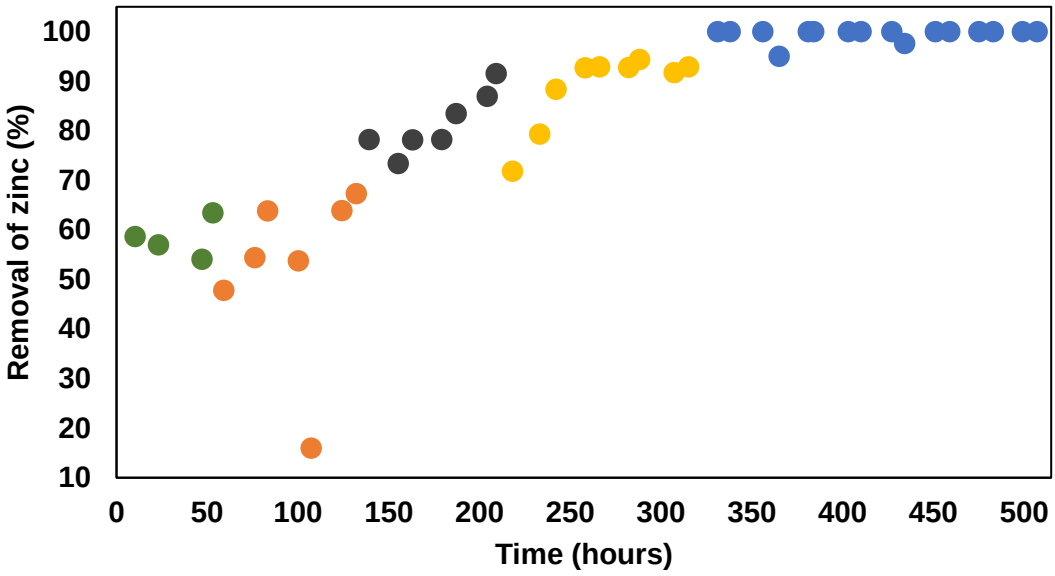

Figure 5.3. Percentage of zinc removed in-line during the priming phase of operating the HSB. Key: experiment 1(●), experiment 2 (●), experiment 3 (●), experiment 4 (●) and experiment 5 (●).

Even though not all of the zinc was removed in-line until the HSB had been operated for about 300 hours, excess H_2S was generated throughout the priming phase, as evidenced by the fact that off-line precipitation of copper occurred throughout this test period (Appendix 5A-E).

Consumption of glycerol increased steadily during experiments 3 and 4 (Fig. 5.4), which coincided with the increase in the concentration of glycerol from 1 mM to 2 mM in the influent liquors in these experiments (Table 5.1). Even so, not all of the glycerol in the feed liquor was consumed, which prompted the return to the initial concentration of 1 mM in experiment 5.

Acetic acid was produced by the HSB throughout the priming phase. The stoichiometry of concentrations of glycerol oxidised and acetic acid generated in experiments 1 and 2 indicated that only part (31 ± 14 % to 38 ± 21%) of the glycerol was oxidised to CO_2 (complete oxidation). In experiments 4 and 5, increased glycerol consumption was not paralleled by a similar increase in the production of acetic acid, indicating that higher percentage (52 ± 15% to 55 ± 18%) of the glycerol was completely oxidised.

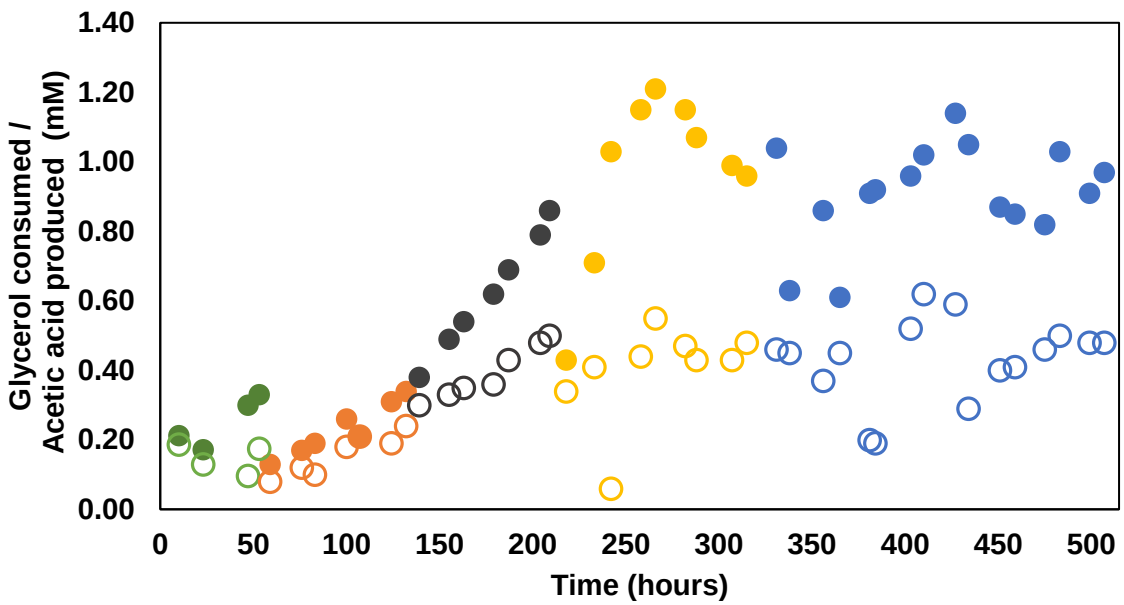

Figure 5.4. Consumption of glycerol (closed circles) and acetic acid produced (open circles) in the priming phase of operating the HSB. Key: experiment 1(●,O), experiment 2 (●,O), experiment 3 (●,O), experiment 4 (●,O) and experiment 5 (●,O).

The pH of the bioreactor liquor varied from 3.4 to 3.7 in experiments 1 to 4, but increased to pH 6.0 during experiment 5 (Fig.5.5). Grouping pH values of experiment 1 to 4 and comparing these to pH values observed at experiment 5, is possible to infer that they were significantly different, when applying independent-samples t-test (Appendix 5F).

Rates of H_2S production showed a great increase from experiment 1 to 3, expressed by the raise in the median and/or upper quartile values of the box and whiskers plot at Figure 5.6. In the final experiment, median and upper quartile values are similar to experiment 3, in which both present higher values than experiment 4, indicating a slight decreased in H_2S generation in the latter. Except experiment 1, numbers are fairly dispersed, notably in the experiments 3 and 5 which present higher interquartile ranges.

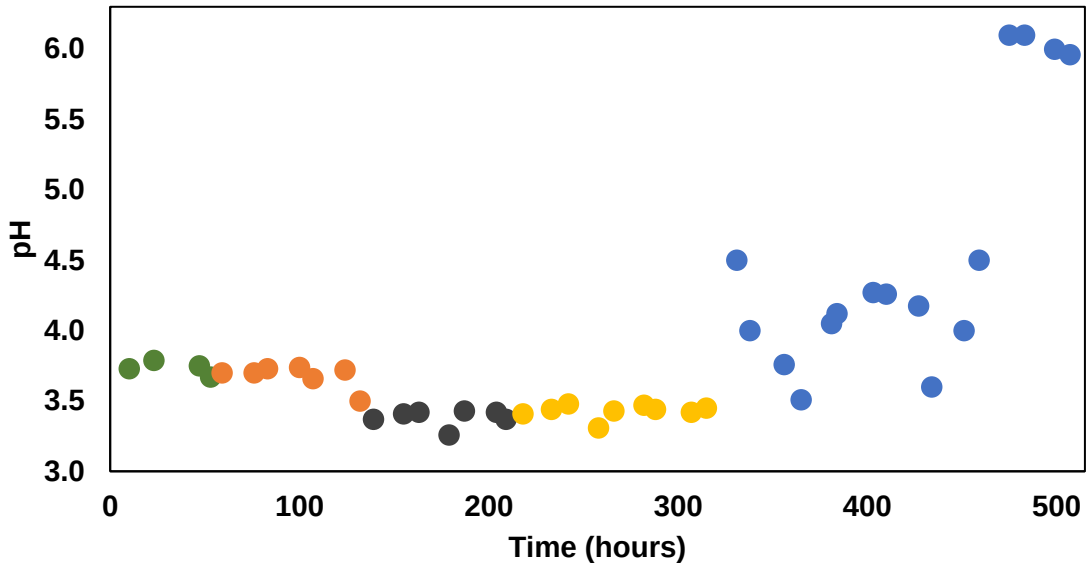

Figure 5.5. Changes in the bioreactor pH during the priming phase of operating the HSB. Key: experiment 1(●), experiment 2 (●), experiment 3 (●), experiment 4 (●) and experiment 5 (●).

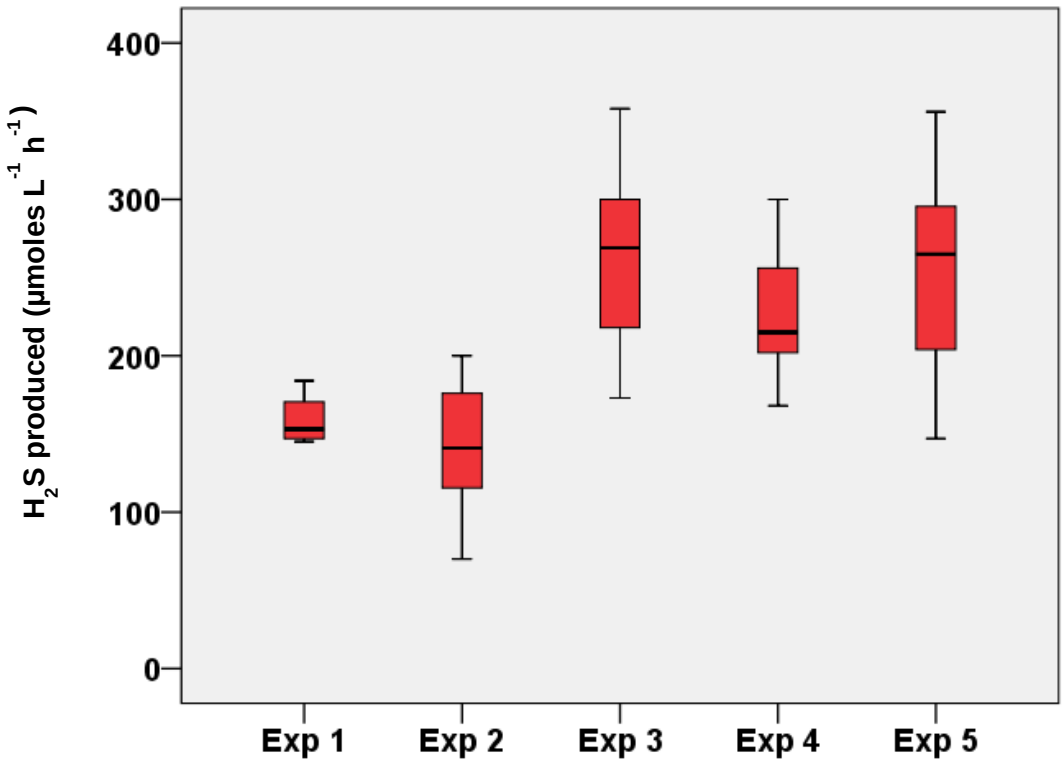

Figure 5.6. Box and whiskers plot of rates of hydrogen sulfide production by the HSB during the priming phase.

Values of net rates of sulfate reduction were very variable during the priming phase, as shown by large interquartile ranges and whiskers (less markedly in experiment 2) in the box and whiskers plot of Figure 5.7. The median values obtained in each experiment indicate that there was a continued increase in sulfate reduction throughout priming phase. Increase relative rates of sulfate reduction, consumes more acidity (reaction 5.1), which is one of the factors that possibly explains the increase of pH in the HSB during experiment 5 (Figure 5.5).

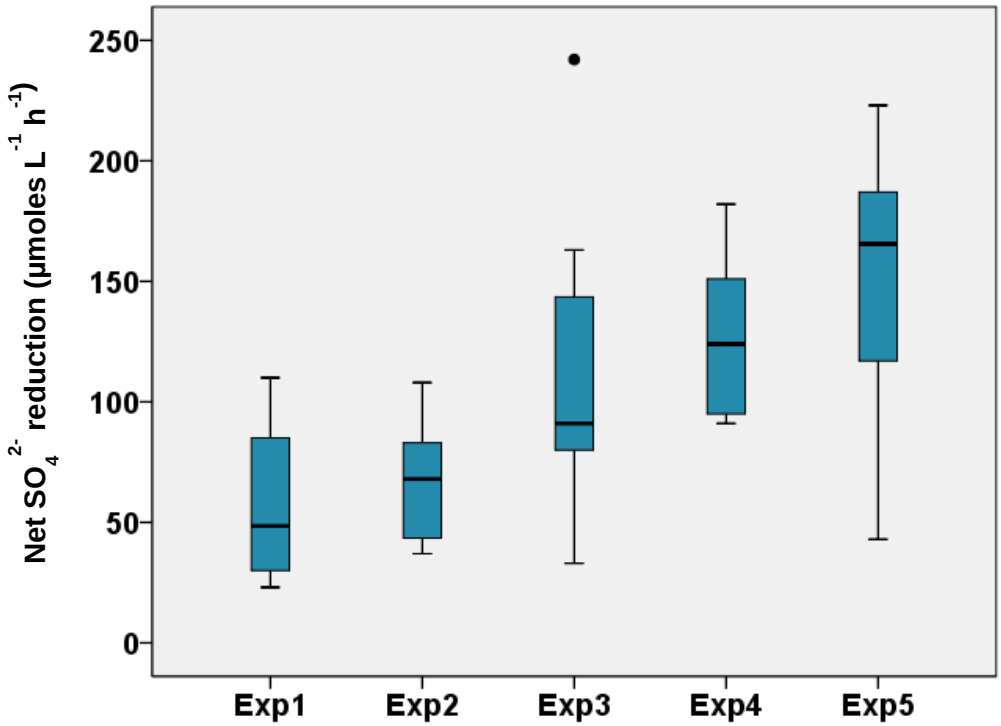

Figure 5.7. Box and whiskers plot of rates of net sulfate reduction by the HSB during the priming phase. The isolated circle is of an outlier data point.

There was a weak correlation between net sulfate reduction and H_2S generation when taking into account values from experiment 1 to 4 (r = 0.56*)*. When all experiments are included (1 to 5) the correlation decreased (r = 0.41; Figure 5.8).

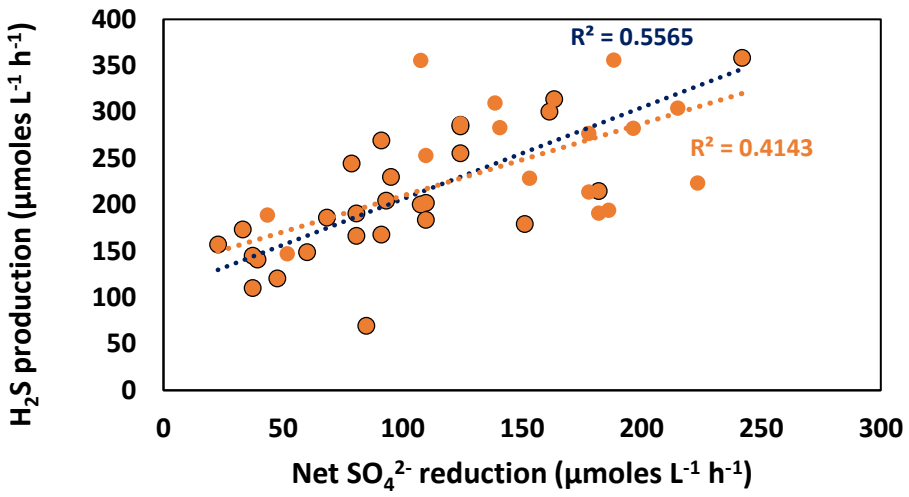

Figure 5.8. Relationship between production of H_2S and net sulfate reduction for all experiments carried out during the priming phase (*r=0.41*) and when taking into account only experiments 1 to 4 only (*r=0.56*).

Mass balance calculations, described in section 5.2.2, indicated that there was a relative increase in the percentage of hydrogen sulfide generated *via* sulfate reduction, as opposed from *via* ZVS reduction, during the priming phase (Figure 5.9).

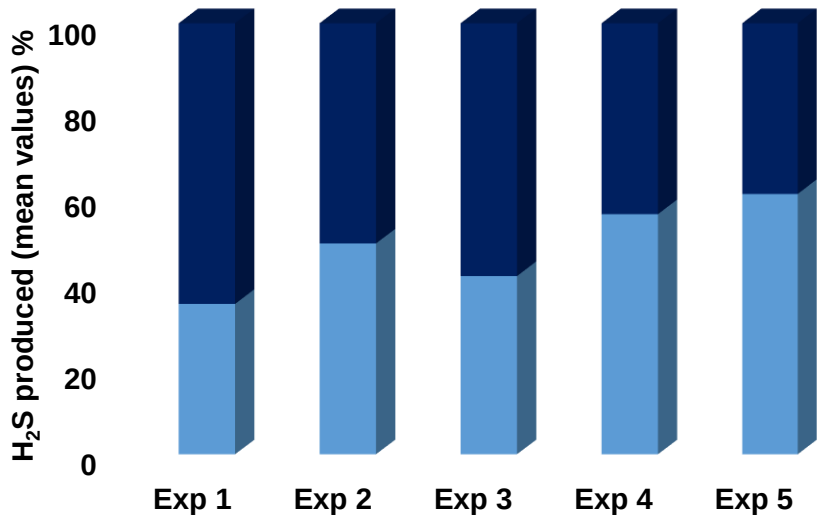

Figure 5.9. Percentage (mean values) of H_2S produced *via* reduction of ZVS (■) and sulfate (■) during the priming phase operation of the HSB.

Numbers of planktonic bacterial cells in the effluent liquors during the priming phase were very variable, but showed a general increasing trend, especially in experiment 5 (Figure 5.10).

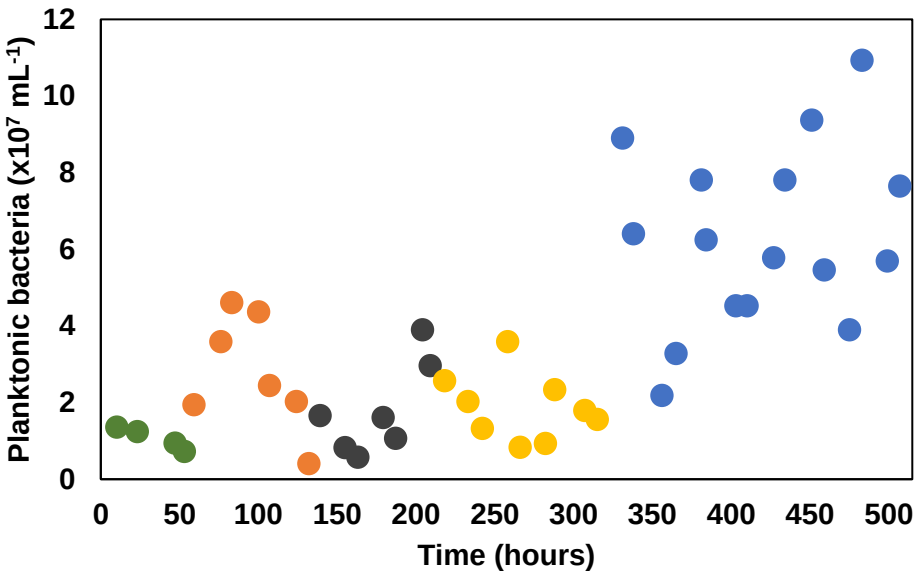

Figure 5.10. Planktonic bacterial cell numbers in the effluent liquors during the priming phase of operating the HSB. Key: experiment 1(●), experiment 2 (●), experiment 3 (●), experiment 4 (●) and experiment 5 (●).

T-RFLP analysis indicated that there was a general increase in the relative abundance of the aSRB strains *Peptococcaceae* CEB3 (138 nt) and *Desulfosporosinus acididurans*^{T} (213 nt), during the priming phase (Figure 5.11). *Firmicute* strain I2511 (266 nt) also increased in relative abundance from experiments 1 to 3, but declined considerably in experiment 5. The acetoclastic acidophilic bacterium *Acidocella aromatica* (228 nt) was abundant during all experiments even though acetic acid was detected throughout the priming phase. *Clostridium* sp. (220 nt) showed significant abundance only in experiments 1 and 5.

Results obtained during the priming phase confirmed the feasibility of the HSB in consistently producing H_2S at relatively low pH. Furthermore, the HSB precipitated > 99% the soluble zinc present in circum-neutral pH influent liquors by the end of the priming phase, at a HRT of < 5 hours. The priming phase confirmed therefore that the HSB had potential use for remediating circum-neutral pH metal contaminated waters.

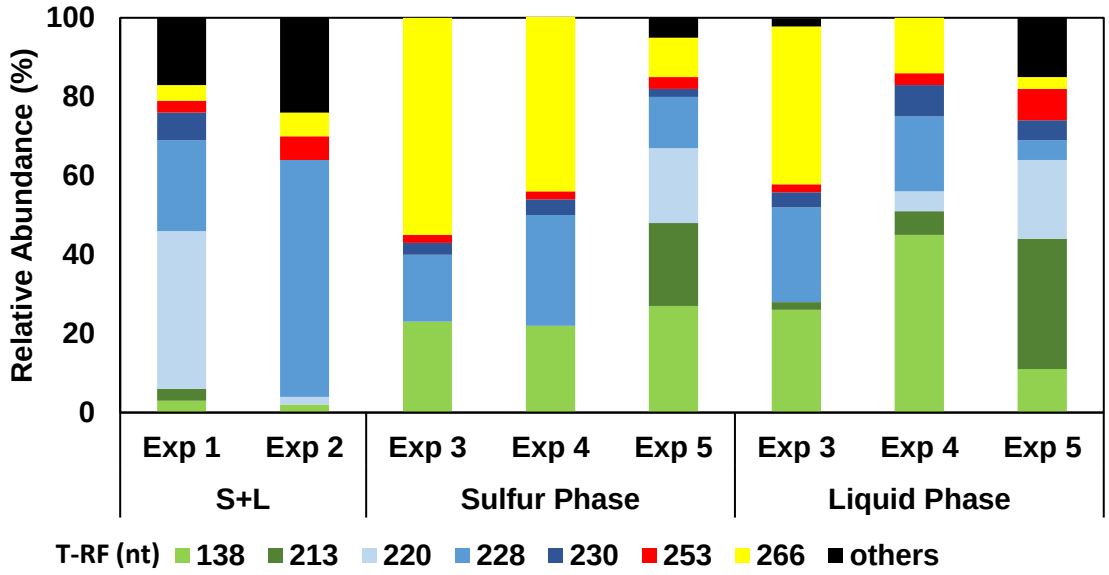

Figure 5.11. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during the priming phase of operating the HSB. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*^{T}, (■) *Clostridium* sp., (■) *Acidocella aromatica*^{T}, (■) actinobacterium sp. IR1, (■) *Acidithiobacillus ferrooxidans*, (■) *Firmicute* strain I2511, (■) unidentified T-RF, (■) low abundance unidentified T-RFs. S+L correspond to DNA extracted from combined sulfur-attached (S) and planktonic (L) cells in experiments 1 and 2. DNA was extracted separately from sulfur biofilms and bioreactor liquor in experiments 3 – 5.

5.3.2 Operating the HSB in batch mode at fixed pH values

5.3.2.1 Methods

Batch culture experiments were established to evaluate production of H_2S from ZVS and sulfate reduction by the HSB at fixed pH values of 3.5, 3.0 and 2.5. The H_2S generated was removed in a continuous OFN gas stream, and used to precipitate soluble copper off-line in a separate vessel containing a 20 mM copper sulfate solution (Fig. 5.2).

Following completion of the priming phase, the HSB was used to test its applicability for remediating synthetic and actual mine water from Minsterley Brook, UK (described in Chapter 6). When these tests had been completed, a zinc-free medium containing approximately 5 mM glycerol, 0.01% (w/v) yeast extract, 10 mM magnesium sulfate and acidophile basal salts was pumped continuously into and out of the bioreactor (at 455 mL h^{-1}) for 3 hours. The pH of the bioreactor liquor was adjusted to 3.5, de-oxygenated by gassing with OFN, and maintained as a batch culture until most of the glycerol had been oxidised. pH homeostasis was maintained in the bioreactor vessel by automated addition of sterile 0.25 M hydrochloric acid and 0.5 M

sodium hydroxide. The liquid phase above the ZVS (~ 1200 mL) was then removed and replaced with fresh 5 mM glycerol, 0.01% (w/v) yeast extract, 10 mM magnesium sulfate and acidophile basal salts medium, the bioreactor pH adjusted to 3.5 and samples removed and analysed at regular intervals, in the first of the "fixed pH/batch mode" experiments. Next, the bioreactor was again partly drained and fresh medium added and the pH adjusted to 3.0. Analytical data from a repeat run at this pH were recorded. A similar two-stage protocol was used to obtain data from the bioreactor when maintained at pH 2.5.

A sampling device was connected to the reactor vessel to enable removal of samples at regular intervals from the middle of the liquid phase (above the layer of ZVS: Fig. 5.2). Samples were withdrawn regularly from the reactor's liquid phase and from the off-line vessel to measure concentrations of glycerol, acetic acid, sulfate and copper.

Various calculations were made from the raw analytical data (Appendix 5G-5J) obtained in the experiments of this section:

(i) *Rates of glycerol oxidation:* the difference in glycerol concentrations at the start and end of an experiment, divided by the length of time of the experiment, and expressed as µmoles L^{-1} h^{-1}.

(ii) *Rates of sulfate reduction*: the difference in sulfate concentrations at the start and end of an experiment, divided by the length of time of the experiment, and expressed as µmoles L^{-1} h^{-1}.

(iii) *Rates of H_2S production*. These were calculated from determining time-related changes in soluble copper in the external vessel (section 5.2.2), and dividing these values by the volume (V) of the bioreactor (2.2 L). Since the stoichiometry H_2S produced to CuS precipitated is 1:1, these data corresponded to rates of H_2S production as µmoles L^{-1} h^{-1}.

(viii) *Relative % of sulfate and ZVS used as electron acceptors*: The percentage of H_2S produced via reduction of sulfate was calculated dividing the final amount of sulfate reduced (µmoles) by the final accumulated amount of H_2S produced (µmoles) over the same time period (x 100). The percentage of H_2S produced via reduction of ZVS was inferred from the difference between this value and the total amount of H_2S produced.

H_2S produced from reduction of sulfate (%) =

{Δ SO_4^{2-} (µmoles)} / total H_2S produced (µmoles) x 100

H_2S produced from reduction of ZVS (%) =

100 - H_2S produced from reduction of sulfate (%).

5.3.2.2 Results

Tests carried out in batch mode showed that the HSB was effective at generating H_2S at pH values fixed between 2.5 and 3.5. Raw and processed data from these experiments are shown in Appendix 5G- 5J, and in Figures 5.12 and 5.13 and Table 5.2 below.

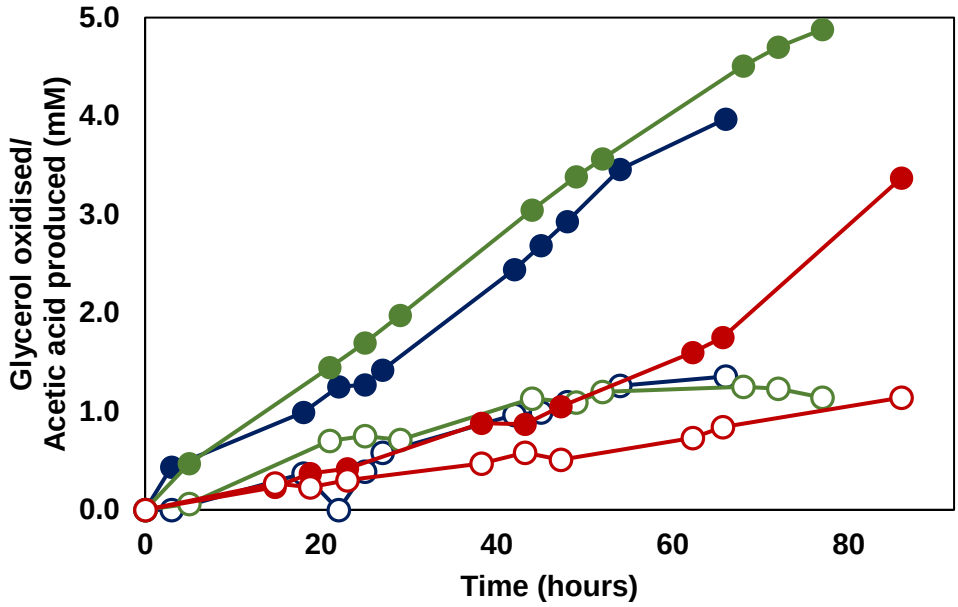

Figure 5.12. Oxidation of glycerol (closed circles) and production of acetic acid (open circles) in tests of the HSB carried out at fixed pH values in batch mode. Key: (●,○) pH 3.5, (●,○) pH 3.0 and (●,○) pH 2.5.

Data in Fig. 5.12 show that acetic acid was generated in the bioreactor at each pH tested, though in no case was the stoichiometry of glycerol oxidised:acetic acid produced 1:1, indicating that some glycerol was oxidised completely to CO_2 and some to acetic acid + CO_2. Figure 5.12 also shows that, in terms of efficiency of glycerol oxidation, the HSB operated optimally at pH 3.0 and least efficiently at pH 2.5.

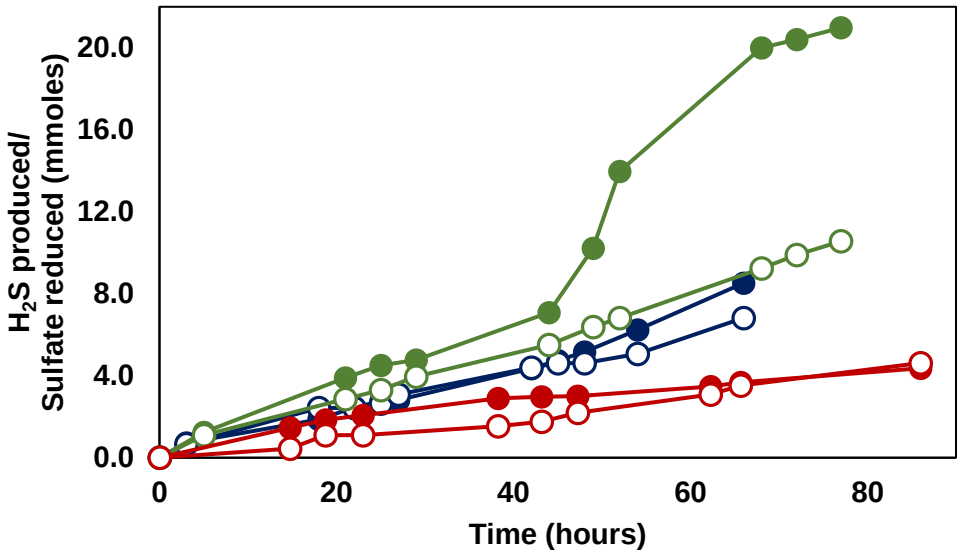

Figure 5.13. Cumulated amounts of hydrogen sulfide produced (closed circles) and sulfate reduced (open circles) during the operation of the HSB in batch mode at fixed pH values. Key: (●,O) pH 3.5, (●,O) pH 3.0 and (●,O) pH 2.5.

Production of H_2S was also more efficient at pH 3.0 than at both pH 3.5 and 2.5 (where rates of H_2S production were the lowest in these tests (Fig. 5.13). Figure 5.13 also shows that the amounts of H_2S produced and sulfate reduced were very similar at both pH 3.5 and 3.0 for the first ~45 h of the batch experiments, indicating that almost all the H_2S generated during this time derived from the dissimilatory reduction of sulfate rather than ZVS. After this time, however, the stoichiometry between the two was no longer ~1:1, indicating the ZVS reduction became more important towards the ends of these batch culture tests, particularly at pH 3.0, where this also corresponded to a marked increase in H_2S production by the HSB. In the test carried out at pH 2.5, the amounts of H_2S produced and sulfate reduced displayed close to 1:1 stoichiometries throughout, suggesting that ZVS reduction contributed little to net H_2S production.

Table 5.2 summarizes data from these three experiments. The rates of glycerol oxidation, sulfate reduction and H_2S production are those calculated from data at the start and end of each experiment, and do not show changes within the time frame of the experiments, such as the marked increase in H_2S production after ~45 h by the HSB maintained at pH 3.0. The same is true for the relative percentages of sulfate and ZVS from which the H_2S was generated during these experiments.

Table 5.2. Results of experiments carried out operating the HSB in batch mode at pH 3.5, 3.0 and 2.5

Bioreactor pH	Glycerol oxidation µmoles L^{-1} h^{-1}	SO_4^{2-} reduction µmoles L^{-1} h^{-1}	H_2S production µmoles L^{-1} h^{-1}	H_2S produced from reduction of: (%)	
				$[SO_4^{2-}]$	ZVS
3.5 +/- 0.1	60	47	59	80	20
3.0 +/- 0.2	64	62	124	50	50
2.5 +/- 0.1	27	24	23	>99	<1

The microbial populations in the bioreactor, as assessed by T-RFLP analysis, showed major changes as the operating pH was lowered (Fig 5.14). A direct comparison of combined planktonic and sulfur-attached bacteria at pH 3.5 and 2.5 shows that there was far greater biodiversity present at the higher pH. Two sulfate-reducing bacteria were detected at pH 3.5 (*Peptococcaceae* CEB3 and *Desulfosporosinus acididurans*^{T}) but only *Peptococcaceae* CEB3 at pH 2.5. Most noticeable was the relatively high relative abundance of the sulfur-reducing strain I2511 at pH 2.5, while this was found as a relatively minor member of the bacterial consortium at pH 3.5. T-RFLP analyses were carried out separately of planktonic and attached bacteria at pH 3.0. The T-RFLP profile of planktonic cells was similar to that found of planktonic + attached cells at pH 3.5, though with notable shifts on the relative abundances of the non-sulfidogenic bacteria *Clostridium* sp. and *Ac. aromatica*. The profile of bacteria attached to sulfur was very different, however, and dominated by a T-RF (300 nt) of a bacterium that was not identified from the BART databank. Given this profile was obtained at the end of an experiment where, as inferred from Fig. 5.13, H_2S production was occurring at a relatively fast rate and seemingly predominantly from the reduction of ZVS rather than of sulfate, the implication was that this bacterium was mostly likely to be an acidophile that generated H_2S by coupling glycerol oxidation to the reduction of ZVS. All attempts to isolate this bacterium or to identify it *via* clone library analysis were, however, unsuccessful. It is worth noting that, while the same T-RF was found when the bioreactor was maintained at pH 3.5, is was not detected at pH 2.5.

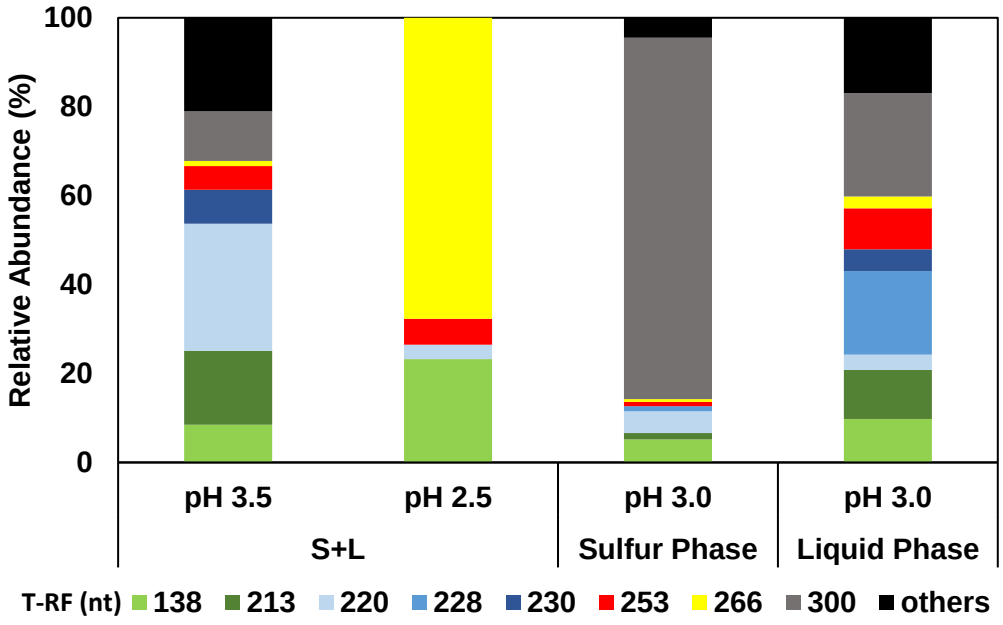

Figure 5.14. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during experiments operating the HSB in batch mode at pH 3.5, 3.0 and 2.5. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*^{T}, (■) *Clostridium* sp., (■) *Acidocella aromatica*^{T}, (■) actinobacterium sp. IR1, (■) *Acidithiobacillus ferrooxidans*, (■) *Firmicute* strain I2511, (■) unidentified T-RF, (■) low abundance unidentified T-RFs. S+L correspond to DNA extracted from combined sulfur-attached (S) and planktonic (L) cells in experiments pH 3.5 and pH 2.5. DNA was extracted separately in the experiment carried out at pH 3.0.

5.3.3 Operation of the HSB at low pH in continuous flow mode

5.3.3.1 Methods

To evaluate hydrogen sulfide generation by the HSB when operated at very low pH in continuous flow rather than in batch mode, the bioreactor was operated in a similar manner to that described in section 5.3.1. The influent liquor contained 2 mM glycerol, 0.01% (w/v) yeast extract and acidophile basal salts, and was adjusted to either pH 2.5 or 2.0 with sulfuric acid. A continuous flow rate of 455 mL h^{-1} was maintained throughout both experiments, and the bioreactor pH was not controlled. Hydrogen sulfide produced was removed in a continuous OFN gas stream and used to precipitate soluble copper off-line in a separate vessel containing 300 mL of 20 mM copper sulfate (Fig. 5.2).

After allowing the HSB to adapt to the new operating conditions (for 20 days for the pH 2.5 influent liquor, and for 15 days for the pH 2.0 influent liquor), samples were withdrawn regularly

from both influent and effluent liquors and the off-line vessel to measure concentrations of glycerol, acetic acid, sulfate and copper, as described in section 5.2.2.

5.3.3.2 Results

Raw and processed data from these experiments are shown in Appendix 5K- 5M, and in Figures 5.15 - 5.17.

The pH of the bioreactor liquors were similar to those of the influent liquors in both experiments: pH 2.6 (throughout) with the pH 2.5 influent liquor and pH 1.98 – 2.05 with the pH 2.0 influent liquor. Some of the glycerol present in the pH 2.5 influent liquor, but none of that in the pH 2.0 influent liquor, was oxidised, though acetic acid was detected in effluents in both experiments (Fig. 5.15). Likewise, some of the sulfate present in the pH 2.5 influent liquor was reduced, though none in the pH 2.0 influent liquor, even though H_2S was generated at both pH values throughout the experiments (Fig. 5.16).

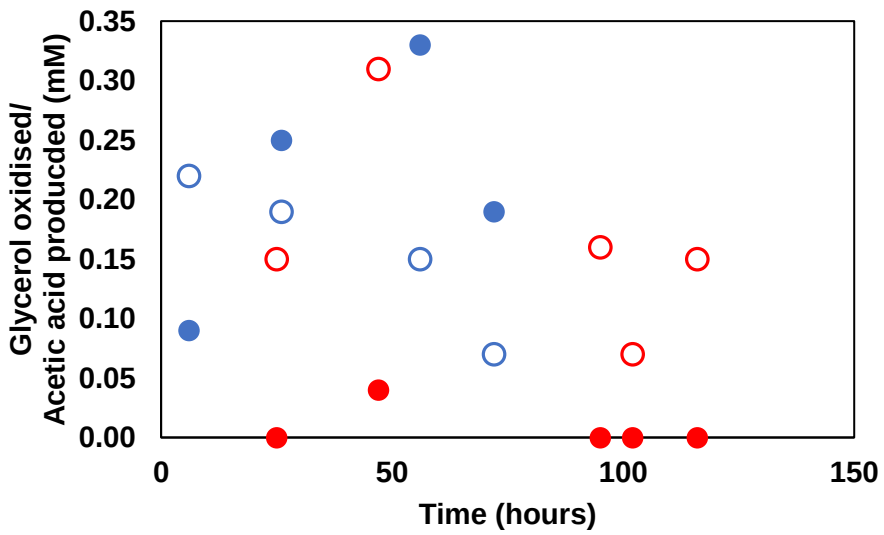

Figure 5.15. Consumption of glycerol (closed circles) and acetic acid produced (open circles) when operating the HSB at low pH in continuous flow mode. Key: (●,○) pH 2.5 influent liquor; (●,○) pH 2.0 influent liquor.

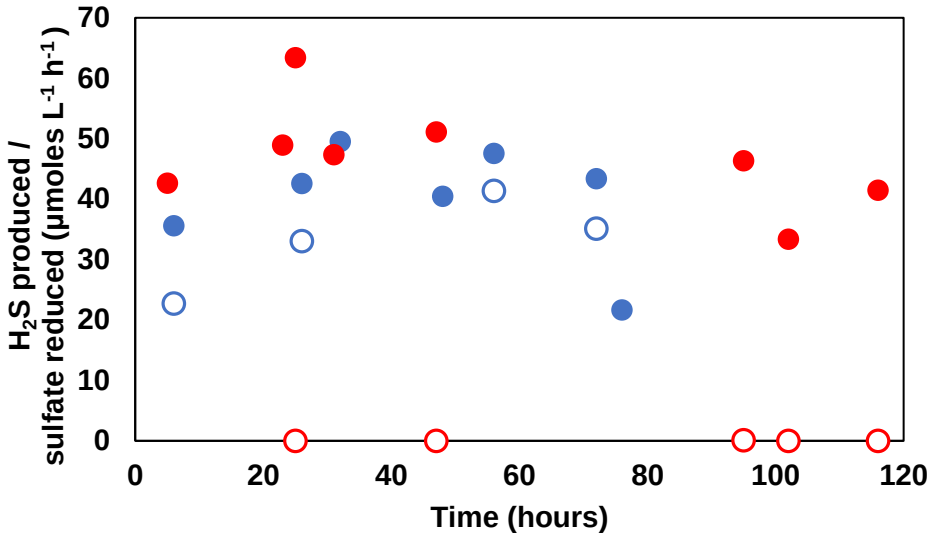

Figure 5.16. Rates of hydrogen sulfide production (closed circles) and rates of sulfate reduction (open circles) when operating the HSB at low pH in continuous flow mode. Key: (●,○) pH 2.5 influent liquor; (●,○) pH 2.0 influent liquor.

Rates of H_2S generation at both pH values showed similar median and interquartile ranges in the box and whiskers plot at Figure 5.17. Applying an independent-samples t-test on values obtained for rates of H_2S production (excluding outliers), results showed no significant differences in rates in both experiments [t(11) = 0.30, p = 0.77] (Appendix 5M).

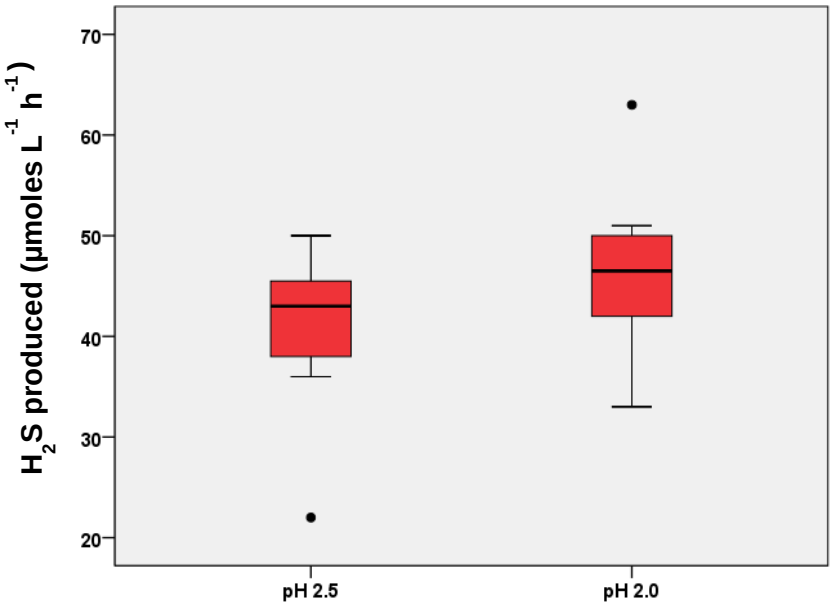

Figure 5.17. Box and whiskers plot of rates of H_2S produced when operating the HSB in continuous flow mode and fed with pH 2.5 and pH 2.0 influent liquors. The isolated circles are of an outlier data points.

Mass balance calculations indicated that most (77 ± 8%) of the H_2S was produced by sulfate reduction with the pH 2.5 influent liquor. However, there was no net sulfate reduction when the influent liquor was lowered to pH 2.0 (Appendix 5L), implying that either ZVS reduction or other mechanisms (described in the Discussion section of this chapter) were responsible for the generation of H_2S.

Analysis of the composition of the microbial communities in the HSB by T-RFLP analysis (Fig. 5. 18) showed that those of sulfur-attached and planktonic bacteria were similar with either the pH 2.5 or pH 2.0 influent liquor, but that the different pH values of the feed liquors had resulted in major changes in the dominant bacteria present. Most notably, neither the known sulfate-reducing bacteria *Peptococcaceae* CEB3 and *D. acididurans*, which together were the dominant bacteria detected at the higher pH, were not found when the HSB was fed with pH 2.0 influent liquor. In the latter case, the most abundant bacterium detected was the ZVS-reducing *Firmicute* strain I2511. The facultative anaerobe *At. ferrooxidans* and the putative fermenter *Clostridium* sp. were also detected in relatively higher proportions in the bacterial communities when the HSB was operated in continuous flow mode at pH ~2. T-RF 300 nt, which was not detected at pH 2.5 when the HSB was operated in batch mode, had abundancy ~ 10% in both experiments (except in sulfur-attached cells at pH 2.5).

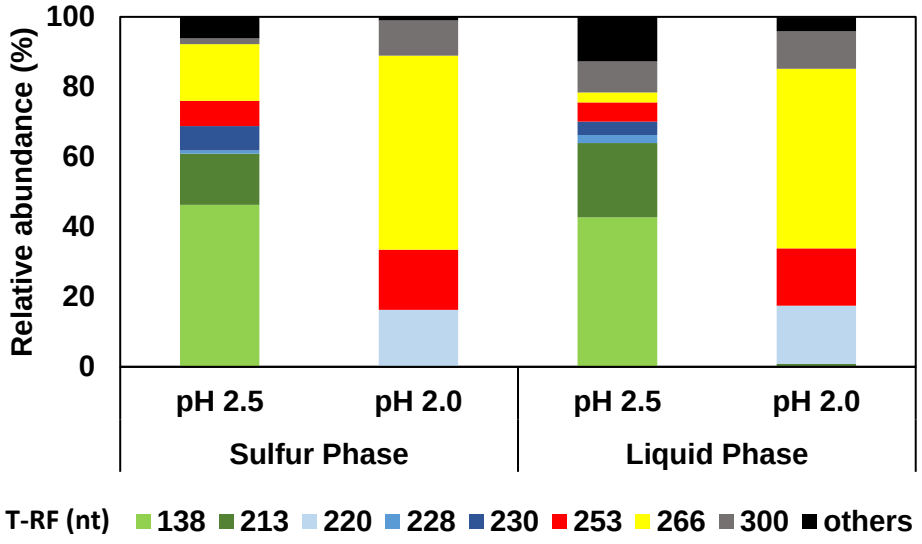

Figure 5.18. T-RFLP profile of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during experiments operating the HSB at low pH in continuous flow mode. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*^{T}, (■) *Clostridium* sp., (■) *Acidocella aromatica*^{T}, (■) actinobacterium sp. IR1, (■) *Acidithiobacillus ferrooxidans*, (■) *Firmicute* strain I2511, (■) unidentified T-RF, (■) low abundancy unidentified T-RFs. Sulfur phase correspond to DNA extracted from sulfur-attached bacterial communities. Liquid phase correspond to DNA extracted from planktonic cells.

5.3.4 Operation of the HSB as a sulfur-only reducing bioreactor

5.3.4.1 Methods

Experiments were carried out in which the HSB was operated exclusively as a sulfur-reducing bioreactor. For this, all sulfate salts present in standard acidophile basal salts were replaced by chloride salts (section 2.2.1) and hydrochloric, rather than sulfuric, acid was used to adjust solution pH values. Each experiment lasted for approximately 10 days, and the HSB was operated in continuous flow mode (at 455 mL h^{-1}) with no internal pH control, similar to the protocol described in section 5.3.1. A zinc-free influent liquor containing 1 mM glycerol, 0.01% (w/v) yeast extract and chloride-based basal salts, adjusted to either pH 4.0 or 3.0 with hydrochloric acid, was used in these experiments. Hydrogen sulfide produced was removed in a continuous OFN gas stream and used to precipitate soluble copper off-line in a separate vessel containing 300 mL of 20 mM copper sulfate (Fig. 5.2).

After adaptation periods of 5 days using sulfate-free influent liquors at either pH 4.0 or pH 3.0, samples were withdrawn at regular intervals from influent and effluent liquors and the off-line vessel to measure concentrations of glycerol, acetic acid and copper (section 5.2.2).

5.3.4.2 Results

Raw and processed data from these experiments are shown in Appendix 5Q- 5U, and in Figures 5.19 - 5.21.

In experiments performed with the pH 4.0 influent liquor, the bioreactor pH was 4.1 – 4.8 and with pH 3.0 influent liquors the pH measured in the HSB liquors was 3.0 – 3.2. Glycerol oxidation and acetic acid production were significantly different between experiments when using pH 4.0 and pH 3.0 influent liquors, confirmed by an independent-samples t-test [glycerol oxidised; $t(8) = 15.64$, $p < 0.001$] [acetic acid produced; $t(8) = 18.23$, $p < 0.001$] (Appendix 5S – 5T). Nearly all of the available glycerol present in the pH 4.0 influent liquor was consumed (0.96 ± 0.09 mM), but very little glycerol was consumed (0.14 ± 0.04 mM) when using pH 3.0 influent liquor. Acetic acid was detected in effluents in both experiments (Fig. 5.19). With the pH 4.0 influent liquor, some glycerol was completely oxidised to CO_2 (23 ± 8 %) , however when using pH 3.0 influent liquors acetic acid concentrations in the effluent liquors exceeded the amounts of glycerol consumed, indicating that all of the latter was incompletely oxidised.

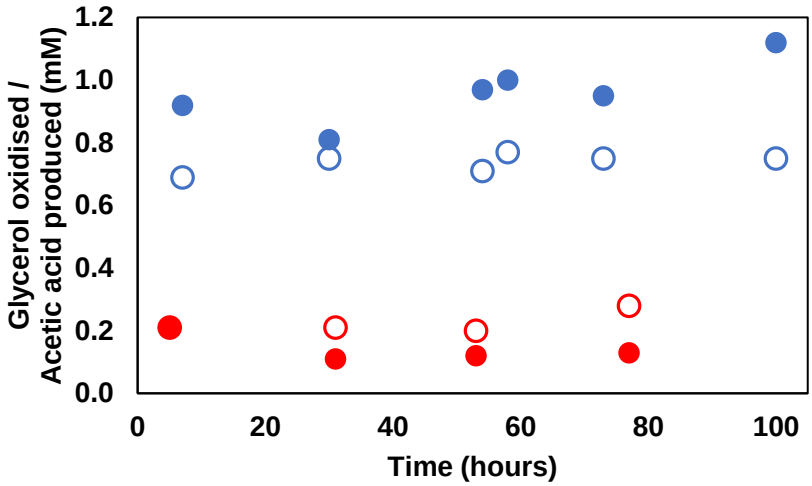

Figure 5.19. Consumption of glycerol (closed circles) and acetic acid produced (open circles) when operating the HSB as a sulfur-only reducing bioreactor in continuous flow mode. Key: (●,○) pH 4.0 influent liquor; (●,○) pH 3.0 influent liquor.

Hydrogen sulfide was produced throughout both experiments, though rates were highly variable (Fig. 5.20). In the box and whiskers plot shown in Figure 5.21, the median and interquartile range of H_2S production were greater using pH 4.0 than pH 3.0 influent liquor. An independent-samples t-test performed on the rates of H_2S production confirmed that differences in the rates in experiments using pH 4.0 and pH 3.0 influent liquor were significant [$t(21) = 3.539$, $p = 0.002$] (Appendix 5U).

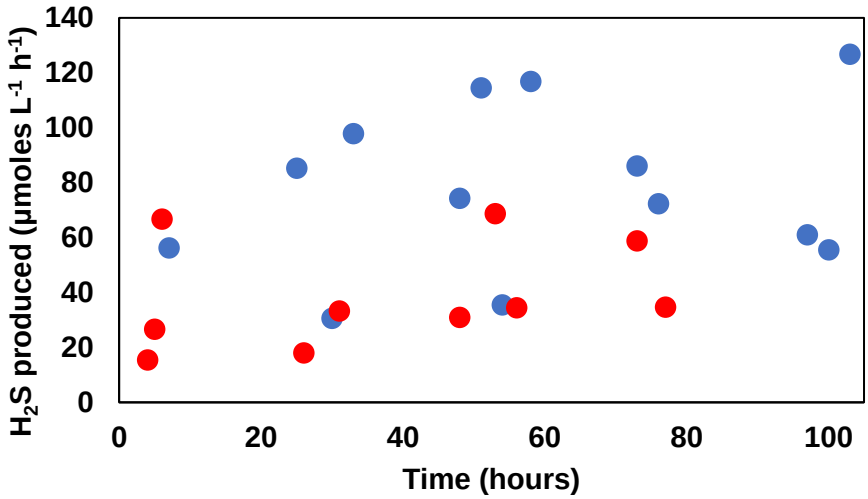

Figure 5.20. Changes in rates of H_2S production over time when operating the HSB as a sulfur-only reducing bioreactor in continuous flow mode. Key: (●) pH 4.0 influent liquor; (●) pH 3.0 influent liquor.

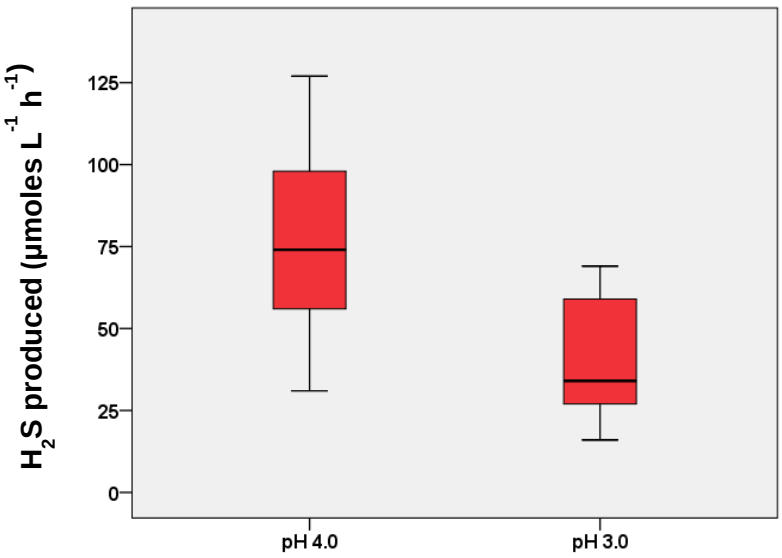

Figure 5.21. Box and whiskers plot of rates of H_2S produced when the HSB was operated with pH 4.0 and pH 3.0 influent liquors as a sulfur-only reducing system in continuous flow mode.

Analysis of microbial communities by T-RFLP analysis showed some, but relatively marginal, differences in attached and planktonic bacterial populations in both experiments (Fig. 5.22). The dominant known sulfate-reducer present was *D. acididurans*, and *Peptococcaceae* CEB3 was only detected as a biofilm bacterium when the HSB was fed with pH 3.0 influent liquor. *Firmicute* strain I2511, a confirmed ZVS-reducer, and the bacterium corresponding to the T-RF of 300 nt (also a putative ZVS reducer) were detected as attached and planktonic bacteria throughout this experiment. In addition, a novel T-RF (310 nt) of a bacterium that was not identified from the BART databank was detected in both pH values tested. This T-RF was relatively more abundant when the HSB was fed with pH 4.0 than with pH 3.0 influent liquor, again suggesting that it is a moderate acidophile. The non-sulfidogenic bacteria *Clostridium* sp., *Ac. aromatica,* and *At. ferrooxidans*, were highly abundant (Σ = 69%) in the planktonic communities when using pH 3.0 influent liquor, but much less (Σ = 27%) when using pH 4.0 influent liquor.

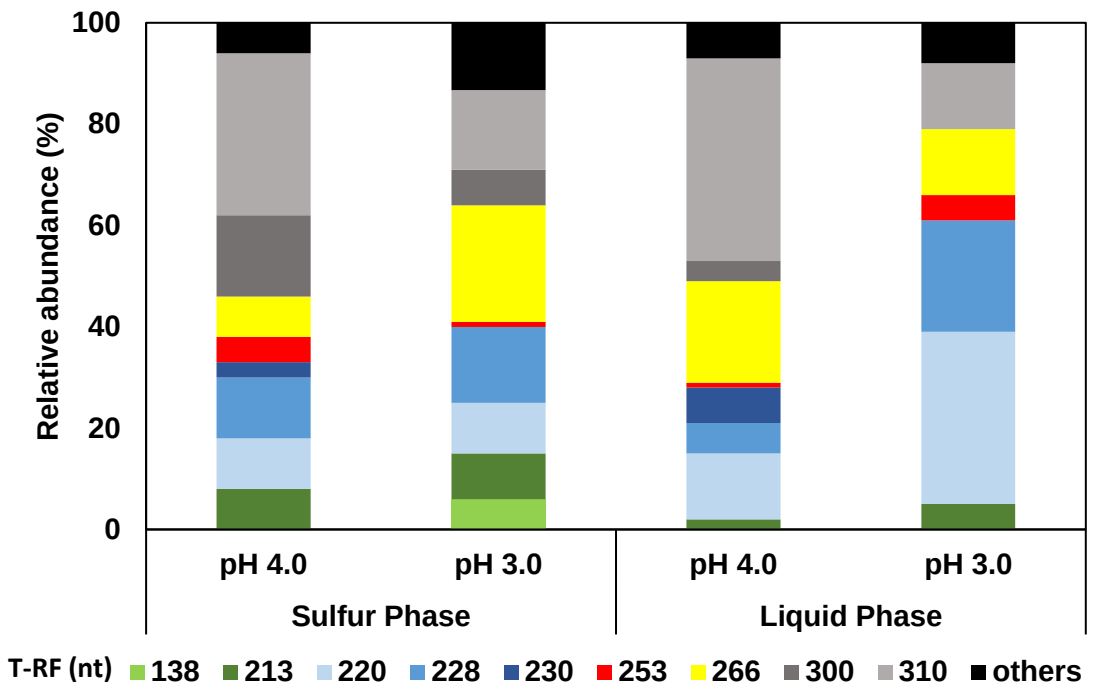

Figure 5.22. T-RFLP profile of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during experiments operating the HSB as a sulfur-only reducing bioreactor in continuous flow mode. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*^{T}, (■) *Clostridium* sp., (■) *Acidocella aromatica*^{T}, (■) actinobacterium sp. IR1, (■) *Acidithiobacillus ferrooxidans*, (■) *Firmicute* strain I2511, (■) unidentified T-RF, (■) unidentified T-RF, (■) low abundance unidentified T-RFs. Sulfur phase correspond to DNA extracted from sulfur-attached bacterial communities. Liquid phase correspond to DNA extracted from planktonic cells.

5.4 Discussion

The hybrid sulfidogenic bioreactor (HSB) generated H_2S consistently under contrasting operational parameters, e.g. over a wide range of pH values (2.0 – 6.0) and variations in the composition of the influent liquor. When provided together, ZVS and sulfate were utilised as electron acceptors concurrently in most of the tests, as evidenced from mass balance calculations, with the exceptions of when the HSB was operated in batch mode at pH 2.5 where sulfate reduction was predominant, and in continuous flow mode with pH 2.0 influent liquor, where H_2S was produced but no sulfate was reduced.

Results obtained during the priming phase confirmed the potential of the HSB for remediating circum-neutral pH metal contaminated waters, precipitating >99% of zinc present in the influent liquor in the final experiment. Glycerol consumption in experiments 1 to 3 was 0.4 ± 0.2 mM, in contrast to data from experiments 4 and 5 (0.9 ± 0.2 mM), indicating a significant

increase during the later stages of the priming phase. Acetic acid was produced throughout the priming phase, though not in 1:1 stoichiometry with glycerol consumed, indicated that part of glycerol was oxidised completely to CO_2 while the rest was incompletely oxidised. Even though the bioreactor pH was not controlled, the upper liquid phase showed only small variations (3.3 - 3.8) in experiments 1 to 4, though was significantly different (pH 3.5 – 6.1) in experiment 5. Figure 5.23 shows variations in H_2S production with bioreactor pH for all five experiments of the priming phase (shown as the pH values of the HSB in these experiments) and the corresponding rates of H_2S generated. One-way analysis of variance showed there was no significant difference in rates with HSB pH values of 3.4, 4.3 and 6.0, but at pH 3.7 rates of H_2S generation were significantly lower than the others (Appendix 5N-5P). These results indicated that, under the conditions of the priming phase experiments, bioreactor pH did not play pivotal role in dictating rates of H_2S generation.

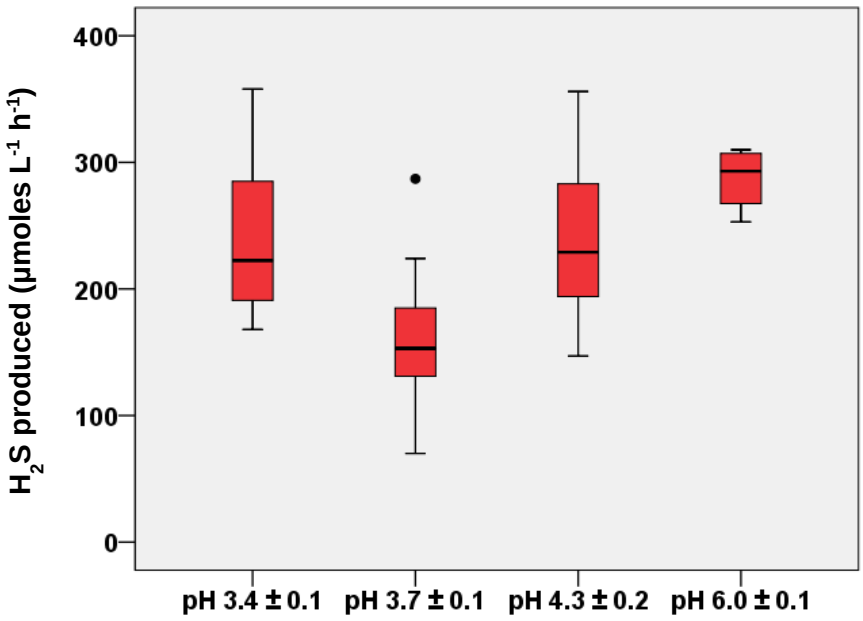

Figure 5.23. Box and whiskers plot of rates of hydrogen sulfide generated with distinct bioreactor pH values when the HSB was operated during the priming phase. The isolated circle is of an outlier data point

Rates of net sulfate reduction increased throughout the priming phase, although values were very variable (Fig. 5.7). Sulfate reduction became increasingly important towards the end of the priming phase experiments, with 60 ± 23 % of H_2S produced in experiment 5 being produced *via* sulfate reduction compared to 35 ± 17 % in experiment 1. An important consequence of the high pH (6.0 - 6.1) observed in the bioreactor liquors in the last 32 h of experiment 5 is that it might have impaired in the measurement of the amount of hydrogen sulfide generated by the HSB. The excess sulfide (i.e. that not used to precipitate metals within

the reactor vessel) produced by the HSB was determined by transferring this as a gas phase (H_2S) into an off-line vessel where it contacted and precipitated copper. Hydrogen sulfide is a weak acid with pK_a values of 6.9 (H_2S/HS^-) and 14.15 (HS^-/S^{2-}). At pH 6.0, 13% of the total sulfide produced would have been present as soluble HS^-, which would be less readily removed in the OFN gas stream and transferred to the off-line vessel. This may explain the correlation between net sulfate reduction and H_2S generation, where the regression coefficient was higher when only experiments 1 to 4 were considered rather than all five experiments (Fig 5.8).

The HSB generated H_2S effectively when operated in batch mode at fixed pH values. However, results obtained at pH 3.0 and pH 2.5 were very different, and those obtained when the HSB was maintained at pH 3.5 were intermediate between them. The most rapid generation of H_2S was observed at pH 3.0, and the lowest at pH 2.5. Additionally, H_2S production *via* ZVS reduction was more pronounced at pH 3.0 than at pH 3.5 and pH 2.5, where it appeared to contribute little to net H_2S generation. Rates of glycerol consumption were similar at pH 3.5 and 3.0, and far greater than observed at pH 2.5. The stoichiometry of glycerol consumed to acetic acid produced in all three pH experiments indicated that, again, only part (70 ± 5 %) of the glycerol consumed was oxidised completely to CO_2.

When operating the HSB with very low pH influent liquors in continuous flow mode, H_2S was produced continuously in both experiments, with no significant differences in the rates of production when using pH 2.5 and pH 2.0 influent liquors. The bioreactor pH was not controlled and showed relatively small variations throughout these experiments, with pH values being similar to those of the influent liquors in both cases. Glycerol and sulfate were only partially metabolised in experiments with pH 2.5 influents liquors, while in experiments with pH 2.0 influent liquors none of the glycerol was oxidised and no net sulfate reduction was detected. Considering the absence of glycerol oxidation with pH 2.0 influent liquor, it is conceivable that the microbial population relied solely on yeast extract as electron donor to generate hydrogen sulfide during that time. The *Firmicute* strain I2511 was the most abundant strain in pH 2.0 influent liquors experiment; it was shown in Chapter 4 that I2511 could grow solely on yeast extract generating acetic acid and hydrogen sulfide. Throughout both experiments, acetic acid was produced in similar amounts 0.16 ± 0.06 mM (pH 2.5 influent liquor) and 0.17 ± 0.08 mM (pH 2.5 influent liquor). With the pH 2.5 influent liquor, 77 ± 8% of H_2S was generated *via* sulfate reduction, but no net sulfate reduction occurred with the pH 2.0 feed. Although when using pH 2.0 influent liquor, all of the H_2S may have been produced solely *via* ZVS reduction, it is possible that there was another source for this gas, the chemical dissolution of ZnS that had accumulated within the HSB during tests carried out with synthetic and mine waters (Chapter 6). Metal sulfides are generally highly insoluble, but their solubilities increase with

decreasing pH. Acid dissolution of ZnS ($ZnS + 2 H^+ \rightarrow Zn^{2+} + H_2S$) increases as solution pH falls below pH 3 though, as indicated, this is a proton-consuming reaction and no corresponding increases were observed in the bioreactor pH, possibly because of the influence of the sulfate/bisulfate buffer ($pK_a = 1.92$) at the pH of the bioreactor during this experiment.

Hydrogen sulfide was produced continuously when the HSB was operated exclusively as a sulfur-only reducing bioreactor in continuous flow mode. This was not unexpected, as several of the sulfidogenic bacteria present in the HSB had been shown to catalyse the dissimilatory reduction of ZVS (Chapter 4). Rates of H_2S generation were higher when using pH 4.0 than pH 3.0 influent liquor. The bioreactor pH was not controlled, and using pH 4.0 influent liquor the pH varied considerably (pH 4.1 – 4.8), which was not the case with the pH 3.0 influent liquor, where the bioreactor pH range was pH 3.0 - 3.2. Equation 5.2 shows that ZVS reduction is a pH-neutral reaction at pH 4.0 and therefore, in theory, no great variations in pH in the bioreactor liquors should occur. The T-RFLP profile of Figure 5.22 shows that putative fermenters as the *Clostridium* sp. and other non-sulfidogens were abundant as both sulfur-attached and planktonic cells during this experiment, and it is therefore possible that fermentation was more pronounced at the higher pH 4.0 which could account for the pH trends observed.

An apparent anomaly in the data generated when operating the HSB as a sulfur-only reducing bioreactor was that, on most sampling occasions, more acetic acid was generated than glycerol consumed. The stoichiometry of these two organic compounds for incomplete oxidation or (the assumed) fermentation of glycerol is 1:1, so the data obtained would appear to be erroneous. However, only 1 mM glycerol (equivalent to 36 mg C L^{-1}) was present in the feed liquor, while 100 mg L^{-1} of yeast extract was used. If the carbon content of yeast extract is considered, as a rough estimate, to be the same as that of glucose (40%), then this is equivalent to 40 mg C L^{-1} present in the feed liquor as yeast extract, which is slightly greater than that of glycerol. In Chapter 4 it was noted that the ZVS-reducing isolate *Firmicute* I2511 generated acetic acid as a waste product when growing only on yeast extract, and the same may well be the case that aSRB, such as the incomplete-oxidiser *D. acididurans*, which were also present in the HSB. This would explain the apparent anomaly, and suggest that yeast extract, rather than glycerol, acted as the main electron donor in this experiment (and in the later experiment where pH 2 feed liquor was used, as discussed below).

Changes in operational parameters of the HSB affected H_2S generation. When the HSB operated with very low pH influent liquors, rates of H_2S generation were much lower than those obtained during priming phase. Decreasing the pH of influent liquors or that of the bioreactor

usually resulted in a downturn hydrogen sulfide production, though the rates were faster (in batch mode) at pH 3.0 than at pH 2.5 or 3.5, and similar (but low) rates of H_2S production were found when using pH 2.5 and pH 2.0 feed liquors.

Rates of H_2S production were generally much greater when the HSB was operated as a hybrid (sulfate- and ZVS-reducing) than as sulfur-only reducing bioreactor (Table 5.3). One possible reason for this is that changing the basal salts composition from sulfate-based to chloride-based had a negative impact on the indigenous sulfidogens. Comparison of rates of H_2S generation during the priming phase with those when the HSB was operated in batch mode (Table 5.3) shows that they were much faster in the former, indicating that continuous flow mode is a superior *modus operandi.*

Table 5.3. Summary of rates of hydrogen sulfide generation in all experiments performed in Chapter 5.

HBS operation mode		**Bioreactor pH[b]**	**H_2S production µmoles L^{-1} h^{-1}**
Priming phase	Exp 1	3.7 - 3.8	159 ± 15
	Exp 2	3.5 - 3.8	142 ± 42
	Exp 3	3.4 - 3.7	262 ± 61
	Exp 4	3.3 - 3.5	227 ± 43
	Exp 5	3.5 - 6.1	256 ± 59
Low pH in continuous flow mode	pH 2.5[a]	2.6	40 ± 9
	pH 2.0[a]	2.0 - 2.1	47 ± 8
Sulfur-only reducing bioreactor in continuous flow mode	pH 4.0[a]	4.1 - 4.8	78 ± 29
	pH 3.0[a]	3.0 - 3.2	39 ± 18
Batch mode at fixed pH	pH 3.5	3.3 - 3.5	59[c]
	pH 3.0	2.9 - 3.2	124[c]
	pH 2.5	2.5 - 2.6	23[c]

[a] pH of Influent liquors; [b] pH range in the bioreactor liquors; [C] calculated from data at the start and end of each experiment.

The HSB is designed to act primarily as a H_2S-generating reactor, in which glycerol is used as an electron donor coupled to the reduction of sulfate and ZVS by acidophilic strains of dissimilatory sulfidogenic bacteria. Glycerol can be oxidised completely to CO_2, or incompletely oxidised to acetic acid plus CO_2 (Kimura *et al.,* 2006). The dissimilatory reduction of sulfate to sulfide is an eight electron transformation, while that of ZVS to sulfide is a two electron transformation. Complete oxidation of glycerol to CO_2 donates fourteen electrons,

while incomplete oxidation to acetic acid plus CO_2 donates only six electrons. Together, this means that the efficiencies of H_2S production coupled to glycerol oxidation could be very variable, depending on the extent to which glycerol was completely oxidised, and which electron acceptor (sulfate or ZVS) was being utilised. These four reactions, two of which have been quoted earlier in this Chapter, are shown below.

$$4\ C_3H_8O_3 + 7\ SO_4^{2-} + 14\ H^+ \rightarrow 12\ CO_2 + 7\ H_2S + 16\ H_2O \qquad \text{Eq. 5.1}$$

$$4\ C_3H_8O_3 + 3\ SO_4^{2-} + 6\ H^+ \rightarrow 4\ CH_3COOH + 4\ CO_2 + 3\ H_2S + 16\ H_2O \qquad \text{Eq. 5.3}$$

$$C_3H_8O_3 + 7\ S^0 + 6\ H_2O \rightarrow 3\ CO_2 + 7\ H_2S \qquad \text{Eq. 5.2}$$

$$C_3H_8O_3 + 3\ S^0 + H_2O \rightarrow CH_3COOH + CO_2 + 3\ H_2S \qquad \text{Eq. 5.4}$$

It is not possible to decipher the extents to which these four reactions were occurring in each of the experiments, though mass balance calculations show that in most circumstances all four would have been operating concurrently, as indicated by (i) the generation of acetic acid within the bioreactor, though mostly in non-stoichiometric (1:1) amounts to those of glycerol oxidised, and (ii) by the fact that the amount of sulfate reduced was, at most times, insufficient to account for all of the H_2S generated. In addition, the microbial communities housed within the HSB included species of bacteria that are non-sulfidogenic, such as the *Clostridium* sp. and actinobacterium sp. IR1 (both of which are considered to survive in the HSB by fermenting glycerol), *Ac. aromatica*, an acetoclastic acidophile that cannot oxidise glycerol, and *At. ferrooxidans*, a chemolithotrophic and facultatively anaerobic acidophile that (among other things) can couple the oxidation of reduced sulfur to the reduction of molecular oxygen or ferric iron. The almost ubiquitous presence of bacteria such as the *Clostridium* sp. and actinobacterium sp. IR1 suggests that some of the glycerol in the feed liquors would have been fermented rather than used as electron donor for sulfidogenic bacteria, while that of *Ac. aromatica* is presumed to be due to the on-going production of acetic acid, both by incomplete oxidation of glycerol by the sulfidogens and as a by-product of fermentation. In the case of *At. ferrooxidans*, the most likely scenario (given the absence of ferric iron in any of the feed liquors) was that this extreme acidophile was using the small amounts of dissolved oxygen (DO) present in the feed liquors as an electron acceptor, coupled to the oxidation of H_2S generated by the sulfidogens, as shown in Equations 5.5 and 5.6.

$$H_2S + 2\ O_2 \rightarrow H^+ + HSO_4^{2-} \qquad \text{Eq. 5.5}$$

$$H_2S + 0.5\ O_2 \rightarrow S^0 + H_2O \qquad \text{Eq. 5.6}$$

None of the influent liquors were de-aerated, as would be the case in a HSB operated at pilot- or full-scale, though sterilisation of these liquids (by autoclaving) would have lowered their

concentrations of dissolved oxygen. Typically, DO in pure water at the temperature of the feed liquor used is ~ 8 mg L^{-1}, or ~250 μM. Given the stoichiometries of H_2S and O_2 in reactions 5.5 and 5.6, this would allow the oxidation of ~ 125 μM or 500 μM hydrogen sulfide, depending on where it is fully or partially oxidised. At a flow rate of 455 mL h^{-1}, this is equivalent to ~ 57 μmoles h^{-1} of H_2S being oxidised to sulfate, or ~ 228 μmoles h^{-1} if oxidised only to ZVS. The maximum amount of H_2S produced by the HSB in the priming phase of operating the HSB was 184 μmoles L^{-1} h^{-1}, which is ~400 μmoles h^{-1} for the 2.2 L volume bioreactor. The hypothetical oxidation of 57 – 228 μmoles h^{-1} of this represents a considerable fraction (14 - 57%) of that produced. However, DO measured in pre-autoclaved feed liquor (pH 3.7) was considerably lower than this (3.50 mg L^{-1}) presumably due to its content of dissolved solutes, and autoclaving these solutions lowered DO still further (to 1.61 mg L^{-1}). In this case, the amount of H_2S that could be oxidised (by *At. ferrooxidans*) is 25 – 100 μmoles h^{-1}, or 11 – 45 μmoles L^{-1} h^{-1} (V = 2.2 L), which is ~ 6.25 - 25% of the maximum produced during priming phase operation of the HSB. Although oxidation of H_2S is a negative attribute, de-aerating feed solutions in an applied operation would not be pragmatic, and sacrificing some of the H_2S generated to consume DO is a far better option.

There are many assumptions in the above calculations, one being that none of the DO in the influent liquors was removed in the OFN gas stream, and the other being that only *At. ferrooxidans* used the available DO. The latter scenario is unlikely as the actinobacterium strain IR1 is also a facultative anaerobe (Ňancucheo and Johnson, 2012). When the HSB was operated with pH 2.0 influent liquor, the bioreactor pH was also about pH 2 which is the optimum growth pH for *At. ferrooxidans* (Hallberg *et al.,* 2010). In this situation it could be supposed that most of the available DO was used by *At. ferrooxidans* rather than strain IR1, and it is interesting to note that, in this phase of the experiment, there was net production, rather than reduction, of sulfate.

An idea of the relative extents to which reactions Eq. 5.1 – 5.4 were operating can be obtained by considering data from operating the HSB in batch mode. In the experiment where the HSB was maintained at pH 3.0, the glycerol concentration declined by 4.9 mM and that of acetic acid increased by 1.1 mM. This is equivalent to 10.78 mmoles of glycerol consumption (V = 2.2 L), 77.5% of which (8.36 mmoles) were oxidised to CO_2 and 22.5% (2.42 mmoles) to acetic acid + CO_2. If all of this had been coupled to the reduction of sulfate, the amount of H_2S generated would theoretically have been 16.45 mmoles (14.63 mmoles from complete and 1.82 mmoles from incomplete glycerol oxidation). If, however, glycerol oxidation had been coupled exclusively to the reduction of ZVS, the equivalent theoretical total amount of H_2S produced would have been 65.78 mmoles (58.52 mmoles from complete and 7.26 mmoles from incomplete oxidation). Mass balance calculations from this experiment indicated that

50.3% of the total H_2S generated had derived from sulfate reduction and 49.7% from the reduction of ZVS. Adjusting the data accordingly, this would account for the net production of 40.96 mmoles of H_2S. The actual amount of H_2S generated in this batch experiment was 20.98 mmoles. Extrapolating, this suggests that only ~51% of the available glycerol was used to generate H_2S in this experiment. The rest of the glycerol could have been: (i) fermented by the *Clostridium* sp. and other non-sulfidogens; (ii) oxidised aerobically by the actinobacterium IR1; (iii) used as a carbon source to produce biomass. It is likely that all three of these options would have been occurring in the HSB. There are a number of assumptions in these calculations, one being that the extent to which glycerol was oxidised completely was the same when metabolised by sulfate- and ZVS-reducing bacteria present bacteria in the consortium. There are some indications that incomplete oxidation is more prevalent among the ZVS-reducers. A calculation based on the hypothesis that glycerol coupled to sulfate reduction is completely oxidised to CO_2, while only a proportion of that coupled to ZVS reduction is completely oxidised and the rest is incompletely oxidised to acetic acid + CO_2, gives the following results:

(i) total H_2S produced = 20.98 mmoles, 50.3% of which (10.55 mmoles) derives from sulfate reduction and 49.7% (10.42 mmoles) from ZVS reduction;

(ii) 10.55 mmoles H_2S requires 6.03 mmoles glycerol as electron donor, assuming complete oxidation;

(iii) assuming that all of the acetic acid (2.42 mmoles) derived from incomplete oxidation of glycerol coupled to ZVS reduction, this would have derived from the same amount (2.42 mmoles) of glycerol, and would have generated 7.26 mmoles of H_2S, leaving a balance of 3.16 mmoles H_2S being generated by complete oxidation of glycerol coupled to ZVS reduction, which would require 0.45 mmoles glycerol;

(iv) the total glycerol required for this scenario is 8.90 mmoles (6.03 mmoles coupled to the reduction sulfate, plus 2.42 mmoles coupled to the reduction of ZVS by incomplete oxidation, plus 0.45 mmoles coupled to the reduction of ZVS by complete oxidation;

(v) since the actual amount of glycerol oxidised was 10.78 mmoles, the efficiency of it use as an electron donor coupled to H_2S generation now becomes 83%.

Bacterial 16S rRNA genes were amplified from sulfur-attached and planktonic cells in the HSB in every test performed, though all attempts to amplify archaeal genes proved negative. Semi-quantitative comparison of selected T-RFLP profiles of bacterial 16S rRNA genes from the different experiments are shown in Figure 5.24. These show that there were major fluctuations in the bacterial community composition. One reason for this is that other environmental

microorganisms would have entered the HSB when non-filtered mine waters were used as feed liquors (that from Minsterley Brook after the priming phase experiments and later that from the abandoned Force Crag mine, as described in Chapter 6).

Nine terminal restriction fragments (T-RFs) were found at >5% relative abundance at different times during these experiments. Three of these are sulfidogens: *D. acididurans*^{T}, *Peptococcaceae* CEB3, and *Firmicute* I2511, in which the latter catalyses the dissimilatory reduction of ZVS but not sulfate (Chapter 4). Four T-RFs (*Clostridium* sp., *Ac. aromatica*, *At.*

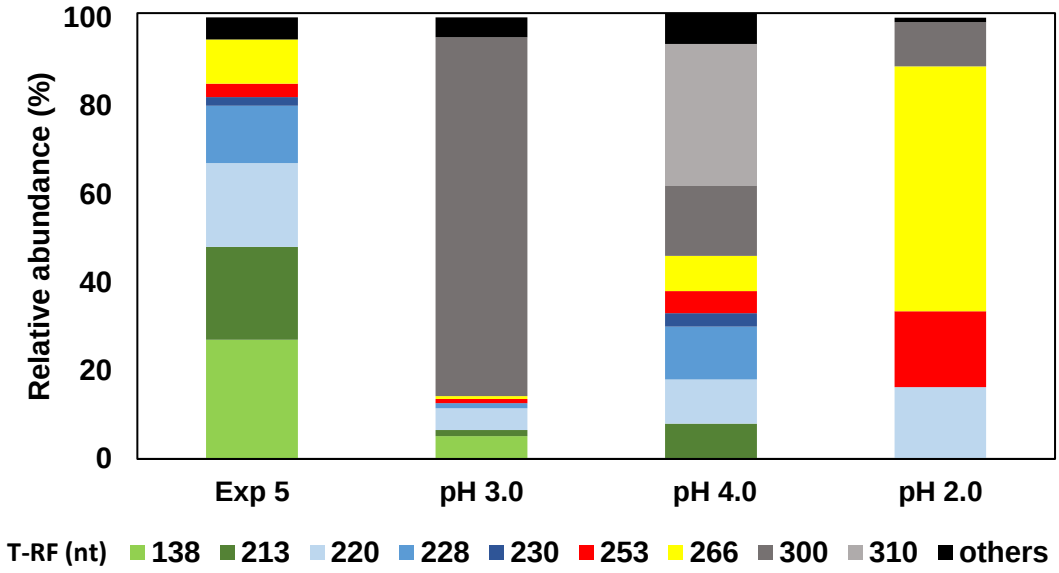

Figure 5.24. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts from sulfur-attached bacterial communities and digested with the restriction enzyme HaeIII. "Exp 5", experiment 5 of the priming phase; "pH 3.0", batch mode operation at pH 3; "pH 4.0", HSB operated as a sulfur-only reducing bioreactor with pH 4 influent liquor; "pH 2.0", HSB operated in continuous flow mode with pH 2 influent liquor. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*^{T}, (■) *Clostridium* sp., (■) *Acidocella aromatica*^{T}, (■) actinobacterium sp. IR1, (■) *Acidithiobacillus ferrooxidans*, (■) *Firmicute* strain I2511, (■) unidentified T-RF, (■) unidentified T-RF, (■) low abundance unidentified T-RFs.

ferrooxidans and actinobacterium sp. IR1) do not catalyse the dissimilatory reduction of sulfate or sulfur. The 300 nt T-RF, which corresponded to a bacterium not present in the BART databank, was found frequently in these profiles, and was sometimes the most dominant sulfur-attached bacterium (Fig. 5.24) when ZVS reduction was prevalent (HSB operating in batch mode at pH 3.0). The 300 nt T-RF is therefore likely to have derived from another acidophilic bacterium that generates H_2S from the reduction of ZVS. Another unidentified bacterium, corresponding to the 310 nt T-RF, was abundant when HSB was operated as a sulfur-only reducing bioreactor (Fig. 5.22) but not in earlier experiments. Attempts to isolate both bacteria or to identify them in clone libraries were unsuccessful.

Changes in the relative abundance of members of the microbial population reflected changes in the HSB operating parameters and performance. For example, in experiment 5 of the priming phase the increase in the pH values (Fig 5.5) and net sulfate reduction (Fig 5.7) coincided with the increase in the relative abundance of aSRB, which accounted for almost 50% of the microbial population at this stage. Complete glycerol oxidation increased in experiment 4 of the priming phase, coinciding with an increase in the relative abundance of planktonic *Peptococcaceae* CEB3 (43%), a bacterium that has been reported to oxidise glycerol to CO_2 (Santos and Johnson, 2016). Changing from sulfate salts to chloride salts in the influent liquors when the HSB was operated as sulfur-only bioreactor resulted in a notable shift in the microbial population, with emergency of the 310 nt T-RF and a decrease in relative abundance of aSRB *D. acididurans*^{T} and *Peptococcaceae* CEB3, and the latter was detected only in the sulfur-attached cells at pH 3.0 influent liquors experiment. In general terms, decreasing the pH of bioreactor liquors caused a large decrease in the biodiversity of the dominant bacteria present in the microbial community. Ñancucheo & Johnson (2012) designed a continuous flow bioreactor operating at low pH (pH 3.6-2.2) to selectively precipitate metals from pH 2.1 synthetic mine water. *Peptococcaceae* CEB3 was found to be the most relatively abundant member (~ 54%) of the bacterial community when the bioreactor pH was 2.4. However, CEB3 was not detected at pH 2.2. In the same work, *D. acididurans*^{T} was detected in relatively low abundance (~6%) when the bioreactor pH was at pH 2.4 and 3.6, but was not detected at pH 2.2. Likewise, when the HSB was operated with pH 2.5 influent liquors, *Peptococcaceae* CEB3 was the most relatively abundant found as both sulfur-attached (46%) and planktonic cells (43%), while corresponding figures for *D. acididurans* were 15% and 21%. However, with pH 2.0 influent liquor neither bacterium was detected, and there was no net sulfate reduction (Fig 5.18), and *Firmicute* strain I2511 accounted for >50% of the total planktonic and sulfur-attached bacteria, and was therefore assumed to be responsible for generating H_2S at that time. As a consequence of the variations in operating parameters and the biodiversity of the microbial population in the HSB, various biological processes occurred simultaneously, making this system quite complex and difficult to evaluate and model. However, data from the experiments showed that the HSB operated effectively at pH 3.0 to pH 4.5 with presence of sulfate salts and when operated in continuous flow mode.

The objectives of experiments performed with the HSB described in this chapter were to provide important background data for operating the novel bioreactor and to test whether it could be used for remediating zinc-contaminated mine waters. The bioreactor was effective in meeting both objectives and results obtained in the experiments underlined the resilience of the system. This provided the backdrop for the experiments described in the following chapter.

Chapter 6. Application of a novel “hybrid” low pH sulfidogenic bioreactor for removing zinc from circum-neutral pH mine-impacted waters.

6.1 Introduction

Throughout the world in many post-industrial mining countries, metal mining has left a legacy of contaminated watercourses, estimated to be several hundred kilometres in the UK alone (Mayes *et al.,* 2009).The latter assessed the extent of the pollution caused by discharges from abandoned non-coal mines in England and Wales, and concluded that 6% of surface water bodies were affected. This represents a significant source of water pollution, generating a flux of hundreds of tons of metals per annum into the streams and rivers into which they flow (Mayes *et al.,* 2013).

Many mine-impacted waters in the UK are circum-neutral pH and, in contrast to extremely acidic mine waters, contain relatively little soluble iron (e.g. in areas with hydrothermal base metal sulfide deposits from which pyrite is often absent) though their concentrations of Zn, Pb and Cd can exceed EU water quality guidelines. Some systems used to remediate these watercourses, such as constructed wetlands, use passive biological technologies (Nuttall and Younger, 2000; Mayes *et al.,* 2009; Warrender *et al.,* 2011; Gandy and Jarvis, 2012; Jarvis *et al.,* 2015; Gandy *et al.,* 2016). However, this approach has several drawbacks. For example, the metals, which are immobilised in a solid matrix (e.g. ochre and spent compost), cannot be recycled and long-term disposal and storage (where remobilisation of metals and metalloids may occur) are required (Johnson and Hallberg, 2005). Changes in the environment can influence the metal removal efficiency of passive system, and the occurrence of secondary contamination to receiving waters caused by a constructed wetland has been reported (Jarvis *et al.,* 2015). Protracted hydraulic residence time and large land surface areas are often required for these systems to be efficient. Many metal mine discharges in England and Wales occur in upland areas with steep topographies, making passive systems non-feasible, and the limited availability of land is also an obstacle to installing constructed wetlands in UK (Mayes *et al.,* 2009).

Full-scale sulfidogenic bioreactors have been demonstrated in different parts of the world (e.g. THIOTEQ™ systems, operated by the Dutch company Paques, and BioSulphide® systems operated by the Canadian company BioteQ). There are several advantages of this approach compared to passive remediation, including them being more controllable systems with predictable performance, selective recovery and recycling of chalcophilic metals such as copper and zinc, and much smaller land footprints than constructed wetlands (Johnson, 2014). In order to maintain optimum conditions for microbial activity, these bioreactors require a

continuous input of reagents and more intensive management than passive systems. Operating costs (OPEX) of active biological systems are usually much greater than passive systems, though this can be at least partially offset by the commercial value of the metals recovered.

This chapter describes tests carried out with the novel low pH “hybrid” sulfidogenic bioreactor (HSB; Chapter 5) for removing zinc from two circum-neutral pH metal-contaminated mine waters in the UK.

6.2 Removal of zinc from circum-neutral pH mine impacted waters

6.2.1 Rationale of the experimental setup

Experiments were carried out in order to remove Zn^{2+} from circum-neutral pH mine-impacted waters from abandoned lead/zinc mines in Shropshire (Minsterley Brook Catchment) and Cumbria (Force Crag Mine), United Kingdom. In both cases, initial tests involved preparing synthetic versions of the mine waters, based in chemical data provided by the Coal Authority (UK). Following this, tests were carried out with waters collected from the two sites.

The HSB was operated as described in section 5.2.2, with some modifications. For both mine waters, both in-line (i.e. within the bioreactor vessel) and off-line (i.e. in a separate vessel attached to the HSB) precipitation of zinc was evaluated (Fig. 6.1). A second off-line vessel, containing copper sulfate, was connected to receive the OFN gas stream from the HSB, to remove any H_2S that was not used to precipitate zinc in the first vessel (Fig. 6.1).

A second modification in operating the HSB was required when testing with actual mine waters. In earlier work it had been possible to add organic materials (glycerol and yeast extract) directly to the feed liquors, as these were sterile. This was not possible with the mine waters as the organic material would have been metabolised before they had entered the HSB. Therefore, an addition influent liquor, containing more concentrated glycerol and yeast extract solution than those used in setting up and trialling the HSB, was pumped into the reactor vessel, using an independent peristaltic pump, through a second L-tube at controlled rates, along with unfiltered mine water (Fig. 6.1).

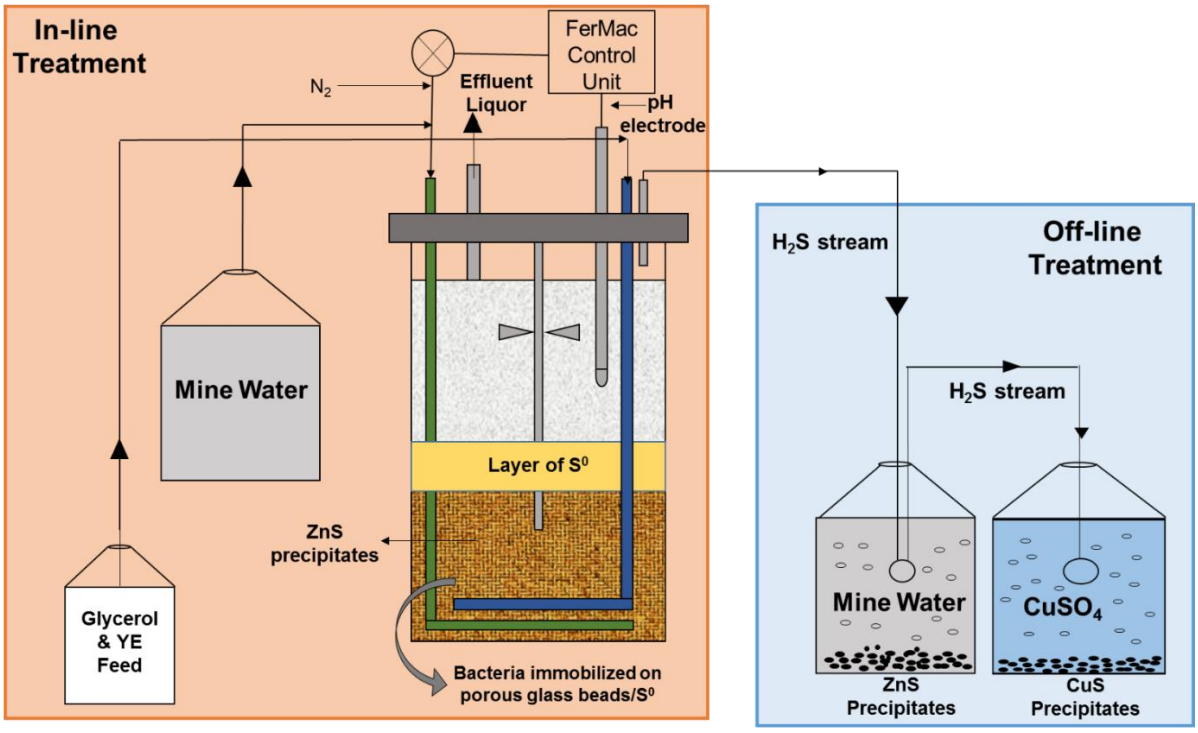

Figure 6.1. Schematic representation of the set-up used to assess removal of soluble zinc from two circum-neutral pH mine waters.

The analytical methods used and microbiological analyses carried out were those described in section 5.2.2. The following additional calculations were also carried out, to take into account the modified *modus operandi* when testing actual mine waters:

(i) *Influent glycerol (mM):* concentrations of glycerol when combining influent mine water and glycerol/yeast extract solutions (GYE feed).
{Glycerol in the feed (mM) x F of GYE feed} / F of influent mine water
(F = flow rate; L h^{-1})

(ii) *Influent yeast extract (mg L^{-1})*: concentrations of yeast extract when combining influent mine water and glycerol/yeast extract solutions (GYE feed).
{YE in the feed (mg L^{-1}) x F of GYE feed} / F of influent mine water
(F = flow rate; L h^{-1})

6.2.2 Minsterley Brook Catchment water

The Minsterley Brook Catchment is part of the Rea Brook which drains into the River Severn, the longest river in the UK, at Shrewsbury (52° 37' 49.6776" N; 2° 56' 2.2596" W).

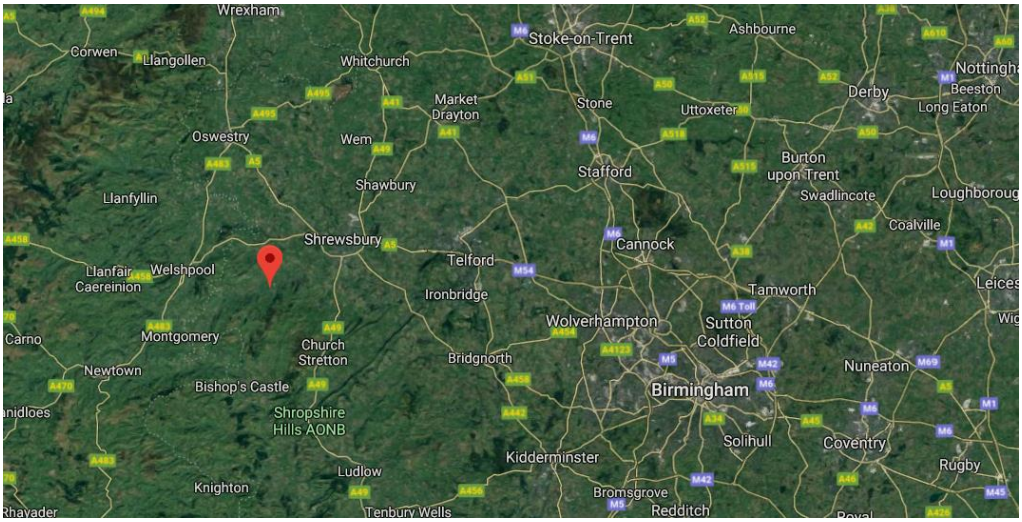

Figure 6.2. Location of Minsterley Brook Catchment, Shropshire, England.

The south Shropshire metal mining area contains a number of abandoned small mines including Snailbeach, Tankerville, and Roman Gravels mines. These were primarily lead mines, but zinc ore and later barites were also mined until 1940s, when it was closed, leaving mine waste deposits and drainage adits that discharge in the catchment in several locations. Waters draining from the waste spoils from these mines represent one of the longest continuous sources of heavy metal pollution in the Severn River Basin (https://restorerivers.eu/wiki/index.php?title=Case_study%3AMinsterley_Brook_Abandoned_Metal_Mines.).

The Minsterley Brook Catchment was ranked the second highest overall impact score in a report of the Environment Agency (UK) on abandoned non-coal mines in England and Wales (Jarvis and Mayes, 2012), with levels of soluble zinc (3 – 66 mg L^{-1}) that exceeded Environmental Quality Standards (EQS). In 2016, this water body failed again in chemical pollution evaluation (http://environment.data.gov.uk/catchment-planning/WaterBody/GB109054049480).

Experiments using the HSB were designed to demonstrate that active biological treatment, based on biosulfidogenesis, could be used to effectively remove soluble zinc from water collected at a particularly highly polluted site within the catchment, Snailbeach Farm,

Shropshire (52° 37' 4.7172" N; 2° 55' 33.726" W). Approximately 60 L of water was collected in October 2016 from a pond at the Farm (Fig. 6.3). Apart from collecting water discharged from the Snailbeach Spoil Tip, the artificial pond also receives domestic effluents and other sources of contamination. For this reason the water collected from Snailbeach Farm is referred to generically as waste water.

Figure 6.3. The pond at Snailbeach Farm, part of Minsterley Brook Catchment, Shropshire, England.

Physico-chemical data of water at Snailbeach Farm was provided by the Coal Authority (UK) and from that a synthetic version was prepared in the laboratory using analytical-grade reagents ($ZnSO_4$, $MgSO_4$, $CaCl_2$, NaCl, K_2SO_4 and NaOH). These, together with the chemical composition of the waste water collected from the Farm, are shown in Table 6.1.

Table 6.1. Chemical composition of the Minsterley Brook Catchment water sample. Concentrations shown are mg L^{-1}.

	Coal Authority report	Synthetic waste water	Waste water sampled
Zn^{2+}	66	66	58
SO_4^{2-}	668	677	768
Cl^-	27	380	33
Ca^{2+}	306	200	ND
Mg^{2+}	12	144	ND
Na^+	19	19	ND
K^+	5	4	ND
Mn^{2+}	0.3	NA	ND
Fe^{2+}	0.3	NA	<0.01
Sr^{2+}	0.2	NA	ND
Cd^{2+}	0.4	NA	<0.01
Pb^{2+}	0.4	NA	<0.01
Cu^{2+}	0.2	NA	<0.01
DOC	ND	74 – 96*	3.4
Alkalinity**	154	12.5	148
pH	6.7	6.7	7.1

*Calculated from the combined glycerol and yeast extract added; **$CaCO_3$ equivalent; ND: not determined; NA: not applicable.

6.2.2.1 Removal of zinc from synthetic Minsterley Brook Catchment (Snailbeach Farm) waste water

Synthetic zinc-free Minsterley Brook Catchment waste water amended with 1 mM glycerol and 0.005% (w/v) yeast extract was prepared and autoclaved and, when cool, zinc sulfate added. This was used as feed liquor for the HSB, operated in continuous-flow mode at 30ºC but with no pH control. Samples were withdrawn from the effluent liquors at regular intervals and used to determine concentrations of glycerol, acetic acid, zinc and sulfate (section 5.2.2). Results of this experiment are described in Figures 6.4 – 6.7 and Appendix 6A. Excess H_2S produced in the HSB was delivered to an off-line vessel containing 1 L of glycerol/yeast extract-free synthetic mine water. Samples were removed at regular intervals to determine concentrations of zinc and to measure pH. Results are presented in Figure 6.8 and Appendix 6A.

Following this, the HSB was modified to include separate feeds for glycerol/yeast extract-free synthetic mine water and the organic substrates (207 mM glycerol and 0.5% (w/v) yeast

extract). The synthetic mine water was injected into and out of the bioreactor vessel using two peristaltic pumps that were integral to the FerMac 360 control unit (Electrolab, UK) at a continuous (maximum) rate of 455 mL h^{-1}, and the organic feed was injected using an independent peristaltic pump (Electrolab) at a flow rate of 4.3 mL h^{-1}. Results of in-line removal of zinc in this set up are shown below in Figures 6.4 – 6.8, and Appendix 6B.

The HSB was found to consistently remove Zn^{2+} from synthetic waste water. Figure 6.4 shows the percentage of zinc removal achieved during in-line treatment of both synthetic waste water directly supplemented with glycerol and yeast extract, and with the organic materials supplied in a separate feed. Over 96% of zinc content in the influent liquors was removed after continuous flow had been in operation for 49 h in the former, and for 52 h in the latter.

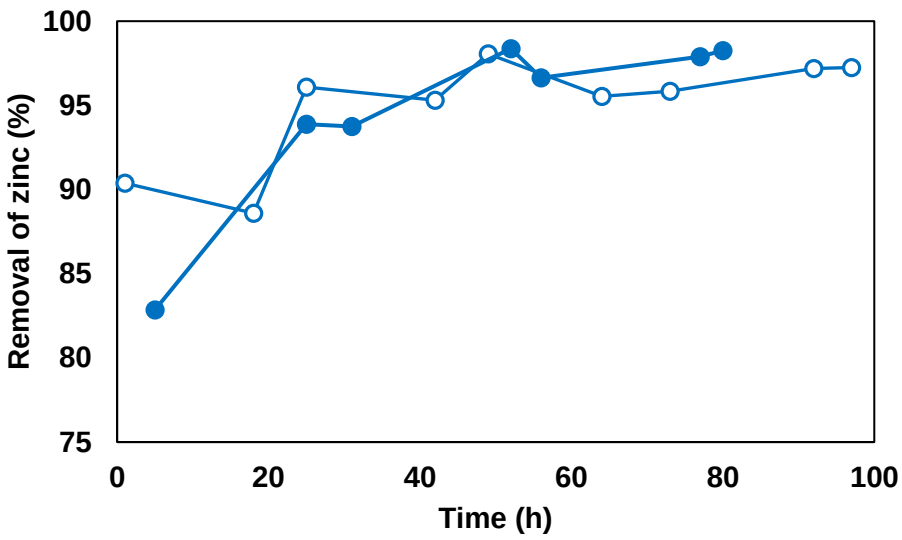

Figure 6.4. Removal of soluble zinc (%) from Minsterley Brook Catchment synthetic waste water during in-line treatment. Key: (○) waste water supplemented directly with glycerol and yeast extract; (●) glycerol and yeast extract supplied as a separate feed.

Bioreactor pH and planktonic bacterial cell numbers showed similar values with both configurations of testing synthetic waste water *via* in-line treatment (Fig 6.5).

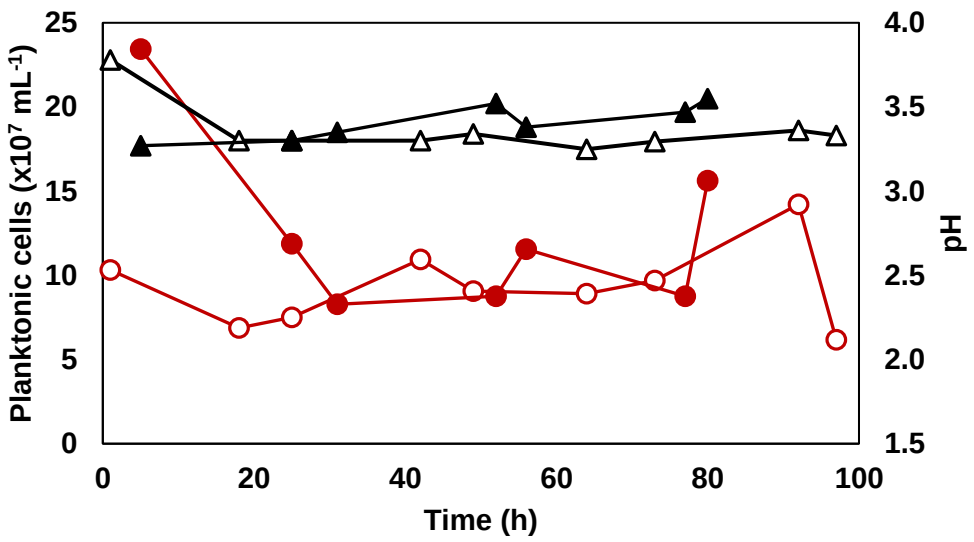

Figure 6.5. Changes in planktonic bacterial cell numbers in the effluent liquors (●, ○) and bioreactor pH (▲, Δ) during in-line treatment of Minsterley Brook Catchment synthetic waste water. Key: (○, Δ) waste water supplemented directly with glycerol and yeast extract; (●, ▲) glycerol and yeast extract supplied in a separate feed.

Concentrations of glycerol oxidised when the synthetic waste water was supplemented directly with glycerol and yeast extract showed only minor variations, with average glycerol consumption of 88 ± 4 % (0.88 ± 0.08 mM). When organic materials were supplied in a separate feed, consumption of glycerol ranged 69 – 98 % (1.41 ± 0.17 mM) (Fig 6.6). Acetic acid was produced throughout in both configurations, and concentrations increased towards the end of the treatment with the separate organic feed. The stoichiometry of glycerol oxidised to acetic acid produced indicated that part of the glycerol was oxidised completely to CO_2.

Rates of net sulfate reduction were very variable with both arrangements (Fig 6.7). Rates of hydrogen sulfide generation also varied considerably, though in general terms these were higher when the synthetic waste water was supplemented directly with glycerol and yeast extract than with the separate feed.

Mass balance calculations, described in section 5.2.2 and data shown in Appendices 6A-6B, indicated that, in terms of relative amounts of H_2S generated *via* sulfate and ZVS reduction, sulfate reduction was apparently more dominant (67 ± 30 %) when the organic supplements were supplied separately than added directly to the synthetic waste water (48 ± 26 %), though when an independent samples t-test was applied, differences were not significant [t(14)=1.34, p=0.2] (Appendix 6C).

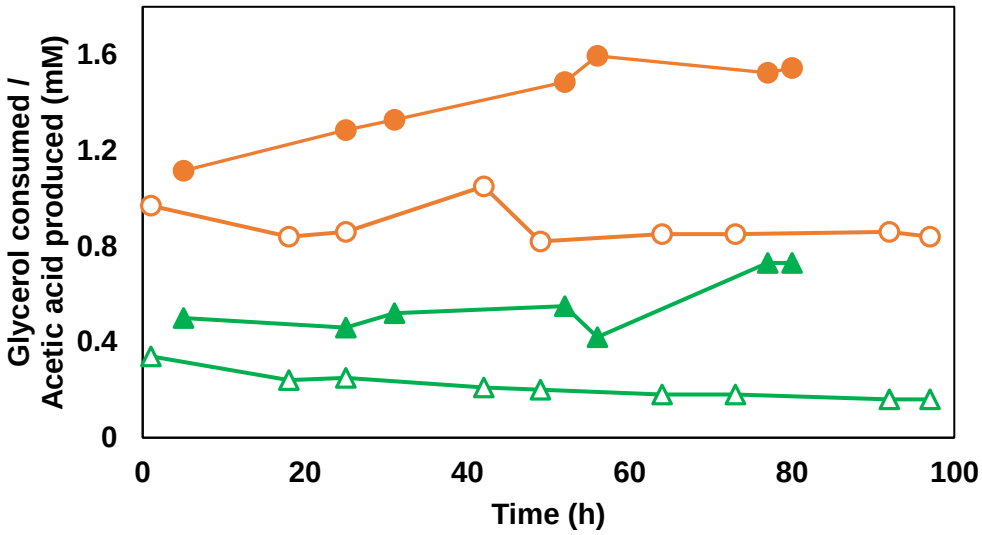

Figure 6.6. Consumption of glycerol (●, ○) and acetic acid produced (▲, Δ) in the in-line treatment of Minsterley Brook Catchment synthetic waste water. Key: (○, Δ) waste water supplemented directly with glycerol and yeast extract; (●, ▲) glycerol and yeast extract supplied in a separate feed.

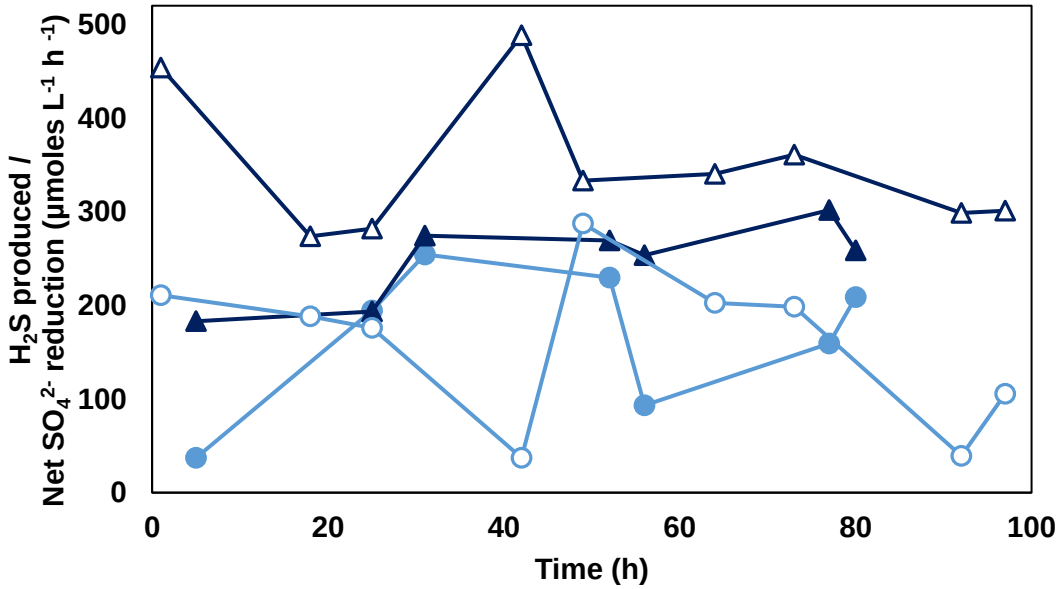

Figure 6.7. Changes in rates of net sulfate reduction (●, ○) and hydrogen sulfide production (▲, Δ) during in-line treatment of Minsterley Brook Catchment synthetic waste water. Key: (○, Δ) waste water supplemented directly with glycerol and yeast extract; (●, ▲) glycerol and yeast extract supplied in a separate feed.

In contrast to the in-line set up, off-line treatment of the synthetic waste water was not very effective in removing zinc, at least in the short-term. After 4 h of the first off-line treatment (Fig. 6.8a), the concentration of soluble zinc had fallen from 1.08 to 0.85 mM (i.e. 21% of the zinc had been precipitated as ZnS). This was not due to an inadequate supply of H_2S, as copper was precipitated in the second off-line vessel that received off-gas that had already passed through the synthetic mine water. The pH of the synthetic waste water in the off-line vessel declined rapidly from 6.6 to 4.5 during the first 30 mins of treatment, and to 3.6 after 4 h. More zinc (75%) was removed from the synthetic waste water in a longer-term (~ 100 h) experiment, and the pH in this case fell to 2.9.

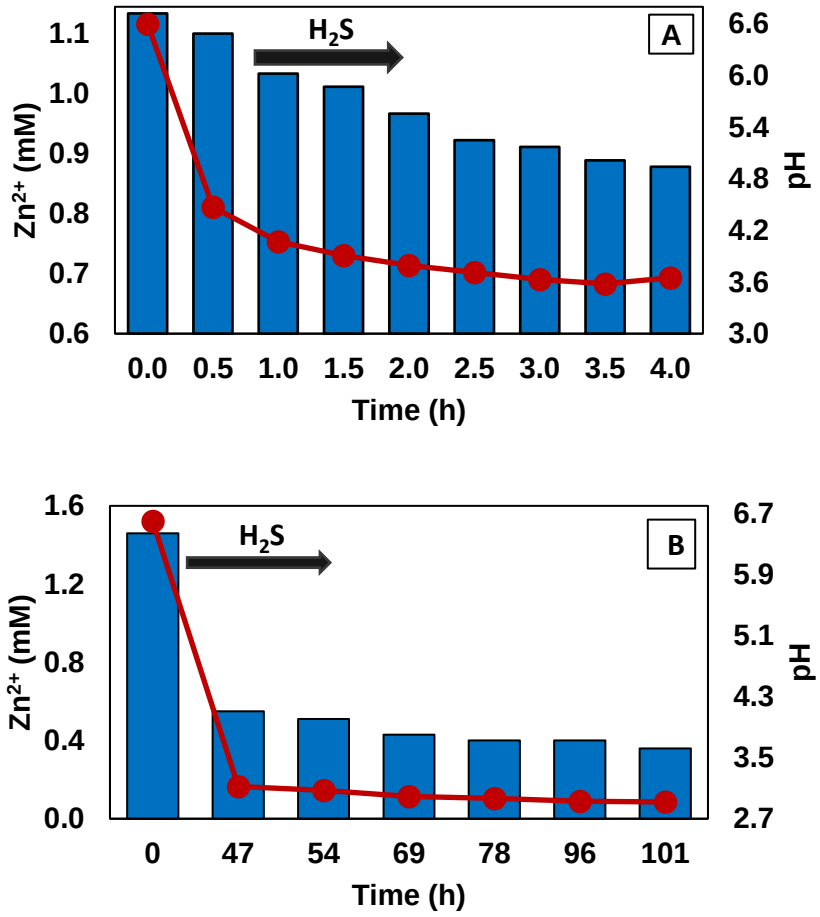

Figure 6.8. Off-line treatment of the Minsterley Brook Catchment synthetic waste water, in (A) a short-term (4 h) and (B) a longer-term (101 h) experiment. Bars denote concentrations of soluble zinc, and the line graphs show changes in pH.

6.2.2.2 Removal of zinc from Minsterley Brook Catchment (Snailbeach Farm) waste water

Following completion of trials with synthetic mine water, 60 L of water was collected from the Snailbeach Farm pond, transferred to the laboratories at Bangor University and used as both influent liquor for the HSB and in the off-line vessel. Analysis of the waste water collected from the Farm showed it had a lower concentration of zinc but that its pH and concentration of sulfate were higher than in data supplied by the Coal Authority (Table 6.1). Metals such as iron, lead, copper and cadmium were below detection limits (< 0.01 mg L^{-1}). Another important difference between the actual waste water and its synthetic equivalent was the higher alkalinity of the first (148 mg L^{-1}) than the latter (12.5 mg L^{-1}). Even though waste water at the site included run off from organic sources, its DOC concentration (3.4 mg L^{-1}) was low and far less than that of the glycerol/yeast extract-amended synthetic and actual waste waters.

The in-line tests carried out with Snailbeach Farm waste water differed from those with the synthetic mine water in that the only configuration used was that where the waste water and organic materials (glycerol plus yeast extract) were pumped into the bioreactor as separate feeds. In-line removal of soluble zinc from Snailbeach Farm waste water was effective, with 99% removed by the first time of sampling at 6 h. Later perturbations in the efficiency of zinc removal (Fig. 6.9) were due to technical problems with the bioreactor. Raw and processed data from this experiment are shown in Appendix 6D.

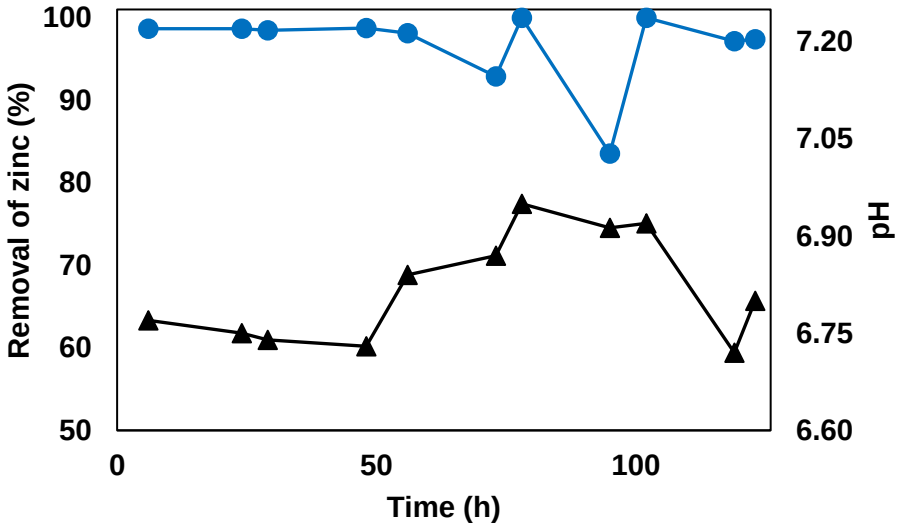

Figure 6.9. Percentage of zinc removal (●) and changes in bioreactor pH (▲) during in-line treatment of Snailbeach Farm waste water.

The bioreactor pH showed only minor changes (between 6.7 and 7.0) during in-line treatment of the actual waste water (Fig 6.9). These values were much higher than those recorded during in-line treatment of synthetic waste water (pH 3.3 - 3.6).

Figure 6.10 shows the consumption of glycerol and production of acetic acid during this test. There was a continuous increase in glycerol consumption during the experiment which was paralleled by a decrease in acetic acid production, though values of the latter increased again by the end of the treatment. The stoichiometry of glycerol oxidised to acetic acid produced indicates that part of the glycerol was oxidised completely to CO_2, and that the relative percentage of complete oxidation increased during the experiment, from 34% at 6 h to 78% at 102 h, and decreased slightly thereafter.

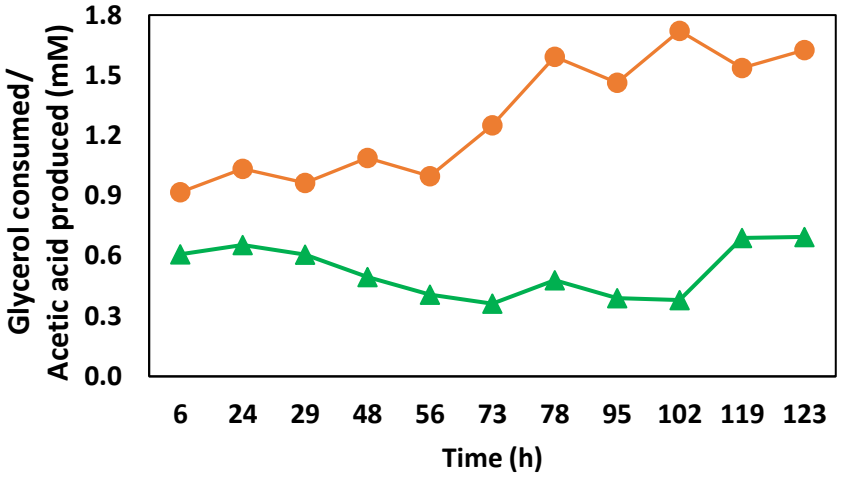

Figure 6.10. Consumption of glycerol (●) and acetic acid produced (▲) during in-line treatment of Snailbeach Farm waste water.

Rates of H_2S generation were reasonably consistent throughout the experiment, while rates of net sulfate reduction varied considerably (Fig 6.11).

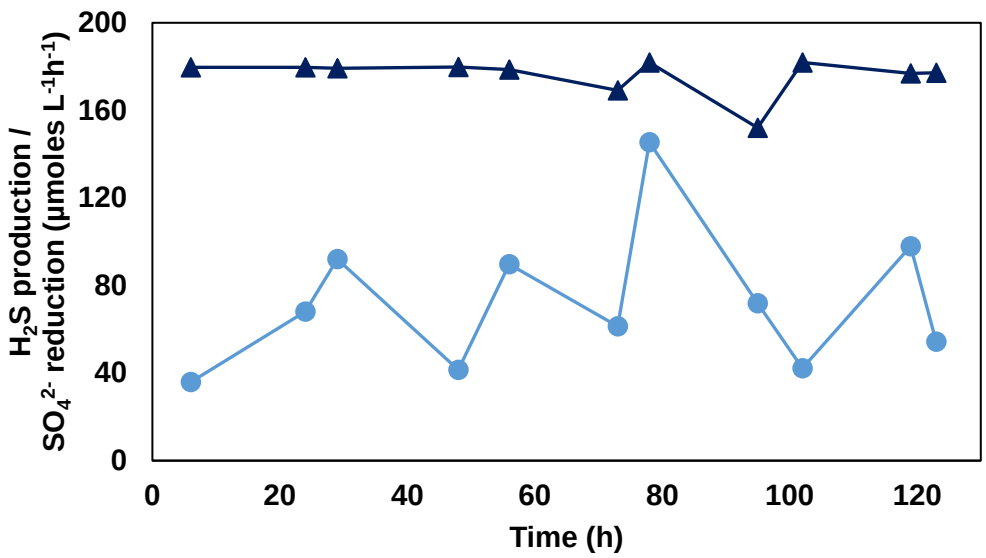

Figure 6.11. Changes in rates of net sulfate reduction (●) and rates of hydrogen sulfide production (▲) during in-line treatment of Snailbeach Farm waste water.

Figure 6.12 shows the relative percentage of H_2S produced *via* sulfate and ZVS reduction during in-line treatment of waste water from Snailbeach Farm. ZVS reduction was more prevalent overall (59 ± 18%), in contrast to the results obtained during tests carried out with synthetic waste water (37 ± 27%).

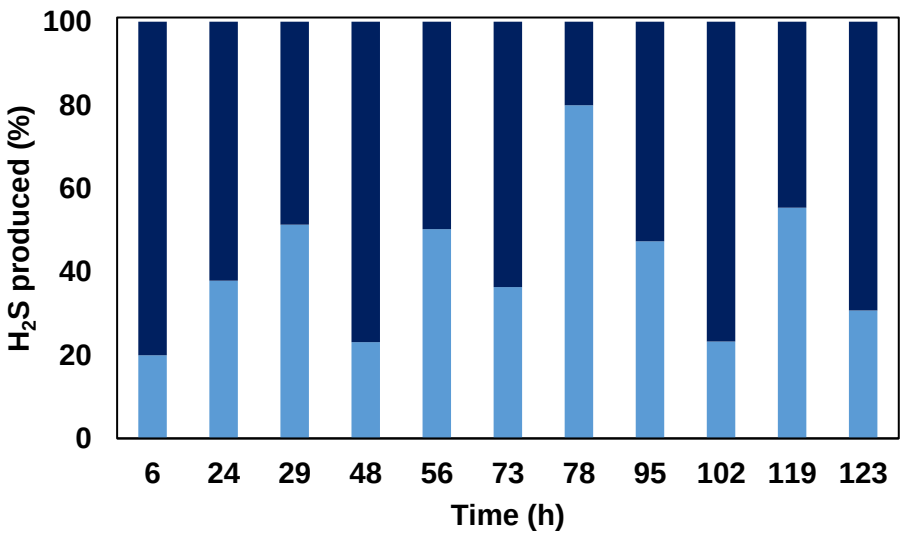

Figure 6.12. Percentage of H_2S produced *via* reduction of ZVS (■) and sulfate (■) during in-line treatment of Snailbeach Farm waste water.

Rapid removal of over 99% of soluble zinc was achieved by off-line treatment of waste water from Snailbeach Farm, generating metal-free effluents with pH of ~ 7.1 (Fig 6.13). The efficiency of off-line removal of zinc using actual waste water was much greater that when using synthetic liquor (Fig 6.8).

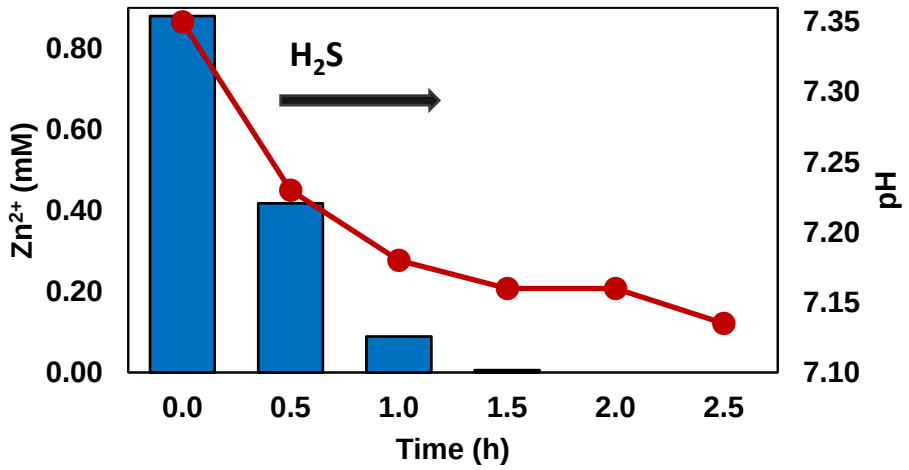

Figure 6.13. Off-line treatment of Snailbeach Farm waste water. Bars shows the decrease of Zn^{2+} (in mM) with time. Scatter plot represents the changes in pH of actual waste water during the off-line treatment.

6.2.2.3 Bacterial populations in the HSB during treatment of Minsterley Brook Catchment (Snailbeach Farm) waste waters

During the visit to Snailbeach Farm, a sample (50 mL) of water from the pond was filtered through a cellulose nitrate membrane filter (pore size, 0.2 µm). In the laboratory, DNA was extracted from the membrane (section 2.6.1), followed by amplification of bacterial 16S rRNA genes and digestion with the restriction enzyme HaeIII. Subsequently, semi-quantitative analysis were carried out using T-RFLP (section 2.6.3). The profile obtained (Fig. 6.14) showed that the bacterial population in the waste water was highly diverse, with no T-RF exceeding >10% relative abundance.

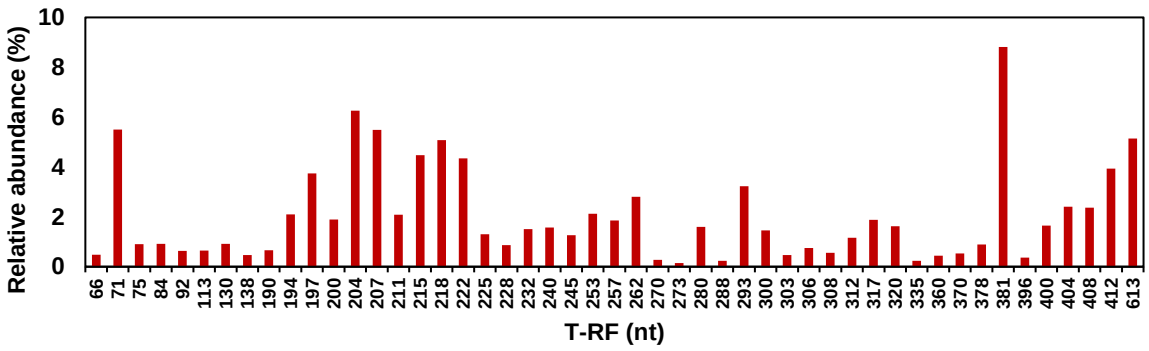

Figure 6.14. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracted from waste water from Snailbeach Farm, and digested with the restriction enzyme HaeIII.

During treatment of synthetic waste water, T-RFLP analysis indicated that the aSRB *D. acididurans*^{T} and *Peptococcaceae* CEB3 accounted for >50% of the bacterial populations in both sulfur-attached and planktonic cells, and that *D. acididurans* (35% and 56%) was far more abundant than CEB3. When the influent liquor changed to actual waste water from Snailbeach Farm, both strains became less abundant (Fig. 6.15) and novel unidentified T-RFs appeared in profiles of sulfur-attached cells, indicated in Fig 6.16 as “others”. At least one of these (the 300 nt T-RF) probably corresponded to a bacterium present in the pond water, as an identical T-RF was found in that profile (Fig. 6.14). Other bacteria identified in the T-RFLP profiles shown in Fig 6.16 were *Clostridium* SBR1, which increased greatly in relative abundance in both attached and planktonic populations when actual waste water was used, *Ac. aromatica,* actinobacterium sp. IR1 and *At. ferrooxidans,* though the latter was detected only in sulfur-attached cells. The ZVS-reducing *Firmicute* strain I2511, was not detected by T-RFLP analysis in any samples analysed in this experiment.

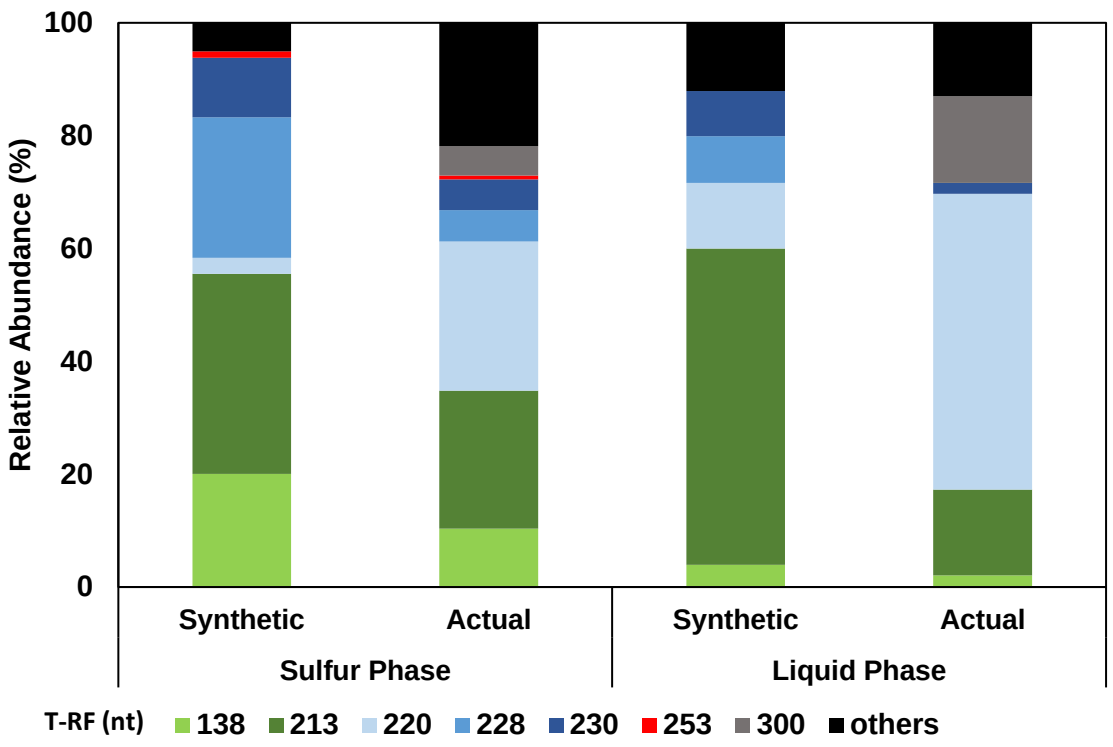

Figure 6.15. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during in-line treatment of Minsterley Brook Catchment waste waters. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*[T], (■) *Clostridium* SBR1., (■) *Acidocella aromatica*[T], (■) actinobacterium sp. IR1, (■) *Acidithiobacillus ferrooxidans*, (■) unidentified T-RF, (■) low abundance unidentified T-RFs. "Sulfur phase" refers to DNA extracted from sulfur-attached bacterial communities, and "liquid phase" to DNA extracted from planktonic cells.

6.2.3 Force Crag Mine

Force Crag is an abandoned lead, zinc and barite mine situated at the head of the Coledale valley, Cumbria, North West England (54° 35' 0.6036" N, 3° 14' 23.1972" W). It had two distinct working areas - the High Force and the Low Force - with early workings extracting barite, sphalerite (ZnS) and galena (PbS) at the Low Force area at the beginning of the 19th century. The mine site is located 275 m above sea level and has abandoned mine workings extending west to an elevation of 600 m (Adams, 1988; Tyler, 2005; Jarvis *et al.,* 2015).

Figure 6.16. Location of Force Crag Mine in Cumbria, England (A) and a view of the abandoned mine site (https://www2.groundstability.com/force-crag-mine-water-treatment-scheme) (B).

Several companies operated the mine intermittently for 157 years until it was finally abandoned in 1992 following a collapse in the lower workings. The workings at Force Crag are formed by nine individual levels, with Level 0 being the lowest level. These working are currently draining two adits, Level 1 (the primary discharge) and Level 0. Long-term monitoring revealed that the Level 1 discharge was the main source of metal contamination to watercourses in the Force Crag area, most notably the Coledale Beck River. The river has very low concentrations of nutrients and DOC and receives metal contamination from diffuse sources from the mine site (Tyler, 2005; Jarvis *et al.,* 2015).

The discharge from Level 1 is characterised as a poorly mineralized circum-neutral pH drainage with low concentrations of inorganic ions, including sulfate. Data provided by the Coal Authority indicated that zinc and copper are the transition metals present at highest concentrations (Table 6.2). The flow-rate of Level 1 discharge varies throughout the year from 8.5 to 24.4 L s^{-1}, chiefly determined by rainfall events, and this affects metal concentrations in the drainage water *via* dilution (Jarvis *et al.,* 2015).

Experiments with Force Crag mine water followed the same sequence as those carried out with Minsterley Brook Catchment waste water. First, a synthetic version of the mine water, with chemical composition based on data provided by the Coal Authority (Table 6.2) was prepared in the laboratory using analytical-grade reagents ($ZnSO_4$, $CuSO_4$, $MgSO_4$, $CaCl_2$, Na_2CO_3, $MnSO_4$ and NaOH) and removal of transition metals (copper and zinc) using the HSB was tested with both in-line and off-line configurations. Following this, a visit was made to the abandoned mine site (in April 2017) and approximately 60 L of mine water was collected from the Level 1 discharge. The chemical compositions of the synthetic and actual mine water discharge from Force Crag are shown in Table 6.2. These differed slightly in their concentrations of sulfate and chloride, and both alkalinity and pH were lower in the synthetic mine water. Most notably, the concentration of soluble copper was below the detection limit (<0.01 mg L^{-1}) in discharge water collected at the mine site.

6.2.3.1 Removal of metals from synthetic Force Crag mine water

To test removal of transition metals using the in-line configuration, glycerol (50 µM) and yeast extract (0.0025% w/v; both final concentrations) were added to sterilised synthetic Force Crag mine water and used as influent liquor for the HSB, operated in continuous-flow mode, at 30ºC and with no pH control. Samples were withdrawn from the effluent liquors at regular intervals and used to determine concentrations of glycerol, acetic acid, zinc and sulfate (section 5.2.2). Results are shown in Fig 6.17– 6.19 and Appendix 6E. For the off-line configuration, H_2S produced in the HSB was delivered to a vessel connected to the HSB, containing 500 mL of synthetic mine water which was not supplemented with glycerol and yeast extract. Samples were removed at regular intervals to determine concentrations of zinc, copper, and to measure pH.

Table 6.2. Chemical composition of the mine water from Level 1 discharge. Concentrations shown are mg L^{-1}.

	Coal Authority Report	Synthetic Mine Water	Mine water collected
Zn^{2+}	3	3	3
Cu^{2+}	4	4	<0.01
SO_4^{2-}	27	28	36
Ca^{2+}	11	11	ND
Cl^-	6	20	26
Mg^{2+}	4	4	ND
Na^+	3	4	ND
Mn^{2+}	0.6	0.6	<0.01
Fe^{2+}	0.3	NA	<0.01
DOC	ND	16-20*	1.0
Alkalinity**	21	12.5	21
pH	6.9	6.9	7.2

*Calculated from the combined glycerol and yeast extract added; **$CaCO_3$ equivalent; ND: not determined; NA: not applicable.

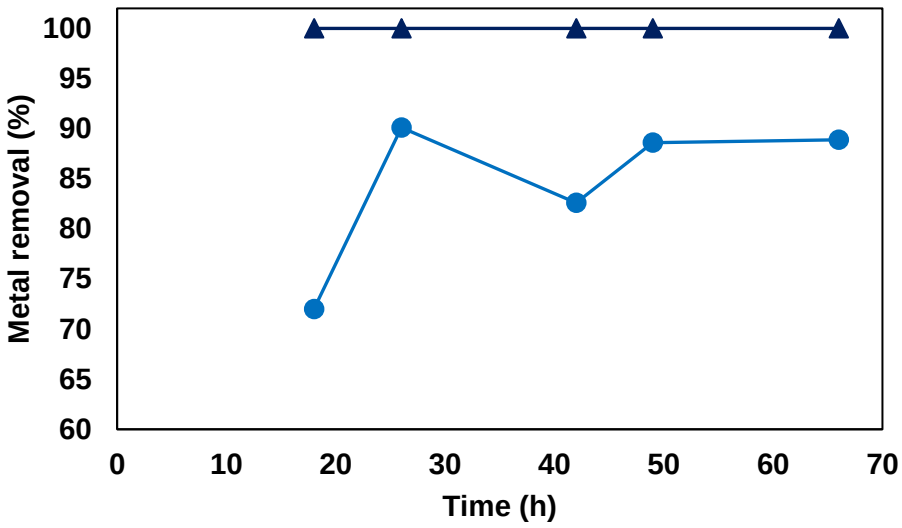

Figure 6.17. Percentages of Cu^{2+} (▲) and Zn^{2+} (●) removed from synthetic Force Crag mine water during in-line treatment.

More than 99% of soluble copper and 72 - 90% of soluble zinc was removed by in-line treatment of synthetic Force Crag mine water (Fig. 6.17). The bioreactor pH remained at 3.6 ± 0.1 throughout the test. Over 99% of glycerol in the influent liquor was consumed and acetic acid was produced in near 1:1 stoichiometric amounts to this (Fig. 6.18).

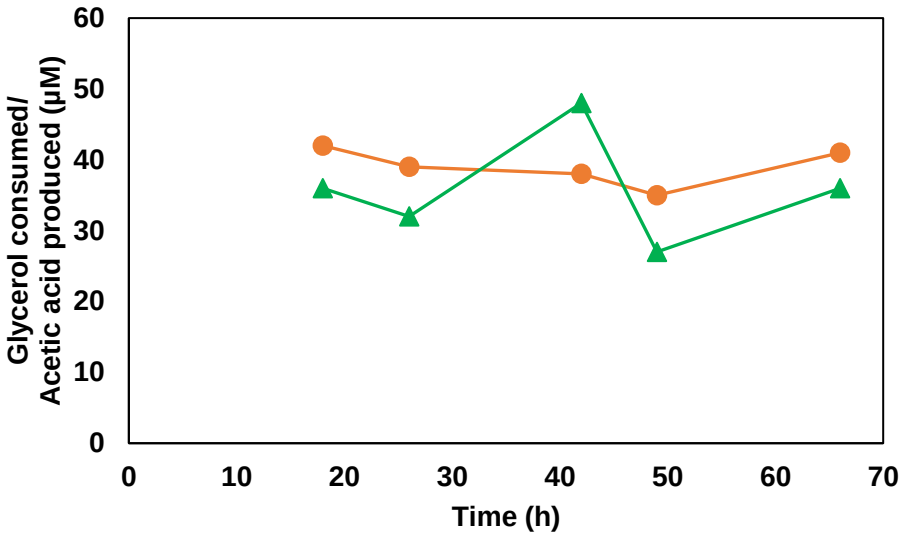

Figure 6.18. Consumption of glycerol (●) and acetic acid produced (▲) during in-line treatment of synthetic Force Crag mine water.

During this experiment, concentrations of sulfate in the effluent liquors were slightly higher than those measured in the influent water and therefore it was concluded that no net reduction of sulfate was occurring (Appendix 6E). In contrast, rates of hydrogen sulfide generation increased during the first 27 h of experiment but decreased progressively after that time (Fig. 6.19).

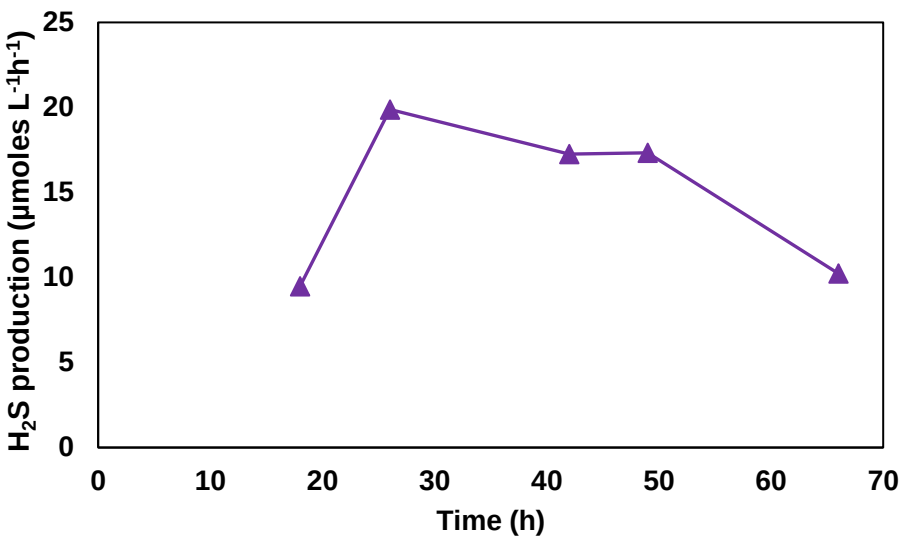

Figure 6.19. Changes in rates of hydrogen sulfide production during in-line treatment of synthetic Force Crag mine water.

In contrast to off-line treatment of synthetic Minsterley Brook Catchment waste water, which was not efficient in removing zinc in the short-term, >99% of Cu^{2+} was removed after 30 minutes and >99% of Zn^{2+} was removed after 16 h with the test using synthetic Force Crag mine water (Fig. 6.20). The pH of the synthetic mine water decreased from 6.9 to 3.7 as a consequence of metal sulfide precipitation.

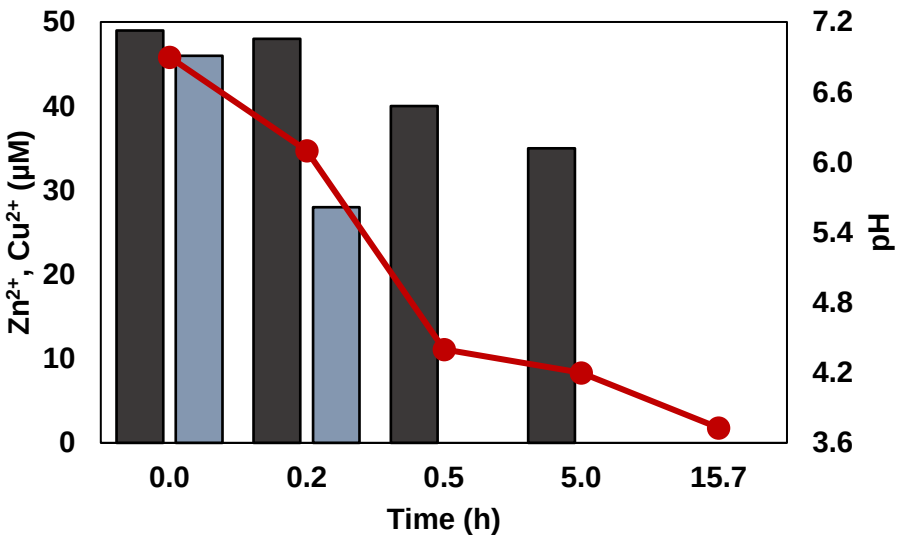

Figure 6.20. Off-line treatment of synthetic Force Crag mine water. Bars show changes in concentrations of Zn^{2+} (■) and Cu^{2+} (■), and the line graph depicts changes in pH.

6.2.3.2 Removal of zinc from Force Crag mine water

In-line treatment remediation of water discharged from the Force Crag mine used separate feeds for the mine water and organic substrates. The latter contained 20 mM (from 0 to 61 h) or 50 mM (from 63 to 78 h) glycerol, and 0.25% (w/v) yeast extract throughout the test period. Initially, mine water was pumped in at a continuous flow rate of 455 mL h^{-1}, the maximum rate supported by the FerMac pumps, while the organic feed was pumped in at 6 mL h^{-1}. Later, the flow rate of the mine water was increased stepwise up to 1520 mL h^{-1} by replacing the FerMac pumps with two independent peristaltic pumps (Ecoline VC-M/CA8-6, ISMATEC) connected to inflow and outflow liquors. The flow rate of the organic substrates also increased stepwise, up to 11 mL h^{-1}. Samples from effluent liquors were withdrawn at regular intervals to measure concentrations of glycerol, acetate, sulfate and zinc. Results of this experiment are shown below in Figures 6.21 – 6.23 and Appendix 6F.

Zinc was very effectively removed from Force Crag mine water by in-line flow through the HSB; 90% at the first two sampling points (at 19 and 21 h) and >99% after that. To test whether faster flow rates would still result in effective removal of zinc, these were increased progressively (up to 1520 mL h^{-1}, equivalent to a dilution rate of 0.69 h^{-1}) though this resulted initially in only 63 % zinc removal (Fig. 6.21). Lowering the flow rate (to 1180 mL h^{-1}) resulted in recovery of ~100% zinc removal, and this persisted when the flow rate was once again increased to 1520 mL h^{-1} (Fig. 6.2). This continued until all of the mine water that had been collected on site had been treated.

More acetic acid was produced than glycerol consumed in the HSB between 18 and 47 h of treatment, though this was reversed from 61 h until the end of the experiment (Fig 6.22).

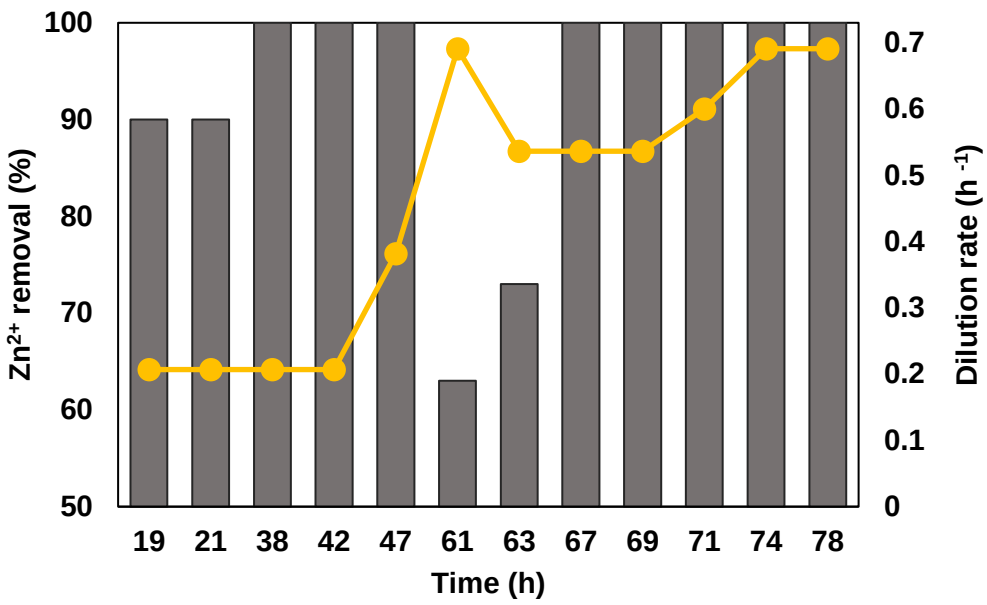

Figure 6.21. In-line treatment of Force Crag mine water. The bars represent percentage removal of Zn^{2+}, and the line graph depicts changes in dilution rate.

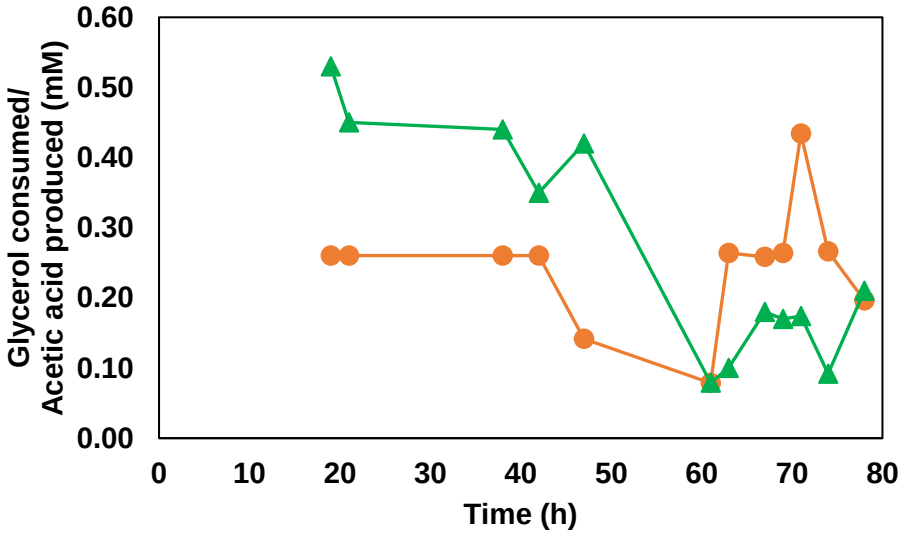

Figure 6.22. Consumption of glycerol (●) and acetic acid produced (▲) during in-line treatment of Force Crag mine water

As with the in-line experiment using synthetic mine water, concentrations of sulfate in the effluent liquors were slightly higher than values of the influent Force Crag mine water, and therefore there was no evidence of net sulfate reduction occurring in the HSB (Appendix 6F). However, hydrogen sulfide was produced in excess of that required to precipitate zinc in-line

up to 47 h of treatment (as evidenced by precipitation of copper as CuS in the connected off-line vessel), and rates of production showed an overall increase with time (Fig 6.23).

The bioreactor pH showed only minor changes (between 5.8 and 6.6) during in-line treatment of Force Crag mine water (Fig 6.23); these values were much higher than those recorded (pH 3.6 ± 0.1) during in-line treatment of the synthetic mine water.

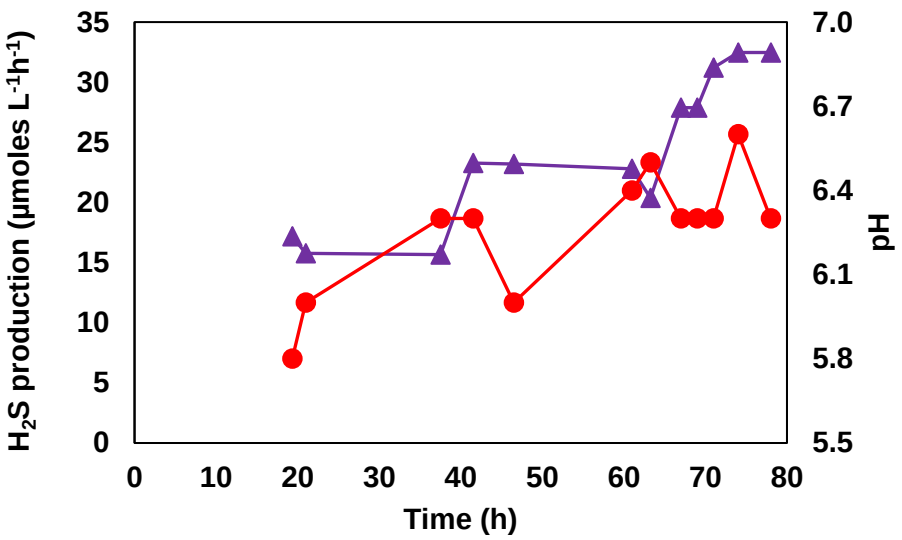

Figure 6.23. Changes in rates of hydrogen sulfide production (▲) and bioreactor pH (●) during in-line treatment of Force Crag mine water.

To test off-line removal of zinc from Force Crag discharge water, 500 mL of mine water was placed in a gas jar which was connected to the bioreactor and received the OFN/H_2S gas stream. Samples were removed at regular intervals to determine concentrations of zinc and to measure pH. Results are shown in the Fig 6.24.

Off-line removal of zinc from Force Crag discharge water was much more rapid than when using the synthetic equivalent, though the latter contained soluble copper in addition to zinc. Over 99% of soluble zinc was removed within 8 minutes of treatment, generating zinc-free effluents with pH 6.8 (Fig 6.21).

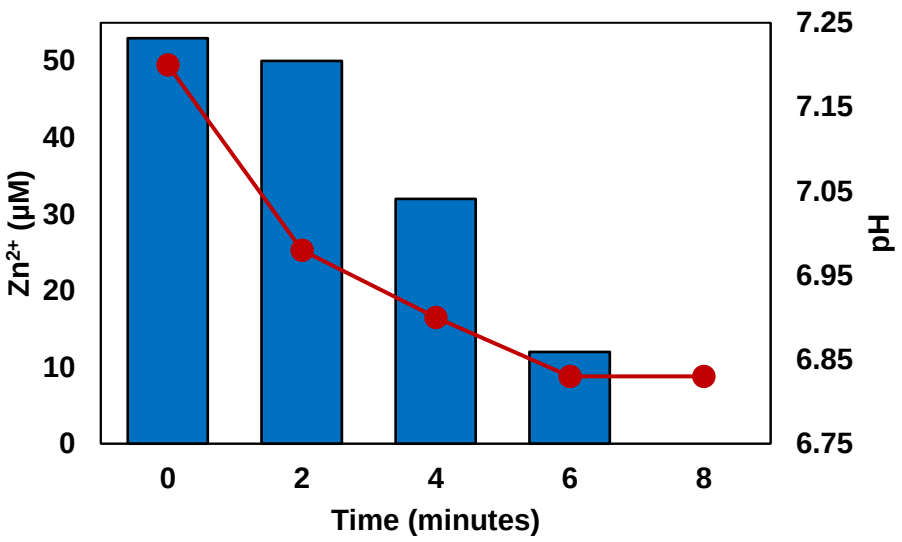

Figure 6.24. Off-line treatment of Force Crag mine water. Bars shows concentrations of Zn^{2+} and line graph depicts changes in pH.

6.2.3.3 Bacterial populations in the HSB during treatment of Force Crag mine waters

A sample (100 mL) from Level 1 discharge in Force Crag mine was filtered through a cellulose nitrate membrane filter (pore size, 0.2 µm). DNA was extracted from the membrane in the laboratory (section 2.6.1) followed by amplification of bacterial 16S rRNA genes and digestion with the restriction enzyme HaeIII. Semi-quantitative analysis was carried out using T-RFLP (section 2.6.3). The bacterial profile of Force Crag mine water (Fig. 6.25) showed that the population was less diverse than Minsterley Brook Catchment and that a relatively highly abundant (33%) T-RF of 220 nt length was present.

Analysis of bacterial communities showed that T-RFLP profiles of sulfur-attached cells remained similar (with some changes in the relative abundance of T-RFs) when the change was made from synthetic to actual mine water. In contrast, the T-RFLP profile of planktonic bacteria showed greater change as a consequence of treating the actual mine water, with known sulfidogens (mostly *D. acididurans*) accounting for only 4% of the total T-RFs. The *Firmicute* strain I2511 was detected in both sulfur-attached and planktonic bacterial populations in the synthetic mine water treatment, at 8% and 13% relative abundance, respectively. However, it was only detected in the sulfur-attached cells (13% relative abundance) following treatment of Force Crag mine water. *At. ferrooxidans* comprised 33% of the planktonic cells population following treatment of actual mine water, compared with 1% during the treatment of synthetic mine water. *Ac. aromatica* was abundant in the sulfur-

attached cells in both treatments (27% with synthetic mine water and 34% with actual). In the planktonic cells, *Ac. aromatica* was more abundant when treating synthetic mine water (38%) than when the actual mine water was tested (7%). The actinobacterium sp. IR1 was not detected by T-RFLP analysis in any samples analysed in this experiment.

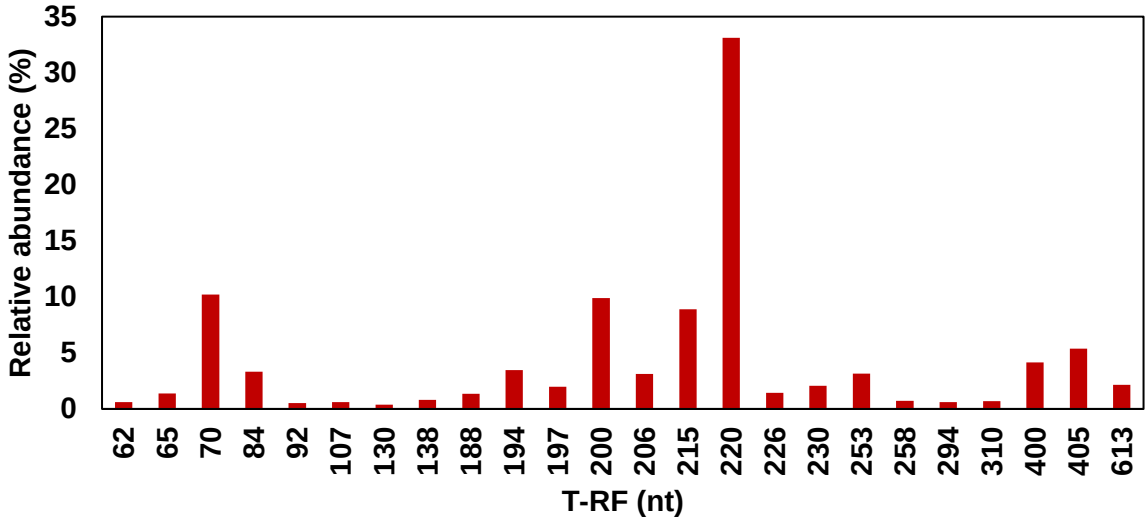

Figure 6.25. T-RFLP profile of bacterial 16S rRNA genes amplified from DNA extracted from Leve 1 discharge from the Force Crag mine, and digested with the restriction enzyme HaeIII.

When using synthetic Force Crag mine water, the unidentified 310 nt T-RF, previously detected during initial tests with the HSB when it was operated with sulfate-free media (section 5.3.4.2) accounted for 24% and 19% of the bacterial population in the sulfur-attached and planktonic cells, respectively. A novel unidentified T-RF (200 nt) appeared in profiles when treating Force Crag synthetic mine water. This T-RF increased in relative abundance paralleled to a decrease in relative abundance of the 310 nt T-RF in both sulfur-attached and planktonic cells (Fig.6.26)

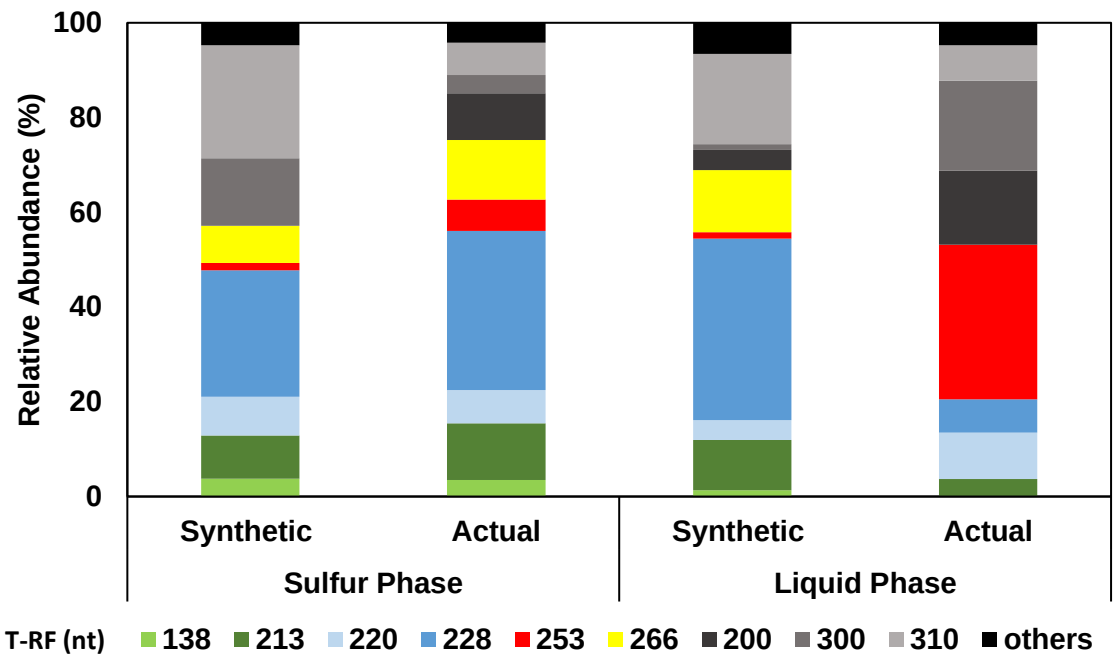

Figure 6.26. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracts and digested with the restriction enzyme HaeIII during in-line treatment of Force Crag mine waters. Key: (■) *Peptococcaceae* CEB3, (■) *Desulfosporosinus acididurans*^{T}, (■) *Clostridium* SBR1., (■) *Acidocella aromatica*^{T}, (■) *Acidithiobacillus ferrooxidans*, (■) *Firmicute* strain I2511, (■) unidentified T-RF, (■) unidentified T-RF, (■) unidentified T-RF, (■) low abundance unidentified T-RFs. "Sulfur phase" refers to DNA extracted from sulfur-attached bacterial communities, and "liquid phase" to DNA extracted from planktonic cells.

6.3 Discussion

Metal contaminated waters with contrasting chemical compositions, collected at two abandoned lead/zinc mines in the United Kingdom, were successfully bioremediated using the HSB. The two configurations used (in-line and off-line) to remove zinc were effective, generating circum-neutral pH liquors with concentrations of Zn^{2+} <0.01 mg L^{-1} (EQS = 0.25 mg L^{-1}). Using non-filtered waters from the two sites, and their synthetic equivalents, caused initial minor perturbations in the performance of the HSB. These perturbations were temporary, and the system functioned consistently well throughout the remainder of the test periods.

In-line treatment of both synthetic and actual Minsterley Brook Catchment waters was effective in removing zinc. When the synthetic waste water was used as influent liquor, the HSB achieved consistent removal of Zn^{2+} (>96%) in either configuration tested (i.e. liquors supplemented directly with glycerol/yeast extract or these supplied in a separate feed) with a

dilution rate of 0.21 h^{-1}. In both configurations, the bioreactor pH was much lower than that of the actual waste water, varying between pH 3.3 and 3.6. Liquors supplemented directly with organic materials displayed low variation in concentrations of glycerol oxidised and acetic acid produced. When organic materials were supplied in a separate feed, glycerol consumption and acetic acid production increased progressively during the experiment, possibly due to the higher concentration of glycerol supplied to the system in this set up.

When waste water collected from the Snailbeach Farm pond was used as an influent liquor, >99% of zinc was being removed in the continuous flow set up (D = 0.21 h^{-1}) by the first sampling time at 6 h, in contrast to the synthetic liquor where initially the zinc removal was 83 - 90%, and >96% zinc removal was not achieved until the system had been operated for 50 h, at the same flow rate. The high pH values (6.7 - 7.0) found when treating actual waste water was due to the greater alkalinity of the actual waste water than the synthetic version. Metal sulfide precipitation is a proton-generating reaction (e.g. $Zn^{2+} + H_2S \rightarrow ZnS + 2\ H^+$), and treating Minsterley Brook Catchment water generated ~2 mM of H^+ from the ~1 mM Zn^{2+} precipitated as a sulfide. The alkalinity of the actual waste water was 148 mg L^{-1} ($CaCO_3$ equivalent) which corresponds to 180 mg L^{-1} of HCO_3^- (for pH < 8.4, concentrations in $CaCO_3$ can be converted to HCO_3^- by multiplying this figure by 1.22, Xue *et al.,* 2017), This is equivalent to 3 mM HCO_3^-, and since each bicarbonate anion can neutralize one proton/hydronium ion ($HCO_3^- + H_3O^+ \rightarrow CO_2 + 2\ H_2O$) the alkalinity of the waste water was greater than that of the acidity generated by complete mineralization of zinc as ZnS. In contrast, the synthetic version of the waste water contained only 12.5 mg L^{-1} alkalinity ($CaCO_3$ equivalent), which corresponds to 15 mg L^{-1} (0.25 mM) HCO_3^-, which is far less than that of the mineral (Zn^{2+}) acidity of the synthetic waste water.

Comparing net sulfate reduction in Minsterley Brook Catchment in-line experiments is possible to infer that values were more variable during treatment of synthetic than of actual waste water, expressed by larger interquartile range and whiskers (Fig 6.27). During experiments using synthetic waste water, rates of net sulfate reduction were not significantly different, therefore not influenced by the method of supply of organic materials. However, these treatments had higher rates of net sulfate reduction than that actual waste water experiment. The bioreactor pH during actual waste water experiment (pH 6.7 - 7.0) was above the pH optima of the acidophilic sulfidogens strains in the HSB (e.g. Sánchez-Andrea *et al.,* 2015), which would have had a negative effect in net sulfate reduction rates.

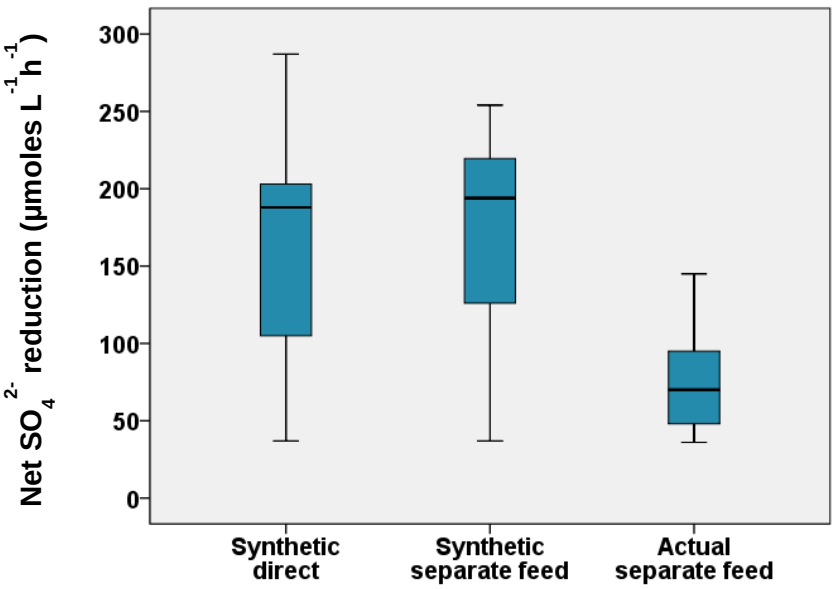

Figure 6.27. Box and whiskers plot of net sulfate reduction of Minsterley Brook Catchment waste water in-line treatments. “Synthetic direct” refers to synthetic waste water supplemented directly with glycerol and yeast extract; “Synthetic separate feed” refers to glycerol and yeast extract supplied as a separate feed, and “Actual separate feed” to waste water collected at Snailbeach Farm also with glycerol and yeast extract supplied as a separate feed.

Hydrogen sulfide generated during Minsterley Brook Catchment experiments varied considerably between tests (Fig 6.28). In the experiment with synthetic waste water, rates of H_2S production were greater when liquors were supplemented directly with glycerol and yeast extract than when a separate feed was used. The experiment with actual waste water showed the lowest rates of H_2S production. A possible explanation for these low rates is the relatively high pH values (6.7 – 7.0) of the bioreactor liquors during actual waste water experiment. As discussed in Chapter 5, at pH 6.9 (the pK_a of H_2S/HS^-), H_2S and HS^- occur in equimolar concentrations and, since HS^- is a water soluble anion rather than a gas, would be less readily transferred to the off-line vessel by OFN gas stream. During the experiment with actual waste water, no H_2S was detected in the off-line vessel (precipitation of CuS did not occur), therefore part of the H_2S generated in excess of that needed to precipitate zinc inside the bioreactor, was probably present in the effluent liquors that were continuously pumped out of the bioreactor. The relative amounts of H_2S produced *via* sulfate reduction (based on mass balance calculations) in experiments using either synthetic liquors or actual waste water were not significantly different (Appendix 6H - 6J).

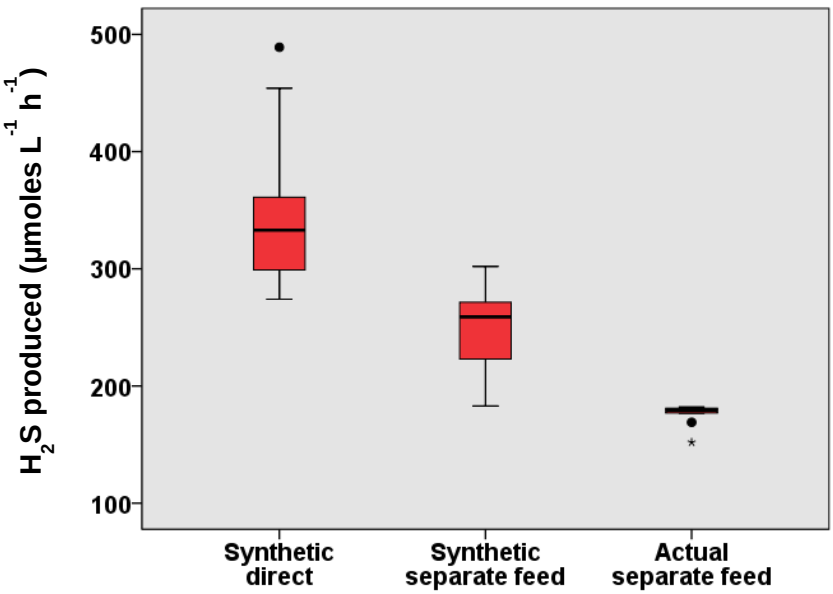

Figure 6.28. Box and whiskers plot of rates of hydrogen sulfide produced during in-line treatments of Minsterley Brook Catchment waste water. “Synthetic direct” refers to synthetic waste water supplemented directly with glycerol and yeast extract; “Synthetic separate feed” refers to glycerol and yeast extract supplied as a separate feed, and “Actual separate feed” to waste water collected at Snailbeach Farm also with glycerol and yeast extract supplied as a separate feed.

Off-line treatment of Minsterley Brook Catchment synthetic waste water was not very effective in the short-term (21% of the zinc was removed within 4 h, equivalent to ~58 µmoles h^{-1} of ZnS precipitation), although H_2S was produced by the HBS in excess of that required to precipitate all of the zinc, as evidenced by copper sulfide precipitation in the second off-line vessel. In contrast, off-line treatment of the actual waste water was far more effective, with >99% of zinc removed within 2 h, equivalent to 440 µmoles of ZnS precipitated h^{-1}. Again these contrasting results can be explained by the higher alkalinity of the actual waste water compared with the synthetic equivalent. Concentrations of S^{2-} become increasingly smaller as pH decreases (pK_a of HS^-/S^{2-} = 14.15), and precipitation of ZnS only occurs when the product of the S^{2-} and Zn^{2+} concentrations exceeds the solubility product (K_{sp} = 1 x 10^{-20} M^2 at 25 °C)

In-line treatment of synthetic Force Crag mine water was removing >99% of the soluble copper by the time the continuous flow system had been operated for 18 h with a dilution rate of 0.21 h^{-1}. However, removal of zinc was less effective, with 72% removal after 18 h of operation, though this increased to 90% from 49 h to the end of the experiment. While data indicated that there was no net reduction of sulfate, hydrogen sulfide was generated at increasing rates up

to 27 h, though this was followed by a progressive decrease. When using actual Force Crag mine water (in-line treatment), zinc removal was 90% from the outset, and increased to >99% after 38 h of continuous operation at a flow rate of 455 mL h^{-1} (D = 0.21 h^{-1}). Increasing the mine water flow rate to 1,520 mL h^{-1} at 61 h had a negative impact on zinc removal (63%), but this recovered (to >99%) when the flow rate decreased to 1,180 mL h^{-1}, and increasing the flow rate once more to 1,520 mL h^{-1} resulted in >99% zinc removal. The increase in the mine water flow rate from 47 h to 61 h, with no corresponding increase in the organic feed, resulted in concentrations of glycerol declining from 0.26 mM to 0.08 mM and yeast extract from 33 mg L^{-1} to 10 mg L^{-1}. However, at 63 h the concentrations were increased to 0.29 mM glycerol and to 15 mg L^{-1} yeast extract, consequently the HSB recovered effectiveness (>99% removal) at 67 h to the end of the experiment. Concentrations of acetic acid produced were higher than those of glycerol oxidised in the early stages of this experiment, though this reversed after 63 h. This could be explained by the relative amounts of organic carbon present in the glycerol and yeast extract provided at these times. From 0 to 61 h of the treatment, the organic feed contained 20 mM of glycerol (720 mg C L^{-1}) and 0.25% (w/v) yeast extract (1,000 mg C L^{-1}, assuming that yeast extract, like glucose, contains 40% C), i.e. there was more organic carbon provided as yeast extract and some of this complex organic material was probably used as electron donor as well as a carbon-source, and acetic acid was generated from it. Later in the experiment the glycerol concentration in the concentrated feed liquor was increased to 50 mM (equivalent to 1,800 mg C L^{-1}) while that of yeast extract remained the same as before, so glycerol was a relatively more abundant electron donor.

As with in-line treatment of the synthetic liquors, there was no evidence of net reduction of sulfate when using actual Force Crag mine water, though hydrogen sulfide was generated throughout the experiment. The concentrations of sulfate in Force Crag mine water was very low (0.3 - 0.4 mM), and the fact that hydrogen sulfide was generated implies that anoxic conditions required for sulfate reduction must have occurred within the bioreactor, albeit possibly in microsites. One possible explanation for the absence of net sulfate reduction is that some of the hydrogen sulfide generated from reduction of sulfate and ZVS was re-oxidized to sulfate, which would have been facilitated by the relatively high flow rates of non-deoxygenated waters.

As was the case Minsterley Brook Catchment waste waters, the bioreactor pH when treating Force Crag actual mine water was higher (pH 5.8 - 6.6) than was the case with the synthetic version (pH 3.6 ± 0.1). Again this can be credited to the greater alkalinity of the first (21 mg L^{-1} of $CaCO_3$, equivalent to 0.42 mM HCO_3^-) than the latter (12.5 mg L^{-1} of $CaCO_3$, equivalent to 0.25 mM HCO_3^-). Zinc sulfide precipitation would have generated ~ 0.1 mM of proton acidity

with the actual mine water (which contained ~0.05 mM Zn^{2+}) and this would have been neutralised by the mine water alkalinity. In the case of the synthetic version of this water, ~0.24 mM proton acidity would have been generated by the combined precipitation of ~0.12 mM Zn^{2+} + Cu^{2+} (as sulfides) which exceeds the alkalinity, hence the pH of the water declined during treatment.

Rates of hydrogen sulfide generation were significantly different with synthetic and actual Force Crag mine waters (Fig 6.29, Appendix 6G). A similar scenario to that described for Snailbeach Farm waste water regarding the greater relative concentration of water-soluble HS^- at the higher bioreactor pH would have applied for Force Crag mine water.

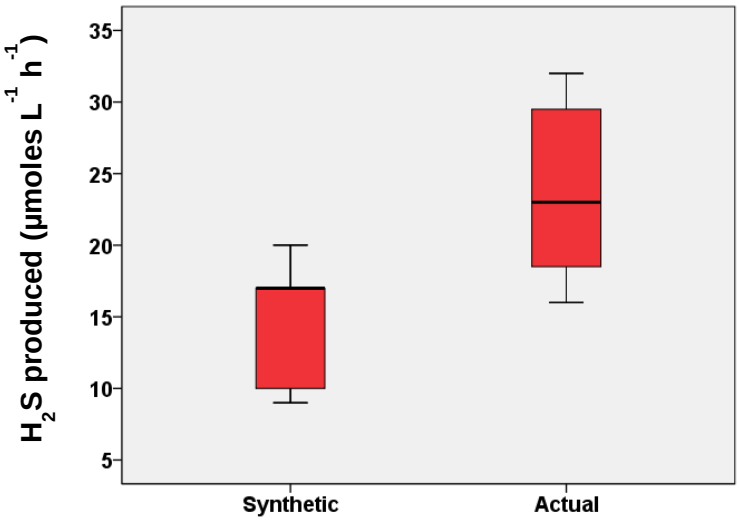

Figure 6.29. Box and whiskers plot of rates of H_2S produced during in-line treatments of Force Crag mine water. "Synthetic" refers to the synthetic equivalent of the mine water and "Actual" refers to the water collected from the mine discharge.

In the off-line treatment of synthetic Force Crag mine water, >99% of Zn^{2+} was precipitated within 16 h , while over 99% of the Cu^{2+} was removed after only 30 minutes. Since zinc and copper have different solubility products (at 25°C the K_{sp} of ZnS is 1 x 10^{-20} M^2 and that of CuS is 8 x 10^{-45} M^2, Stumm and Morgan, 1996) copper sulfide can precipitate in the presence of lower concentrations of S^{2-} (which vary with pH) than zinc sulfide. In contrast, off-line treatment of actual Force Crag mine water removed >99% of soluble zinc within 8 min. Comparing rates of metal sulfide precipitation for synthetic and actual Force Crag mine waters, 30 µmoles of metals had been precipitated within 5 h (23 µmoles of Cu^{2+} plus 7 µmoles Zn^{2+}), equivalent to a rate of 6 µmoles h^{-1} (synthetic) while 26.5 µmoles of Zn^{2+} were precipitated within 8 minutes

with actual mine water (204 µmoles h^{-1}). This compares to 58 µmoles h^{-1} in synthetic and 440 µmoles h^{-1} for actual Minsterley Brook Catchment waste waters.

Comparison of the T-RFLP profiles from Snailbeach Farm waste water and Force Crag mine water indicated that bacterial diversity was far greater in the former, and that a single T-RF (220 nt length, which could have corresponded to one or more bacterial species) accounted for 33% of the total relative abundance in the latter. The reason for this is probably because the pond at Snailbeach Farm received a variety of waste waters, including that from mine tailings while that from Force Crag was impacted solely by the abandoned mine workings. As would be anticipated, using unfiltered waters from both sites as influent liquors for the HSB resulted in changes to the bacterial T-RFLP profiles, especially those of planktonic populations. However, in both cases the HSB continued to function as a sulfidogenic bioreactor. The large increase (from 1% to 33%) in relative abundance of *At. ferrooxidans* in planktonic bacteria populations during in-line treatment of Force Crag mine water was probably a consequence of the much higher flow rates used, and hence greater concentrations of dissolved oxygen (DO). Since it would be anticipated that this chemolithotrophic acidophile was using this oxygen to oxidize ZVS and/or hydrogen sulfide, this would be a negative attribute.

The waters collected from Snailbeach Farm and Force Crag were similar in some respects (e.g. in being circum-neutral pH) but were different in others, most notably in that the Snailbeach Farm waste water had a greater metal (zinc) loading, and larger concentrations of sulfate and alkalinity. This was reflected in different rates of hydrogen sulfide generation by the HSB (Table 6.3).

Table 6.3. Summary of rates of hydrogen sulfide generation of in-line treatment if Minsterley Brook Catchment and Force Crag waters.

In-line treatment		pH[d]	H_2S production (µmoles L^{-1} h^{-1})
Minsterley Brook Catchment	Synthetic direct[a]	3.7 - 3.8	348 ± 76
	Synthetic separate feed[b]	3.5 - 3.8	248 ± 44
	Actual separate feed[c]	6.7 - 7.0	176 ± 9
Force Crag	Synthetic[a]	3.3 - 3.5	15 ± 5
	Actual[c]	5.8 - 6.6	24 ± 6

[a] Synthetic waste water supplemented directly with glycerol and yeast extract; [b] Synthetic waste water with organic material supplied as a separate feed; [c] actual mine water with glycerol and yeast extract supplied as a separate feed; [d]bioreactor pH.

With Snailbeach Farm waste water and Force Crag mine water, both in-line and off-line treatments were effective at removing zinc, and the pH values of the liquors produced were not greatly different from those of the waters treated. The choice of whether an in-line or off-line approach would be better for pilot-or full-scale treatment would need to take into account other factors, including:

- In-line treatment would require all of the water to flow through a bioreactor, which would require large energy inputs to maintain temperatures suitable for the HSB bacteria.
- No removal of sulfate is possible with off-line treatment. While this is not really relevant for Force Crag water, partial removal of sulfate from Minsterley Brook Catchment waters would be beneficial;
- In-line treatment is less complex, requiring only a single unit bioreactor and associated pumping equipment, while off-line treatment requires one or more additional vessels where metals would be removed from the contaminated waters;
- In off-line treatment, mine waters do not receive any input of organic materials, therefore there would be no potential for secondary contamination (with DOC);
- Metal precipitation rates during off-line treatment can be adjusted to be very rapid without impairing microbial communities, since this avoids washout of cells which might occur in high-throughput bioreactors;
- Off-line treatment produces pure metal sulfide precipitates, whereas in-line treatment produces a mixture of metal sulfides, residual ZVS and microbial biomass. The former would be more readily recovered and reprocessed;
- Flow gas costs would be greater for an off-line system to effect better transfer of H_2S from the HSB into the contactor vessel.

The Minsterley Brook Catchment water at Snailbeach Farm is highly contaminated waste due to it being inputted by of several sources of contamination. In-line treatment of might be a more suitable alternative than off-line configuration, since waters could be remediated not only in terms of zinc removal but also to remove some of the sulfate present. There would also be a potential benefit if some or all of the DOC in this water was used as electron donor or carbon source by bacteria in the HSB, though the DOC concentration was very small and would still require an additional extraneous supplement with, for example, glycerol. Recovery of zinc sulfide could generate revenue to off-set energy costs required to increase temperatures of the influent waters with both in-line and off-line treatments. Force Crag mine water not only has a lower Zn^{2+} concentration, but also low concentrations of other inorganic ions and DOC, and off-line treatment would be more suitable given the very large volumes of water that require treatment.

A full-scale passive system for mine discharge treatment was installed at the Force Crag site in 2014 (Fig. 6.30). The passive system is a downward-flow compost bioreactor, comprising two Vertical Flow Ponds (VFP) in parallel, based on microbial dissimilatory sulfate reduction to precipitate metals within the compost substrate (PAS100 and woodchips), possibly as sulfides (Jarvis *et al.,* 2015). Each VFP comprise a treatment substrate area of 760 m^2 and volume of 400m^3. Jarvis *et al.* (2015) reported that during the first 6 months of operation the passive system was a source of secondary contamination to the River Coledale Beck, generating effluents with elevated biochemical oxygen demands (BODs) and chemical oxygen demand (COD), and concentrations of ammonium and phosphate. After 6 months of operation, concentrations of these contaminants decreased but the constructed wetland was reported to still be a source of phosphate contamination to the river (Table 6.4). The passive system removed ~96% of zinc in the mine water, and sulfate concentrations decreased during treatment. However, zinc sulfide was not identified in the solid phase of the compost.

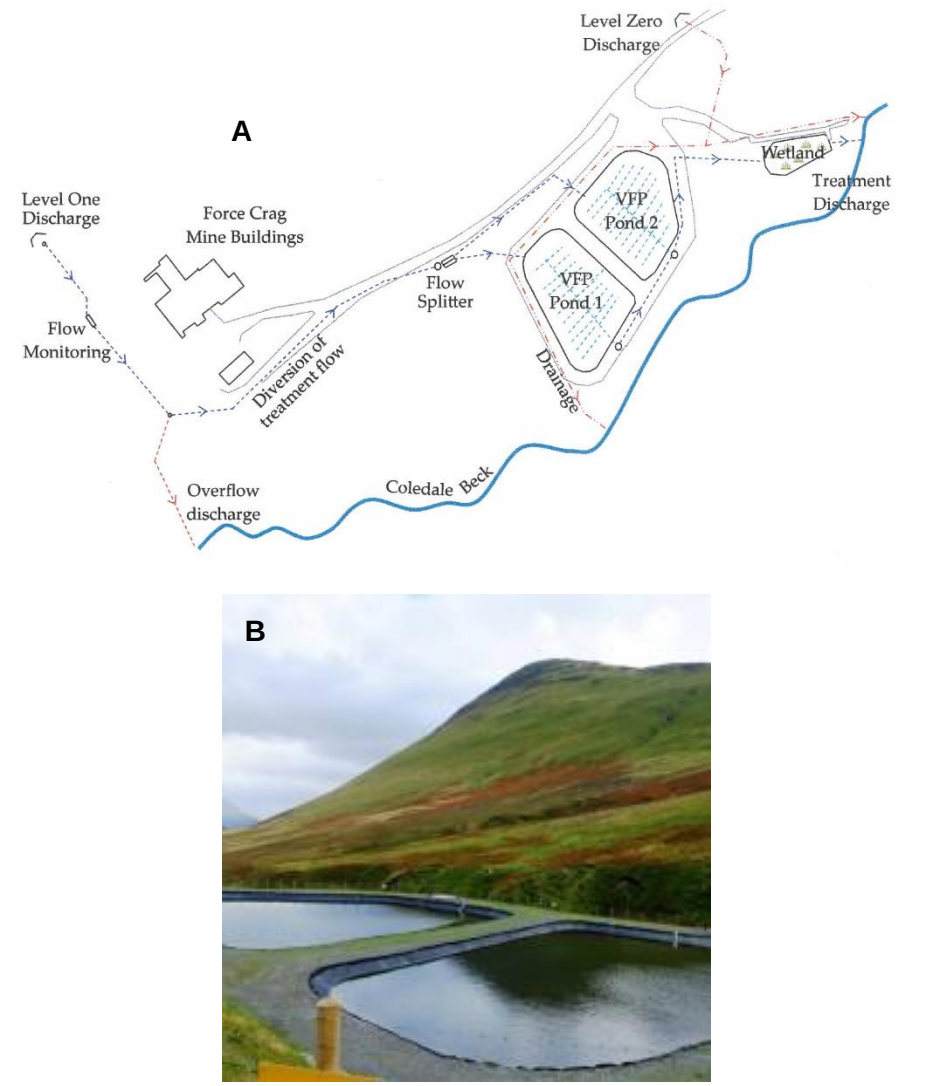

Figure 6.30. Schematic representation of the passive system (Jarvis *et al.,* 2015) (A) and a view of Vertical Flow Ponds (https://www2.groundstability.com/force-crag-mine-water-treatment-scheme) (B).

Results from the Force Crag mine experiments provided a good example of the major differences between using sulfidogenic bioreactors ("active biological treatment") and passive biological technologies, such as the constructed wetland in place in the mine. Comparing the two systems, off-line HSB treatment was superior in terms of zinc removal, no generation of secondary contamination, and in generating zinc sulfide precipitates that would be amenable for recovery as a potentially saleable product. Recovery of zinc sulfide is not possible in the passive system, since the solid matrix generated in the VFP (ZnS plus compost) requires dedicated disposal and long-term storage strategies.

Table 6.4. Performance of VFP System and HSB treatments for remediating Force Crag mine water.

	VFP System		HSB	
			In-line	**Off-line**
Time	10 days[a]	6 months[b]	NA	NA
BOD	100 mg/L	2 mg/L	(27 mg/L)[c]	(< 1.0 mg/L) [e]
NH_4	82mg/L	<1 mg/L	ND	(<0.01 mg/L)[e]
PO_4	28 mg/L	4.3mg/L	ND	(1.8 mg/L)[e]
HRT (h)	15-20		1.5 – 4.8	NA
Zn (%)[d]	90[f]	96	>99	>99
Composition of precipitates	Colloidal zinc and other precipitation phases		Zinc sulfide	
Destination of precipitates	ZnS+compost require adequate disposal/storage		ZnS recovery is applicable	

[a] First 10 days of operation; [b] After 6 months of operation; [c] Calculated as ~3x DOC (mg L^{-1}) (Quayle *et al.,* 2009), DOC was calculated as average of combined concentrations of glycerol and acetic acid in the effluent liquors; [d]% of Zn precipitated in the treatment; [e]concentrations in the mine discharge reported by Jarvis *et al.* (2015); [f]overall % of Zn precipitation during the first 6 months of operation; ND: not determined; NA: not applicable.

The operating costs (OPEX) of using a scaled-up HSB to remediate waters at the two sites can be estimated in terms of the three consumables (glycerol, yeast extract and sulfur) used. The costs of these commodities are shown in Table 6.5 (values quoted during February 2018).

Considering first the in-line treatment of Snailbeach Farm waste water. This contains ~1 mM (65 mg L^{-1}) zinc (65 g m^{-3}). Each cubic metre would require 1 mole of H_2S to completely remove the zinc. If this was generated exclusively from complete glycerol oxidation being coupled to the reduction of ZVS, this would require 14.3 moles (1.32 kg) of glycerol for each 100 m^3 of water treated (the stoichiometry being 1 glycerol:7 ZVS; Eq.5.2) which would cost ~ $0.63 (US). One hundred moles (3.2 kg) of ZVS would be consumed (at a cost of ~$2.66 (US)/100 m^3, making a combined cost of $3.29 (US). If yeast extract was added at a similar rate as in the current experiments (equivalent to about 300 g /100 m^3 this would add a further $0.90 (US) to the consumables cost, which then totals $4.19 (US)/100 m^3 waste water. If, on the other hand, all of the H_2S was generated from reduction of sulfate present (in excess of that required) in the waste water, then the glycerol costs increase (to $2.54 (US)/100 m^3 water, since the stoichiometry of glycerol:sulfate is 4:7; Eq.5.1) but there is no cost for ZVS

consumed. Assuming a similar amount of yeast extract was used as in the previous scenario, the cost of treating 100 m^3 of water is now slightly lower ($3.44 (US)). In the experiments carried out, the roughly similar amounts of H_2S were generated from reduction of sulfate and ZVS, and an average OPEX (consumable) cost of $3.81 (US)/100 m^3 water would therefore be more realistic. This figure assumes, however, that 100% of the principle electron donor (glycerol) is used to generate H_2S, which was clearly not the case. Assuming a more realistic figure of 50% efficiency, the estimated consumable cost would be adjusted to $7.63 (US)/100 m^3 water treated.

Table 6.5. Estimated costs and values of commodities involved in the proposed treatment protocols.

Commodities	Estimated costs $ (US) kg^{-1}
Glycerol	0.48[a]
Yeast extract	3.00[b]
ZVS	0.83[c]
Zinc metal	3.50[d]

[a]http://www.hbi.fr/; [b]Hunan Arshine Biotechnology Co. Limited, China; [c]Montana Sulphur Company, USA; [d] https://www.lme.com/Metals/Non-ferrous/Zinc#tabIndex=0

Since the same amount of H_2S would be required to precipitate zinc from Snailbeach Farm waste water off-line, consumable costs would be similar to in-line treatment. More pertinent here though would be an estimate of the size of a bioreactor required. Considering 1 m^3 of waste water in an off-line tank receiving H_2S-enriched gas from a 1 m^3 HSB and using the figure of 175 μmoles L^{-1} h^{-1} of H_2S generated in the laboratory tests with Snailbeach Farm waste water, 5.7 h would be required to achieve complete precipitation of soluble zinc. By increasing the production of H_2S 10-fold in the HSB (e.g. by operating at lower pH and using higher glycerol and yeast extract concentrations) the contact time required would be lowered to 34 minutes.

Force Crag mine water contains ~0.05 mM zinc (3.25 g m^{-3}), and each cubic metre would require 0.05 mole of H_2S to completely remove the zinc. Considering complete glycerol oxidation being coupled solely to the reduction of ZVS (since there was no evidence of net reduction of sulfate during Force Crag experiments), this would require 0.7 moles (0.06 kg) of glycerol for each 100 m^3 of water treated (stoichiometry of 1 glycerol:7 ZVS; Eq.5.2) which would cost ~ $0.03 (US). Five moles (0.16 kg) of ZVS would be consumed (at a cost of ~$0.13

(US)/100 m^3). The yeast extract required (equivalent to about 14 g/100 m^3) would add a further $0.04 (US), which then totals $0.20 (US) for each 100 m^3 of treated mine water. Assuming that the efficiency to generate H_2S, in terms of glycerol utilisation, would be possibly ~50%, the estimated consumable costs would need to be adjusted to $0.40 (US) for each 100 m^3 of water treated.

To estimate the time required to a 1 m^3 HSB remediate 1 m^3 of Force Crag mine water using off-line treatment, the figure of 24 µmoles L^{-1} h^{-1} of H_2S (that generated in laboratory tests) was used. This would require 2 h of treatment to achieve complete precipitation of soluble zinc. Again this time could be lowered to 12.5 min by 10-fold increase in rates of H_2S generation, for instance, if glycerol and yeast extract concentrations were increased and the HSB was operated at a lower pH.

In order to estimate the revenue generated from zinc recovery, the zinc metal commodity price of $3.5 (US) kg^{-1} (Table 6.5) was considered. Soluble zinc concentration in the waters collected at Snailbeach Farm was 65 mg L^{-1} and Force Crag 3 mg L^{-1}, which would be equivalent to a recovery of 6.5 kg and 0.3 kg of ZnS (from 100 m^3 of each), respectively. Therefore, the estimated revenue produced from the treatment of 100 m^3 of water would be $22.75 (US) for Snailbeach Farm and $1.05 (US) for Force Crag. The first would cover the estimated operation costs of treatment for Snailbeach Farm waters with an excess of $15.12 (US), while the latter would exceed the $0.65 (US) estimated consumable OPEX for Force Crag mine water treatment. Since OPEX was calculated only in terms of glycerol, yeast extract and sulfur consumption, other significant costs of operating a pilot-or full-scale HSB, such as heating, flow gas and equipment maintenance, also need to be considered. Nonetheless, these figures strongly suggest that the HSB system (both in-line and off-line configurations) could be an effective alternative approach for remediating both of these circum-neutral pH waters.

Chapter 7. Generation of hydrogen sulfide in a non-inoculated low pH reactor.

7.1 Introduction

In hydrometallurgy, precipitation of metals as sulfides and hydroxides are processes widely applied in the treatment of effluents (Lewis, 2010). Sulfide precipitation has several advantages compared to hydroxide precipitation, e.g. potential for selective metal removal, rapid reactions rates resulting in low hydraulic retention times (HRT) and lower solubility of metal sufide precipitates. Therefore, processes can achieve high degrees of metal removal over a broad pH range, superior settling properties; also, recycling of metal sulfides by smelting is possible (Fu and Wang, 2011; Lewis, 2010; Kim and Amodeo, 1983). Metal sulfide precipitation can be achieved in several ways by providing sources of sulfide (S^{2-}) as solid (CaS), aqueous (Na_2S, $(NH_4)_2S$, NaHS) or gaseous (H_2S) forms. In addition, other sources of sulfide, such as thiourea, thioacetamide, polyphenylene sulfide and carbon disulfide can be used to precipitate metal sulfides nanocrystals (Lewis, 2010).

There have been a number of reports describing the use of biogenic H_2S to precipitate transition metals in metal-impacted waters, most of them involving microbial sulfate reduction, although other sulfur compounds (e.g. zero-valent sulfur; ZVS) have also used in biological treatments to generate H_2S (Chapters 5 and 6). Using biogenic H_2S to recover metals from very low grade solutions is particularly advantageous, as other technologies (e.g. chemical precipitation, solvent extraction-electrowinning (SX-EW), adsorption and membrane filtration) are often not economic or effective in these situations. Even with higher grade solutions, abiotic approaches may not be cost-effective if they rely on expensive chemicals and/or more complex methodologies.

Although there are several approaches that can be used to generate sulfide biologically for metal precipitation, there are no reports describing abiotic systems using reduction of ZVS. Jameson *et al.* (2010) described a novel low pH bioreactor in which *Acidithiobacillus ferrooxidans* was used to generate H_2S using ZVS as electron acceptor and H_2 as electron donor (generated by acid dissolution of zero-valent iron; ZVI) when operated under anaerobic conditions and maintained at pH 2.5 and 30ºC. This report also described results from a non-inoculated control reactor operated under similar conditions (pH 2.5 - 4.0, and 30 ºC) which also, unexpectedly, generated H_2S, but at lower rates than the inoculated reactor. Results from the non-inoculated reactor suggested that ZVS reacted chemically with ZVI under the imposed acidic conditions, producing H_2S. This reaction possibly represents an alternative to generate sulfide in a cost-effective way that avoids the limitations of using microorganisms.

This chapter describes experiments which follow up the report of Jameson *et al.* (2010) in which the possibility of generating H_2S abiotically in an ambient temperature, low pH reactor containing both ZVS and ZVI was investigated.

7.2 Operation of the sulfidogenic reactor

Six experiments were set up to evaluate the generation of H_2S *via* chemical reduction of ZVS in acidic media. A reactor (FerMac 310/60 unit, Electrolab Ltd., UK), fitted with a 2.3 L glass vessel and pH and temperature control, was commissioned for these experiments (Fig. 7.1). Tests were carried out in batch mode and involved preparing a non-sterile suspension of 100 – 150 g of ZVS, adjusted to pH 1.5 – 2.2 with sulfuric acid. Two different ZVS suspensions were prepared: (a) microbially pre-wetted ZVS, and (b) “biosulfur”, described in section 2.2.3. The reactor pH was controlled by automated addition of 1 M sulfuric acid. Before the start of each experiment, ZVI (sterilized at 160 ºC for 4 h, when specified) was added to the reactor vessel. Three different forms of sources of ZVI were used: iron powder (97% pure, 0.04 mm; Sigma-Aldrich); iron granules (99.98% pure, 1 - 2 mm; Alfa Aesar) and iron filings (1.2 – 1.7 mm, general purpose grade; Fisher Scientific). The reactor was operated under anaerobic conditions by gassing with a continuous stream of oxygen-free nitrogen (OFN ~100 mL min^{-1}) which was also used to deliver H_2S generated inside the reactor to an off-line glass vessel that contained 400 mL of $CuSO_4$ (0.02 to 0.3 M).

Samples from the reactor and off-line vessel were withdrawn at regular intervals to measure concentrations of ferrous iron in the reactor vessel using the Ferrozine assay (section 2.3.1.2) and copper concentrations (colorimetrically; section 2.3.1.3) in the off-line vessel. The volumes of sulfuric acid required to maintain the pH of the reactor were also recorded.

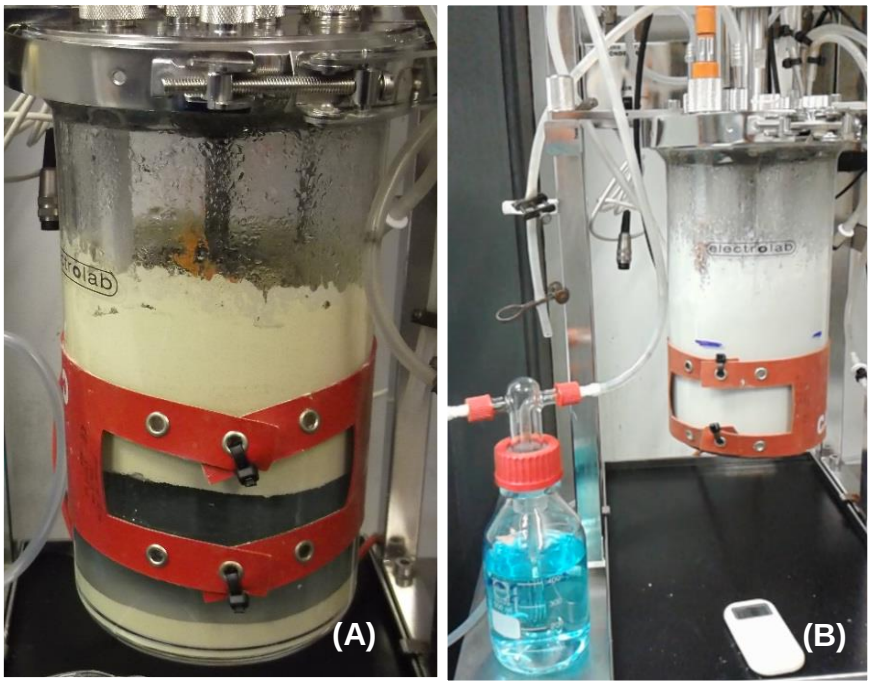

Figure 7.1. (A) The reactor vessel (in experiment 3) with a suspension of pre-wetted ZVS and iron powder; (B) the reactor vessel (in experiment 5) containing a suspension of biosulfur and iron filings.

Calculations: Data from experiments are listed in Appendix 7A – 7E. Stoichiometric calculations were made from the raw analytical data to obtain:

(i) *Rates of H_3O^+ consumption:*

The reaction of ZVS with ZVI to generate H_2S is a proton consuming reaction, therefore a 1 M solution of H_2SO_4 was used to maintain the reactor pH at a set value. Therefore, to calculate the amount (mmoles L^{-1}) of hydronium ion (H_3O^+) added into the system, sulfate and bisulfate speciation needed to be taken into account. Sulfuric acid has two dissociation constants (Equations 7.1 and 7.2):

$$H_2SO_4 + H_2O \rightarrow HSO_4^- + H_3O^+ \qquad (pK_a = -3.0) \qquad \text{Eq.7.1}$$

$$HSO_4^- + H_2O \rightarrow SO_4^{2-} + H_3O^+ \qquad (pK_a = 1.9) \qquad \text{Eq.7.2}$$

The measured pH of 1 M sulfuric acid is ~0.16, which means that most of the sulfate is present as bisulfate, with equimolar concentrations of hydronium ions. One millilitre of 1 M sulfuric acid therefore contains ~ 1 mmole of hydronium ions. When this is added to a reactor maintained at pH 1.5, some of the bisulfate will dissociate (Eq.7.2), generating additional hydronium ions. At pH 1.5, soluble sulfate is present 73% as HSO_4^- and 27% as SO_4^{2-}. This means that the total

amount of hydronium ions added increases to 1.27 mmoles. Since more of the added bisulfate dissociates in higher pH liquids, the equivalent amount of hydronium ions added to the reactor maintained at pH 1.7 and 2.2 (per mL of 1 M sulfuric acid) are 1.37 mmoles and 1.65 mmoles, respectively (Table 7.1).

Table 7.1. Amounts of H_3O^+ added to the sulfidogenic reactor (per mL of 1 M) H_2SO_4 to maintain the reactor at different pH values.

Expt.	Reactor pH	HSO_4^- (%)	SO_4^{2-} (%)	ΣH_3O^+ (mmoles)
1 / 2	1.5	73	27	1.27
3	1.7	63	37	1.37
4 / 5	2.2	35	65	1.65

Rates of H_3O^+ consumption were calculated from the volume of acid added to the reactor and data from Table 7.1, and were expressed as mmoles L^{-1} h^{-1}.

(i) *Rates of H_2S production*. These were calculated by measuring changes in concentrations of soluble copper in the off-line vessel, and assumed that there was no leakage of H_2S gas from the system. The amount of copper precipitated (mmoles) was then calculated by multiplying this number by the volume of copper sulfate solution present in the off-line vessel (400 mL). Since the stoichiometry H_2S to CuS precipitated is 1:1, these data corresponded to H_2S production. Rates of H_2S production were calculated by dividing amount of copper precipitated in µmoles by the time period concerned and by the working volume of the reactor, and expressed as µmoles L^{-1} h^{-1}.

(ii) *Rates of Fe^{2+} generation:* These were calculated from determining changes in soluble Fe^{2+} (µmoles) in the reactor vessel (from data at the start and end of each experiment) divided by the length of time of the experiment and by the working volume of the reactor vessel, and expressed as µmoles L^{-1} h^{-1}.

(ii) *Efficiency of H_2S generation (%)*: To assess efficiency in H_2S production, it was calculated the relative amount of ZVI used to generate H_2S, which corresponded to the amount of Fe^{2+} (mmoles) generated against H_2S produced (mmoles).The value obtained was then multiplied by 100.

7.2.1 Experiment 1

In experiment 1, the reactor was operated with a working volume of 2 L, at pH 1.5 and 25°C, and was stirred continuously at 25 rpm. At the start of the experiment, 5 g of non-sterile iron granules were added to a suspension of 200 g of microbially pre-wetted ZVS in 2 L solution of RO-grade water pH 1.5. At 141 h after the start of the experiment, a further 5 g of non-sterile iron powder was added to the reactor.

Figure 7.2 shows the consumption of H_3O^+ and H_2S production during experiment 1. Consumption of H_3O^+ increased continuously during the first 141 h of the experiment, but no H_2S was produced. Following the addition of 5 g of ZVI powder at this time, there was a large increase in the rate of H_3O^+ consumption and H_2S was generated.

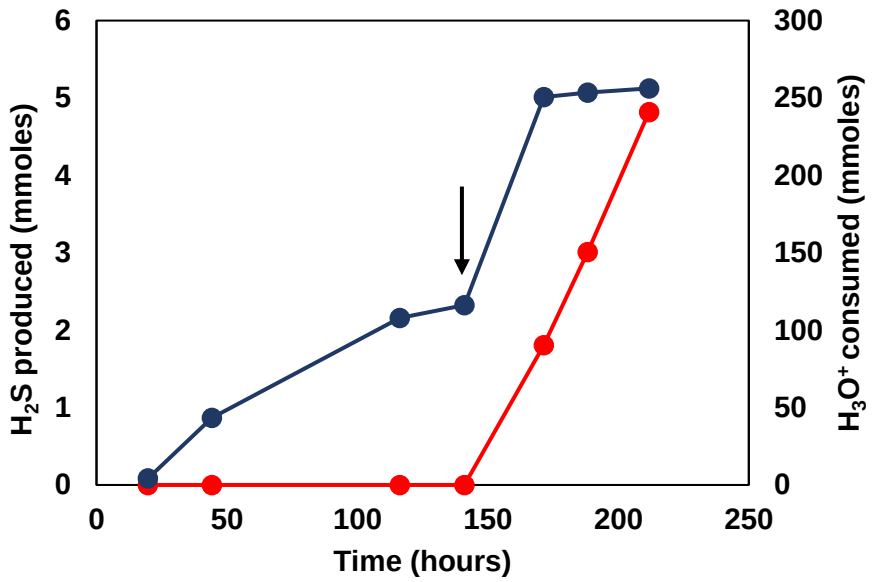

Figure 7.2. Cumulative consumption of H_3O^+ (●) and production of H_2S (●) during experiment 1. The arrow indicates the point at which a further 5 g of ZVI (as iron powder) was added to the reactor.

7.2.2 Experiment 2

The second experiment used similar operating conditions to experiment 1 (pH and temperature fixed at 1.5 and 25°C, respectively) and the reactor was stirred continuously at 25 rpm, though this time, 2 g of non-sterile iron powder was added at the start of the experiment. The reactor also contained 100 g of microbially pre-wetted ZVS, and the volume of the reactor was made up to 1 L with RO-grade water.

Consumption of H_3O^+ proceeded at a relatively rapid rate for the first 22 hours of this experiment, but slowed down thereafter. Small amounts of H_2S were generated from time 0 to 22 h, which was followed by a sudden sharp increase (between 22 and 24 h) after which there was little further production of H_2S (Figure 7.3).

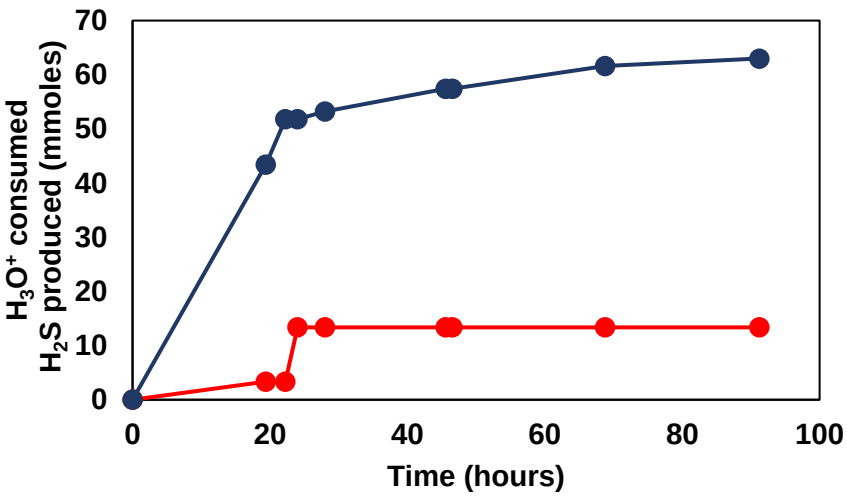

Figure 7.3. Cumulative consumption of H_3O^+ (●) and production of H_2S (●) during experiment 2.

7.2.3 Experiment 3

Experiment 3 utilised the residual ZVS suspension from experiment 2, and the reactor was operated as in experiment 2, except that the pH was maintained at 1.7. Non-sterile iron powder (2 g) was added to the reactor the start of the experiment.

No H_2S was generated in experiment 3. Acid consumption was intense during the first 6 h of this experiment, but slowed down considerably thereafter. Changes in concentrations of ferrous iron paralleled that of acid consumption (Figure 7.4). At the end of the experiment, 98 mmoles of hydronium ion acidity was calculated to have been consumed, and 52 mmoles of ferrous iron generated (from the ZVI).

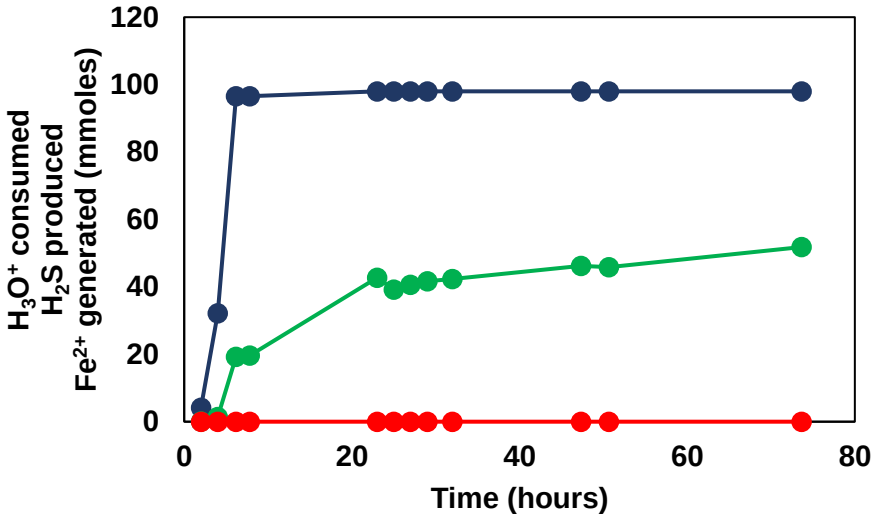

Figure 7.4. Cumulative consumption of H_3O^+ (●), production of H_2S (●) and generation of Fe^{2+} (●) during experiment 3.

7.2.4 Experiment 4

Operating conditions were changed for experiment 4. The reactor pH was increased to 2.2, and the temperature increased incrementally (25°C for 0 – 212 h; 35°C for 212 - 284 h; 45°C for 284 – 358 h). The working volume was again 1 L, and 100 g of microbially pre-wetted ZVS and 20 g of sterile iron filings were added at the start of the experiment. The regime used for sampling was different in this case to that used in experiments 1 – 3. Before samples were withdrawn, pH control and the OFN stream were both turned off and the stirring speed was increased to 600 rpm for 5 minutes. Following this, the agitation speed was reduced to 25 rpm

and the OFN stream increased to ~400 mL min^{-1} for another 5 minutes. Following this, pH control reinstated (the pH increased to ~2.8 during high-speed agitation).

During experiment 4, a black precipitate was noted to form in the bottom of the reactor vessel at between 25h and 28 h of the experiment, and the modified approach used (rapid agitation followed by intense gasification) at this time caused H_2S to be produced for the first time in this experiment. The black precipitate appeared to continue to form after 28 h and H_2S was produced far more intensively (as evidenced by visual formation of CuS in the off-line vessel) during the sampling times than when the reactor was in "rest" mode. In experiment 4, acid consumption and generation of Fe^{2+} increased progressively, with the former increasing much more rapidly after the temperature was incrementally increased (Figure 7.5). Hydrogen sulfide production also increased with time but was less influenced by higher temperatures.

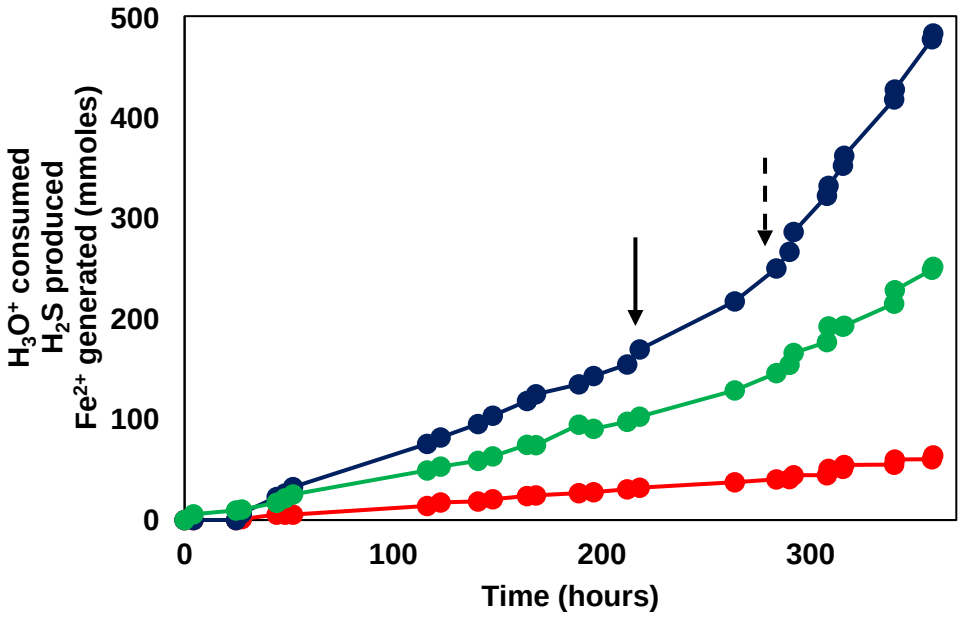

Figure 7.5. Cumulative consumption of H_3O^+ (●), production of H_2S (●) and generation of Fe^{2+} (●) during experiment 4. Solid arrow indicates increase of temperature to 35°C and the broken arrow increase to 45°C.

7.2.5 Experiment 5

The operating conditions used in experiment 5 were similar to those in experiment 4: the 1 L (working volume) reactor was maintained at pH 2.2, with continuous stirring at 25 rpm, and 20 g of sterile iron filings were added at the start of the experiment. However, 150 g of biosulfur suspension (rather than 100 g of microbially-wetted ZVS) was used in experiment 5. The temperature was set at 25°C from 0 -162 h, and increased first to 35°C (162 – 211 h) and finally to 45°C (211 - 267 h).

During experiment 5, samples from the reactor and off-line vessel were withdrawn immediately before and after using the same approach (suspending pH control, high speed stirring, followed by intensive gassing) as that described for experiment 4. Results from samples taken before performing the protocol displayed marginal changes in acid consumption and generation of Fe^{2+} and H_2S (Figure 7.6). Acid consumption and generation of Fe^{2+} increased more rapidly after the temperature was incrementally increased but H_2S production was less affected by increased temperatures.

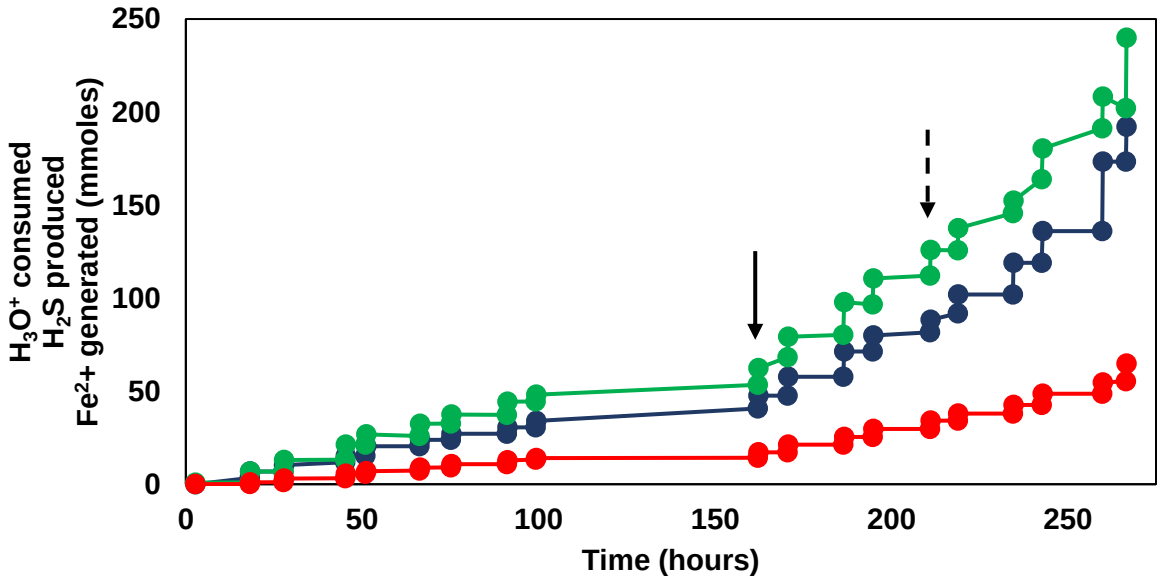

Figure 7.6. Cumulative consumption of H_3O^+ (●), production of H_2S (●) and generation of Fe^{2+} (●) during experiment 5. The solid arrow indicates increase of temperature to 35°C and the broken arrow when it was increased to 45°C.

Figure 7.7 illustrates the rapid rates of H_2S production during one of the times when the reactor (in experiment 5) was intensively gassed with OFN and pH control reinstated. At 219 h, the off-line vessel contained 48 mM Cu^{2+}, and after intensive gassing for ~10 mins this had decreased to 38 mM. This means that 4 mmoles of CuS had precipitated in the off-line vessel,

which is equivalent to 24 mmoles H_2S produced L^{-1} h^{-1} in the reactor. The maximum rates of H_2S generated biologically in this study (chapters 5 and 6) were ~0.4 mmoles L^{-1} h^{-1}. However, the latter were obtained during continuous production whereas the high rates obtained abiotically were only obtained during relatively short periods for a reactor operated as a stop/go system.

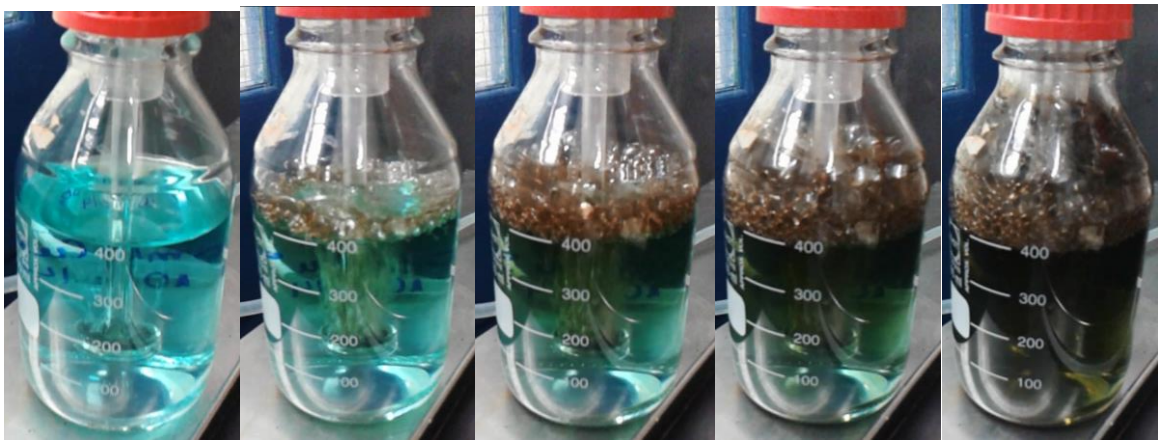

Figure 7.7. Precipitation of copper sulfide in the off-line vessel in experiment 5. Images were captured during intensive gassing with OFN. The sequence of images represent a time-frame of 11 seconds from the start of high intensity gassing of the reactor.

7.2.6 Experiment 6

The aim of experiment 6 was to assess whether H_2S could be produced in acidic media at low temperature from the chemical reduction of ZVS by hydrogen gas.

A suspension of microbially pre-wetted ZVS (100 g) was prepared by adding 1 L of RO-grade H_2O and used in the reactor, which was operated at pH 2.7 and 25°C, stirred continuously at 25 rpm. In contrast to previous experiments (1 – 5), no ZVI was added to the ZVS suspension prior to the start of this experiment, instead, H_2 was gassed continuously into the system (~150 mL min^{-1} of 10% H_2, 5% CO_2 and 85% N_2; BOC Group Ltd, UK).

After 96 h of continuous gassing of H_2, there was no precipitation of CuS in the off-line vessel, therefore H_2S production did not occur, indicating that ZVS was not chemically reduced by H_2.

Figures 7.8 to 7.10 compare data from experiments 1 to 5. Generation of hydrogen sulfide varied considerably between experiments, and was much greater in experiments 4 and 5 (Fig. 7.8). Net acid consumption in the five experiments was more similar than H_2S production (Fig. 7.9), and Figure 7.10 shows that dissolution of ZVI was also similar in experiments 3 - 5.

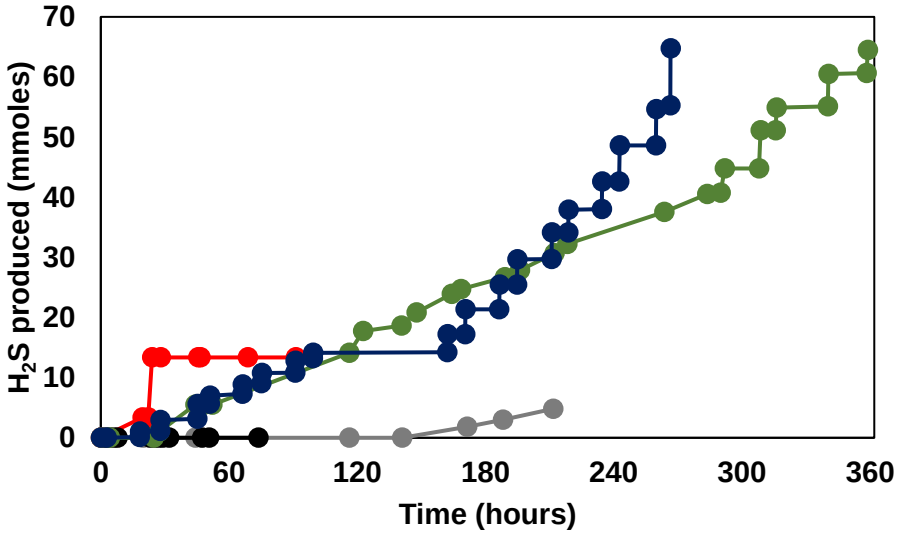

Figure 7.8. Production of H_2S in experiment 1 (●), experiment 2 (●), experiment 3 (●), experiment 4 (●) and experiment 5 (●).

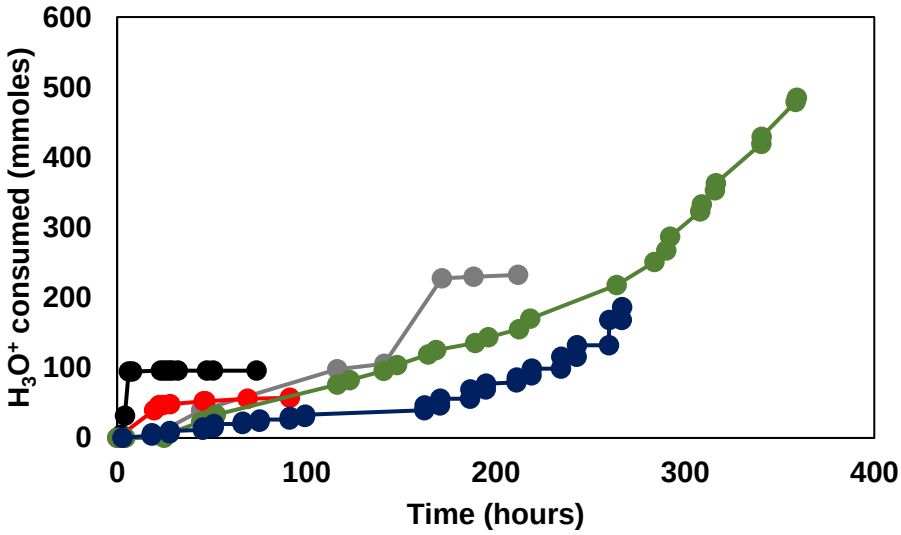

Figure 7.9. Consumption of H_3O^+ in experiment 1 (●), experiment 2 (●), experiment 3 (●), experiment 4 (●) and experiment 5 (●).

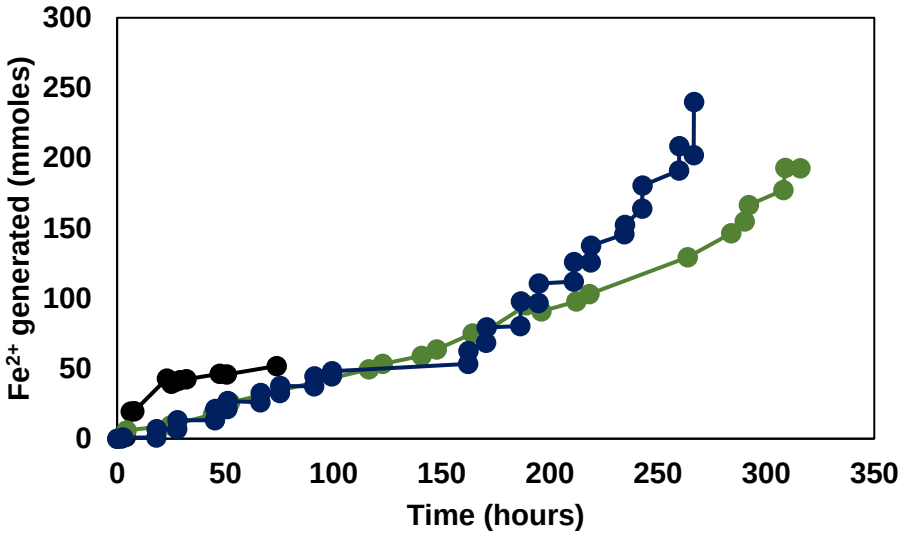

Figure 7.10. Generation of Fe^{2+} in experiment 3 (●), experiment 4 (●) and experiment 5 (●).

The effect of temperature on the generation of H_2S was examined in experiments 4 and 5. To compare rates of H_2S production in these experiments at temperatures of 25°C, 35°C and 45°C, average rates were calculated by dividing the total amount by the time over which it was generated and the working volume of the reactor (V= 1 L; Appendix 7D – 7E). Figure 7.11 shows that higher temperatures increased rates of H_2S generation, though in experiment 4 increasing the temperature from 25º to 35°C did not induce an increase in the overall rate. Generation of H_2S in experiments 4 and 5 was marginal when the reactor was in "rest" mode (more evident in experiment 5, Figure 7.8); acid-catalyzed dissolution of putative FeS was not a continuous reaction, being promoted mostly by the rapid agitation and intensive gassing applied in the modified sampling approach. Consequently, the frequency at which rapid agitation and gassing was carried out appeared to affect rates of H_2S production.

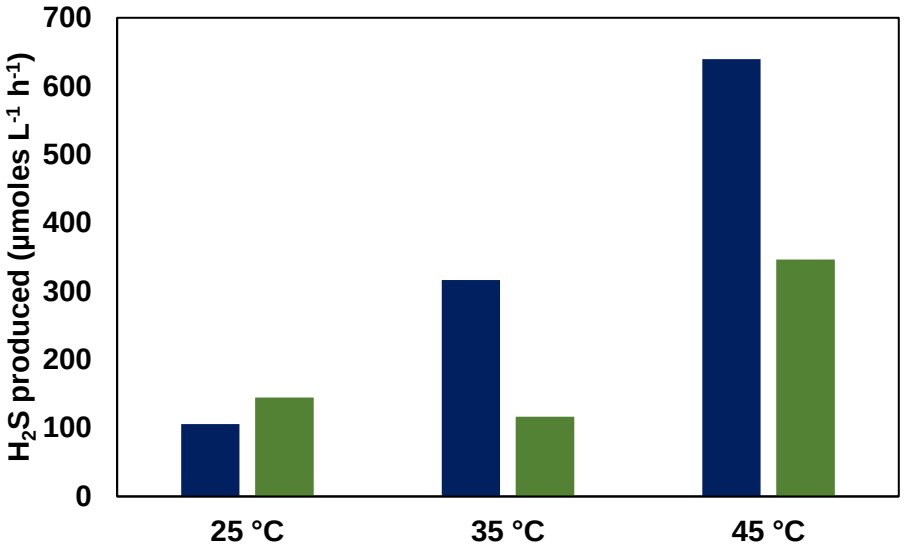

Figure 7.11. Effect of temperature in the rates of H_2S generation in experiment 4 (■) and experiment 5 (■).

7.3 Discussion

Experiments carried out with the sulfidogenic reactor confirmed the feasibility of generating H_2S from the chemical reaction between ZVS and ZVI in acidic liquids. Four reactions were considered to potentially take place within the system (Equations 7.3 – 7.6, showing Gibbs free-energy values at 25°C).

$Fe^0 + 2\ H_3O^+ \rightarrow Fe^{2+} + H_2 + 2\ H_2O$	ΔG^0 = +0.8 kJ mol $^{-1}$ (pH 7.0) ΔG^0 = -56 kJ mol $^{-1}$ (pH 2.0)	Eq.7.3
$Fe^0 + S^0 \rightarrow FeS$	ΔG^0 = -79 kJ mol $^{-1}$	Eq.7.4
$FeS + 2\ H_3O^+ \rightarrow H_2S + Fe^{2+} + 2\ H_2O$	ΔG^0 = +52 kJ mol $^{-1}$ (pH 7.0) ΔG^0 = -5 kJ mol $^{-1}$ (pH 2.0)	Eq.7.5
$S^0 + H_2 \rightarrow H_2S$	ΔG^0 = -28 kJ mol $^{-1}$	Eq.7.6

As shown in the reaction data above, pH has a major influence in determining the thermodynamic viability of two of the Equations 7.3 and 7.5 considered to be important in the current context; negative ΔG^0 values at pH 2.0 indicate that the reactions would occur spontaneously while positive ΔG^0 values at pH 7.0 suggest the opposite. Gibbs free energy values can also be modified by rapid removal of one or more or the products of a reaction, such as occurred in the experiments described where H_2S was removed from the reactor vessel in the stream of OFN and delivered to the off-line vessel (Eq.7.5).

Even in cases where ΔG^0 values are negative, rates of reactions may be so slow as to be barely detectable. This is because large activation energy barriers can have major impacts on reaction rates. However, activation energy barriers can also be modified, e.g. by increasing reaction temperatures or in the presence of catalysts, including microbial enzymes. This is illustrated by Equation 7.6, which was not detected in the abiotic experiment set up, but which is known to be catalysed by some species of *Acidithiobacillus* under near-identical conditions (e.g. Hedrich and Johnson, 2013).

The series of experiments carried out gave very different results in terms of H_2S production. Although the sulfidogenic reactor was generally considered to be an abiotic system (no microbial inocula were used, and reagents, liquids and equipment were sterilised before use), experiments were not carried out under sterile conditions. In experiment 1, acid was consumed during the first 141 hours but no H_2S was produced, implying that only Equation 7.1 was occurring (acid dissolution of metallic iron granules). When additional iron was added as a powder, H_2S was generated, confirming that the form of ZVI used was critical to generating FeS (the perceived precursor of H_2S) at low temperatures. More H_2S was produced during experiment 2 (~13 mmoles, most within a relatively short time frame) than in experiment 1 (~ 5 mmoles) even though less ZVI was added in the former (2 g of iron powder) than in the latter (5 g of iron granules and 5 g of powder). In experiment 3, the reactor pH was increased from pH 1.5 to 1.7 to assess if, at higher pH, H_2S would be produced more consistently than in previous experiments, while at the same time reducing acid-catalyzed dissolution of ZVI. The same amount and type of ZVI used in experiment 2 (2 g of iron powder) was used in experiment 3, but no H_2S was produced. One possible reason for this was that the residual ZVS suspension from the previous experiment was used in experiment 3.

Taken together, the results from the first three experiments suggested that very low pH (1.5 – 1.7) and the use of ZVI powder were not very suitable for H_2S production, which occurred only intermittently, and more of the ZVI was dissolved by acid that was used to generate FeS. The design of experiments 4 and 5 was different in that: (i) a different sampling regime was used; (ii) the reactor pH was increased further; (iii) iron filings replaced iron powder; (iv) the amount of ZVI used was increased 10-fold; (v) biosulfur was used (experiment 5 only). In both of these experiments, black material (putative FeS) accumulated in the reactor vessel and H_2S was generated more effectively (Fig. 7.8).

Support for the hypothesis that FeS acts as an intermediate in H_2S production at low temperatures

A combination of Equations 7.4 and 7.5 could lead to the abiotic generation of hydrogen sulfide. However, even though Equation 7.4 has a large negative ΔG^0 value, it also has a large activation energy barrier, and conventionally requires temperatures of >200ºC to occur spontaneously (Waldner and Pelton, 2004). However, black material was found to accumulate in the bioreactor when operated at much lower temperatures (25°C) in experiments 4 and 5, which was considered likely to be iron monosulfide, and this dissolved in acid when the bioreactor was subsequently agitated strongly, generating H_2S (Eq.7.5). This was similar to the observations reported by Jameson *et al.,* (2010) in an experiment carried out under similar conditions, and implies that the activation energy barrier which conventionally restricts Equation 7.4 at low temperatures was lowered under the conditions used in the abiotic reactor vessel.

MacDonald *et al.* (1978), and Fang *et al.* (2008 and 2011) reported ZVS as an important corrosion agent of mild steel at 25°C. They set up experiments in systems containing ZVS, water and mild steel at 25°C to evaluate corrosion under anaerobic conditions. The main findings of MacDonald *et al.* (1978) were: (a) corrosion products were H_2S and mackinawite ($Fe_{1+x}S$); (b) direct contact of ZVS and steel was a necessary condition to occur corrosion; (c) pH increased with time as reaction progress; (d) ZVS particle size influenced on the reaction, whereby the smaller the particle the shorter the time to initiate the corrosion process; (e) corrosion was autocatalytic, catalysed by FeS; (f) the reaction occurred by direct electron transfer from Fe to ZVS; (g) corrosion rates were higher at pH 5-6, values below and above exhibited much lower rates due to formation of passive films. It was concluded that the sulfur species $S_{y-1}.S^{2-}$ (or polythionates formed due to the reaction of ZVS with water) were the oxidant agents in the cathodic reaction.

$$S_{y-1}.S^{2-} + 2H^+ + 2(x - 1)e^- \rightarrow xH_2S + S_{y-x} \qquad \text{(cathodic reaction)}$$

$$\left.\begin{array}{l} (x - 1)\ Fe^0 \rightarrow (x - 1)Fe^{2+} + 2(x - 1)e^- \\ (x - 1)Fe^{2+} + (x - 1)H_2S \rightarrow (x - 1)FeS + 2(x - 1)H^+ \end{array}\right\} \qquad \text{(anodic reaction)}$$

Fang *et al.* (2008 and 2011) and Fang (2012) reported FeS film formation (with pitting corrosion underneath) in the area of direct contact of ZVS and a steel coupon surface. When the system was subjected to a solution of 0.175 M NaCl, a FeS film was formed also in the surrounding area outside of the contact point of ZVS and steel. When a conductive medium (a perforated carbon sheet) was placed between the ZVS and a steel surface, a FeS film was

formed over the whole area comprising the carbon sheet, but was thickest underneath the holes. In the absence of water, ZVS and steel in direct contact at 125°C for 6 h, no corrosion was detected. Regarding the formation of a FeS film at the contact points of ZVS and steel, use of water at different pH (4.0, 5.0 and 7.0) did not affect the corrosion rate. Direct electron transfer from iron to sulfur was defined as the main mechanism of ZVS corrosion.

These findings suggests that, in aqueous systems, ZVS and ZVI are likely to react in an oxido-reduction reaction. MacDonald *et al.* (1978) suggested that an ionic sulfur specie ($S_{y-1}.S^{2-}$) rather than neutral ZVS reacted with ZVI. Fang *et al.* (2008 and 2011) used molten ZVS (heated at 115 °C) in their experiments, which possibly allowed some increase in reactivity of sulfur. The microbially pre-wetted ZVS and biosulfur used in the sulfidogenic reactor were hydrophilic, possibly due to the presence organic polymers (e.g. polyhydroxy fatty acids, phosphatidylinositol, ornithine derivatives) secreted by bacteria (Steudel and Holdt, 1988). These organic polymers possess non-polar and polar regions (i.e. they are amphiphile compounds) where in the case of phosphatidylinositol the polar region comprises a phosphate group substituted with an inositol polar head group. Attachment of these compounds to ZVS could 'activate' it in some way and thereby increase the reactivity of the sulfur. In addition, at low pH, ZVI undergoes acid dissolution, exposing fresh ("nascent") surfaces would also be more reactive than more aged surface layers. Both of these factors could contribute to lowering the activation energy barrier of the reactions between ZVS and ZVI, allowing the reaction to occur at relatively low temperatures (25 - 45ºC) as observed in the present study and in the report by Jameson *et al.* (2010).

Efficiency, rates and ratios of reactions in the experiments carried out

The ratios of total H_2S formed:H_3O^+ consumed, and of total Fe^{2+} generated:H_3O^+ consumed in the experiments are shown in Table 7.2, which also lists overall rates of H_3O^+ consumption, and generation of Fe^{2+} and H_2S. These were calculated from data at the start and end of each test, and do not show changes within the experiments. Ratios of Fe^{2+}:H_3O^+ in experiments 3 and 4 (Table 7.2) were in accord with combined Equations 7.3 and 7.5, while experiment 5 exhibited variability, as the amount of Fe^{2+} released was greater than the amount of acid consumed. Conversely, ratios of H_2S formed:H_3O^+ consumed were very different in the experiments, and was always lower values than that corresponding only to Equation 7.5 (ratio of 0.5), as a consequence of acid-catalysed dissolution of ZVI, which is an undesirable reaction. On this basis, the efficiencies of H_2S production (i.e. the relative amount of ZVI used to indirectly generate H_2S) was evaluated in the experiments 4 and 5 and were 26 - 27% in both cases.

Table 7.2. Rates, ratios and efficiency calculated for experiments carried out with the sulfidogenic reactor.

Exp	$H_3O^{+\,a}$ µmoles L^{-1} h^{-1}	$Fe^{2+\,b}$ µmoles L^{-1} h^{-1}	H_2S^{c} µmoles L^{-1} h^{-1}	$Fe^{2+}:H_3O^{+\,d}$	$H_2S:H_3O^{+\,e}$	E (%) H_2S production[f]
1	548	ND	11	NA	0.02	NA
2	626	ND	147	NA	0.24	NA
3	1297	703	0	0.54	NA	NA
4	1351	702	180	0.52	0.13	26
5	699	899	243	1.29	0.35	27

[a] rates of H_3O^+ consumed; [b] rates of Fe^{2+} generated; [c] rates of H_2S produced; [d] ratio of Fe^{2+} generated to H_3O^+ consumed; [e] ratio of H_2S produced to H_3O^+ consumed; [f] efficiency of H_2S production in terms of Fe^{2+} generated; ND: not determined; NA: not applicable.

Jameson *et al.* (2010) described the generation of H_2S in two low pH systems, both containing ZVS and ZVI: (a) a bioreactor inoculated with *Acidithiobacillus ferrooxidans,* and (b) a control reactor (non-inoculated). Selected data from their report are shown in Figures 7.12 and 7.13. In the control reactor (non-inoculated, Fig. 7.12), ~40% of ZVI was used for H_2S production (~200 mmoles of Cu^{2+} precipitated and ~500 mmoles of Fe^{2+} generated), therefore ~60% of ZVI was subject to acid-catalyzed dissolution. In the bioreactor inoculated with *At. ferrooxidans*, only ~30% of ZVI was used to generate H_2S (~300 mmoles of Cu^{2+-} precipitated and 1000 mmoles of Fe^{2+} generated). The net efficiency of both of those systems was greater than that found in the present study (26 – 27%), possibly because the amount of ZVI used in the experiments of Jameson *et al.* (2010) was over 10-fold greater (200 g) than the amount used in the present study (2 - 20 g).

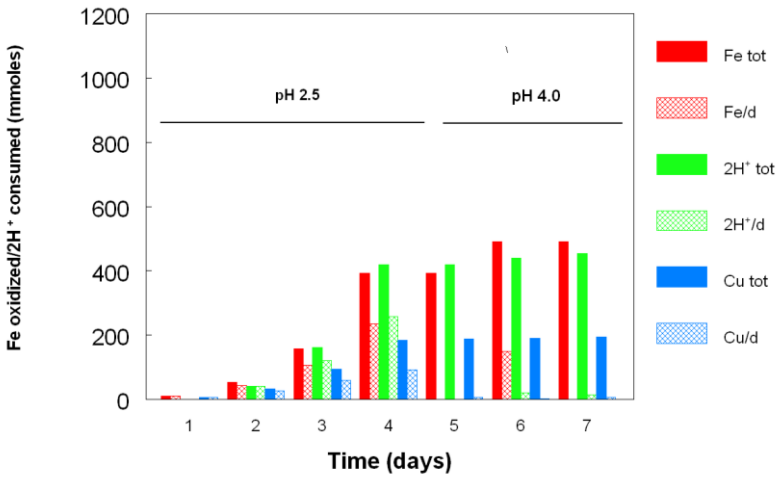

Figure 7.12. Data from the report of Jameson *et al.* (2010) of H_2S generation by a non-inoculated low pH reactor containing ZVS and ZVI. "Fe tot" refers to total Fe^{2+} concentrations, "Fe/d" refers to Fe^{2+} generated per day, "Cu tot" refers to total Cu^{2+} precipitated, "Cu/d" refers to Cu^{2+} precipitated per day, "$2H^+$ tot" refers to $2H^+$ consumed, "$2H^+$/d" refers to $2H^+$ consumed per day.

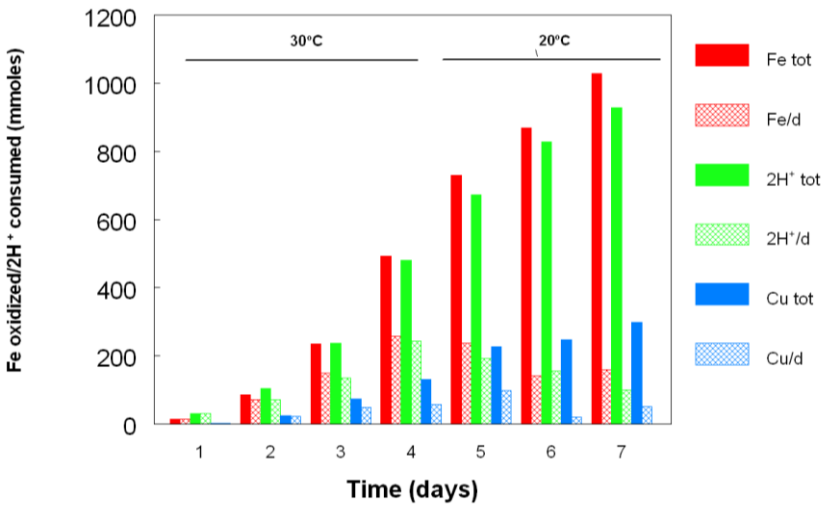

Figure 7.13. Data from the report of Jameson *et al.* (2010) of H_2S generation by a low pH reactor containing ZVS and ZVI. inoculated with *Acidithiobacillus ferrooxidans*. "Fe tot" refers to total Fe^{2+} concentrations, "Fe/d" refers to Fe^{2+} generated per day, "Cu tot" refers to total Cu^{2+} precipitated, "Cu/d" refers to Cu^{2+} precipitated per day, "$2H^+$ tot" refers to $2H^+$ consumed, "$2H^+$/d" refers to $2H^+$ consumed per day.

Conditions identified as being most favourable for H_2S production by the sulfidogenic reactor in the present study were 45°C, pH 2.2, biosulfur as ZVS source and ZVI provided as iron filings, and using the modified sampling regime for intense intermittent production of in order to generate H_2S. While results confirmed the feasibility of generating H_2S by the chemical

reaction of ZVS with ZVI at low pH, some important aspects need to be addressed in future work:

- optimising the modified sampling regime in terms of length and frequency. In order to increase system efficiency, the effect of varying the times for the generation of H_2S need to be evaluated. Additionally, experiments should be performed to define the minimum time required for the reaction between ZVS and ZVI to produce FeS, therefore to determine the optimum frequency for the sampling regime.
- assessing the efficiency and cost effectiveness of alternative ZVI sources from recycled material (e.g. scrap metal from factory waste)
- defining the optimum parameters (e.g. reactor pH, temperature, ZVS and ZVI stoichiometries) in order to minimise ZVI dissolution and decrease consumption of acid, while maintaining adequate rates of H_2S production. Since experiments on corrosion by ZVS that were reported by MacDonald *et al.* (1978) and Fang *et al.* (2008 and 2011) were carried out at circum-neutral pH, it would be interesting to assess H_2S generation over a similar pH range.

Estimative of operating costs for abiotic generation of H_2S

Operation costs (OPEX) involved in scaling-up the reactor for the generation of H_2S can be estimated in terms of the three consumables (ZVI, ZVS and sulfuric acid) used. The costs of these commodities are shown in Table 7.3 (values quoted during May 2018). It should be noted that these figures are based on 100% efficiency of ZVI being used indirectly to generate H_2S, which was not achieved in the current work or by that described by Jameson *et al.* (2010).

To generate 100 moles of H_2S at pH 2.2 requires 100 moles of ZVI, 100 moles of ZVS and 121 moles of H_2SO_4 (Eq.7.4 - 7.5 and Table 7.1), which is equivalent to 5.6 kg of ZVI, 3.2 kg of ZVS and 11.9 kg of H_2SO_4.These have the following costs: $0.39 (US) of ZVI, $0.26 (US) of ZVS and $1.9 (US) of H_2SO_4, making a combined cost of $2.55 (US). Taking into account that the average efficiency of the system from the current experiments is ~30%, the estimated cost of consumables would increase to $8.50 (US) per 100 moles of H_2S generated. From 100 moles of H_2S produced, 100 moles of $FeSO_4$ was also produced as by-product, which is a potentially saleable product (commodity price quoted at ~ $70 – 190/t; May 2018), therefore it can offset operation costs. One hundred moles of $FeSO_4$ (15.2 kg) could generate a revenue of $2.90 (US) per 100 moles of H_2S generated, giving a final estimated OPEX of $5.60 (US) per 100 moles of H_2S produced. It is important to underline that other significant costs of

operating a pilot-or full-scale reactor, such as heating, flow gas and equipment maintenance, also need to be considered in future work.

Table 7.3. Estimated values of commodities involved in the proposed system.

Commodities	Estimated value $ (US) kg^{-1}
ZVI	0.07[a]
ZVS	0.08[b]
Sulfuric acid	0.16[c]
Ferrous sulfate	0.19[d]

[a] www.metalbulletin.com; [b]Buoyancy Impex Private Ltd., India; [c] Shijiazhuang Xinlongwei Chemical Co., China; [d] Guangxi Hezhou Yongfeng Mineral Feed Co., Ltd..

An alternative way of generating H_2S is by acid dissolution of sodium sulfide (Eq.7.7):

$$Na_2S + 2\ H_3O^+ + SO_4^{2-} \rightarrow H_2S + Na_2SO_4 + 2\ H_2O \qquad \text{Eq.7.7}$$

The cost of sodium sulfide (60% pure) quoted on the commodity markets (May 2018) is ~ $500 (US)/tonne, which is equivalent to about $6.5/100 moles Na_2S. The cost of sulfuric acid to generate 100 moles H_2S by Equation 7.7 is $1.57, giving a total reagent cost of $8.07 (i.e. $0.43 less than the ZVI/ZVS route). In addition the other product of reaction 7.7 (sodium sulfate) is a highly soluble salt with a similar commodity value (for the anhydrous salt) to ferrous sulfate (typically about $100/tonne; May 2018).

If a larger capacity (1 m^3) abiotic sulfidogenic reactor, operating at the same rate of H_2S production, is considered, it is possible to calculate the approximate time that would be required to remediate 1 m^3 of metal-impacted waste water from the Force Crag mine (Chapter 6). Using the highest rate of H_2S produced in all experiments carried out, 639 μmoles L^{-1} h^{-1} (experiment 5), and 50 mmoles of Zn^{2+} in each m^3 of Force Crag mine water, to decrease the concentration of soluble zinc to below detection limits (< 0.01 mg L^{-1}) would require a 5 min treatment.

Results from this study suggest that the system developed to generate H_2S chemically could be both a pragmatic and cost-effective alternative, in situations that require high rates of metal removal and highly controlled systems. Additionally, the sulfidogenic reactor is simpler to set up and operate than other chemical methods (some of which utilise hazardous substances) when applied for metal sulfide precipitation.

Chapter 8. General discussion and suggestions for future research

Extremely acidophilic *Firmicutes* are ubiquitous in both natural and man-made acidic environments. Although many novel strains have been isolated in the last 30 years (e.g. Norris *et al*., 1996; Johnson *et al*., 2008; Goto *et al*., 2007; Lopez *et al.,* 2018), this phylum has, up to now, included only two validated genera of extreme acidophiles: *Sulfobacillus* and *Alicyclobacillus*. The research reported in this thesis examined the characteristics of several novel isolates of both moderately and extremely acidophilic *Firmicutes.* Laboratory-scale tests carried out have suggested that the novel bacteria could have significant roles in biohydrometallurgical operations. The isolates studied showed to be only distantly related to currently validated species, forming separate monophyletic clades within the *Firmicutes* phylum (Fig. 8.1). Consequently, a novel genus was described and a novel isolate representing a novel candidate genus was characterised in this thesis.

Eight novel isolates, isolated prior to this study from a variety of different global locations, were shown to belong to the proposed novel genus, "*Acidibacillus".* These were shown to be obligately heterotrophic acidophilic bacteria that catalysed the oxido-reduction of iron. One proposed species ("*Ab. sulfuroxidans*") was shown to also catalyse the oxidation of ZVS, and was moderately thermophilic, while the other ("*Ab. ferrooxidans*") did not oxidise sulfur and was mesophilic. Since then, a third novel species of the genus ("*Acidibacillus ambivalens*") has been described (Johnson *et al*., 2018). This is an iron/sulfur-oxidising mesophile which grows at much lower pH (1.0 and above) to the two species described in detail in this thesis. Additional research is required to assess potential roles of these bacteria in microbial consortia used for mineral bio-processing and also possibly in removing soluble iron from acidic ferruginous mine waters.

In contrast to the "*Acidibacillus*" isolates, which were facultative anaerobes, a novel obligately anaerobic acidophilic *Firmicute* (strain I2511) was isolated and characterised during the course of this study. This bacterium catalysed the dissimilatory reduction of ZVS and ferric iron but was not able to reduce sulfate or reduced inorganic sulfur compounds such as thiosulfate and tetrathionate. This isolate appeared to be able to generate H_2S over a wide pH range (1.8 - 5.0) and coupled the reduction of ZVS to the oxidation of a variety of organic compounds. In addition, this isolate showed unique traits (i.e. being a strict anaerobe and ZVS-reducer) not yet described in other species of acidophilic *Firmicutes*. In order to name and validate isolate I2511, other (chemotaxonomic) analysis are required (as reported in this

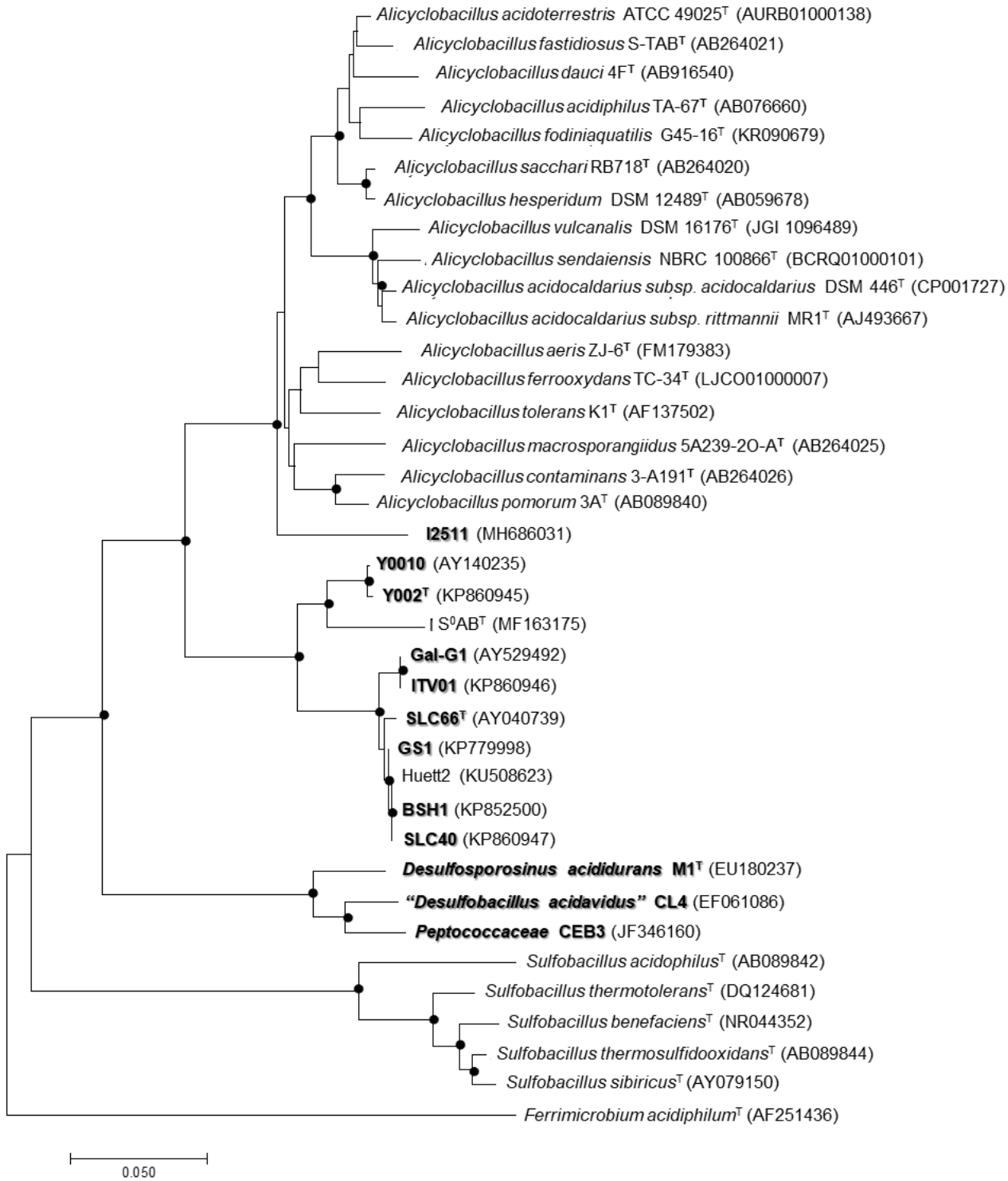

Figure 8.1. Rooted neighbour-joining tree based on 16S rRNA gene sequences showing the relationship of the novel isolates examined in this thesis (in bold) to species of *Alicyclobacillus* and *Sulfobacillus*. The support in bootstrap analysis (1000 replicates) with values ≥70% are indicated by "●". The bar represents 0.05 substitutions per site. The 16S rRNA gene sequence of *Ferrimicrobium acidiphilum*^{T} (AF251436) was used as the outgroup.

thesis for the two type strains of novel "*Acidibacillus"* spp. (e.g. fatty acids, polar acids, peptidoglycan analysis) and deposition in two national culture collections. In addition, future work should focus on improving the functional annotation of the genome to identify putative genes involved in ZVS- and ferric iron-respiration in order to clarify energy conservation pathways utilised by this isolate.

A novel variant of low pH sulfidogenic bioreactors was developed. The "hybrid" sulfidogenic bioreactor (HSB) was populated with selected strains of aSRB, isolate I2511 and other acidophilic bacteria. Hydrogen sulfide was generated in each of the experiments performed with the HSB and in most cases aSRB were detected in the T-RFLP profile of bacterial communities, except when the bioreactor was operated at pH 2.0 in continuous flow mode, isolate I2511 represented 55% of the population and was the only sulfidogenic bacterium detected. The only experiment in which the isolate I2511 was not detected was during the treatment of Minsterley Brook Catchment, using both synthetic and actual wastewaters. Analysis of the T-RFLP profiles of the bacterial population obtained in the experiments with the HSB indicated that, in most cases, isolate I2511 was detected in both sulfur-attached and planktonic cells, with similar abundances (only in few experiments this isolate was more abundant in sulfur-attached cells, e.g. experiment 4 section 5.3.1.2). Likewise, the aSRB showed similar abundance in sulfur-attached and planktonic cells. Mass balance calculations indicated that ZVS and sulfate were reduced concurrently during most of experiments with the HSB. Some results suggested that aSRB may have catalysed both the dissimilatory reduction of ZVS and sulfate during these experiments. Results obtained showed the aSRB were able to reduce ZVS as well as sulfate. In addition, during experiments using the HSB to remediate polluted water from Minsterley Brook, aSRB were the only known sulfidogens detected and ZVS reduction represented 37% and 59% of the total H_2S generated (mean values of synthetic and actual wastewater treatments, respectively). Thirdly, aSRB were detected when HSB operated as sulfur-only reducing bioreactor.

The HSB was developed to remediate and recover transition metals from circum-neutral pH mine-impacted waters and was successfully tested with zinc-rich wastewaters with different chemical compositions collected from two abandoned lead/zinc mines in the United Kingdom. The HSB has different features from previous described low pH sulfidogenic bioreactors:

(i) potential for using two electron acceptors (ZVS and sulfate) while previous low pH sulfidogenic bioreactors have operated only with sulfate.

(ii) the inflow of liquors inside the bioreactor was not controlled by pH, and instead fixed flow rates were used, (i.e. constant hydraulic retention times, HRTs), which allowed to test the effectiveness of the system in removing zinc at different HRT.

(iii) previous systems were tested only with synthetic mine waters (sterile influent liquors) while the HSB treated successfully unfiltered mine waters. While this did not negatively impact the generation of H_2S, this caused changes in the microbial community composition in the HSB.

(iv) the HSB used a different set up to deliver electron donors to the system while treating unfiltered mine waters;

(v) the HSB generated H_2S over a wide pH rage (2 – 7);

(vi) the HSB functioned effectively with short HRTs (1.5 h to 5 h), while reports using similar low pH bioreactor treating synthetic mine water displayed much higher HRTs (e.g. 42 h, Hedrich and Johnson, 2014; 33 h, Santos and Johnson, 2016 and 18 h, Ňancucheo and Johnson, 2012);

Consistent abiotic generation of hydrogen sulfide was also achieved by the chemical reaction between ZVS and zero-valent iron (ZVI) in acidic liquors. The highest rates of H_2S generation were obtained when the reactor was operated at 45°C and at pH 2.2 using biosulfur (ZVS source) and iron filings (ZVI source) while performing a modified sampling regime. The abiotic reactor and the HSB had similar estimated operating costs (the calculated cost to generate 100 moles of H_2S was ~$8 (US) for the HSB and ~$6 (US) for the abiotic reactor). However, these systems have important differences, for instance the HSB was operated at continuous flow mode with variable HRTs, while the abiotic reactor as a stop/go system. In addition, removal of metals from wastewaters by the HSB could be performed by either in-line (a single unit system) or off-line (a two unit system: bioreactor vessel and off-line vessel) treatment, while in the abiotic reactor only off-line treatment would be pragmatic. Rates of H_2S production were much higher using the abiotic reactor than with the HSB, which can be attributed at least in part for the stop/go operation of the former, in which a large amount of H_2S was generated in a very short period. Although generation of H_2S by the HSB was consistent and the removal of zinc was effective and reasonably stable throughout all tests (with, in most cases, >90% metal removed), the use of an abiotic system can be more advantageous for being more predictable and reproducible, in particular, if the costs of the biological and the abiotic processes were similar. In addition, further research focusing on increasing the efficiency of the abiotic reactor (from current 27% to at least 50%) would decrease considerably operating costs and make this system an attractive alternative for metal recovery in hydrometallurgy.

The results obtained in the diverse experimental approaches used in the present study have characterised novel species of *Firmicutes* and highlighted their potential uses for mobilising and immobilising metals at low pH, and also confirmed that abiotic generation of hydrogen sulfide is possible at ambient temperatures in low pH liquors. This research should also be

the basis of follow up work that could consolidate and expand this area, and provide further data required to scale-up the novel bioremediation systems described. Suggested areas for future related research include:

- investigating the bioleaching of metal ores and electronic waste using "*Acidibacillus"* spp. as part of the microbial consortia;
- examining the bio-oxidation and selective precipitation of iron for bioremediation of extremely acidic ferruginous mine waters using "*Acidibacillus"* spp.;
- attempting to isolate other novel extremely acidophilic ZVS-reducing prokaryotes from sulfur-rich low pH environments by applying techniques developed in Chapter 4 (e.g. Src liquid medium and S^0o solid medium);
- optimising abiotic production of hydrogen sulfide using ZVS and ZVI in low pH and low temperature reactors;
- to develop and characterise optimised biological systems to process and adding value to the products obtained from the remediation of mine waters.

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Appendix 3A. Metabolism of organic carbon compounds by strains of "*Acidibacillus*".

	Growth on*											
Isolate	**Yeast extract**	**Tryptone**	**Casein hydrolysate**	**Ethanol**	**Glycerol**	**Mannitol**	**Fructose**	**Glucose**	**Maltose**	**Citric acid**	**Lysine**	**Benzyl alcohol**
SLC66^T	+	+	+	-	+	-	+	+	+	-	-	I
SLC40	+	+	+	-	-	-	+	+	+	-	-	I
BSH1	+	+	+	-	-	+	+	+	+	I	-	I
GS1	+	+	+	-	-	-	+	+	+	I	I	I
ITV01	+	+	+	-	-	-	+	+	+	I	I	I
Gal-G1	+	+	+	-	+	-	+	+	+	-	-	-
Y002^T	+	+	+	-	-	-	+	+	+	-	I	I
Y0010	+	+	+	-	-	+	-	+	+	+	I	I

+, cell numbers >25% more than in control cultures; -, cell numbers not significantly different (+/- 25%) than in control cultures; I, cell numbers >50% less than in control cultures, indicating inhibition of growth; *in cultures containing 0.005% (w/v) yeast extract

Appendix 3B. List of closely related isolates to "*Acidibacillus*" type strains obtained with BLAST analysis at the NCBI database (http://ncbi.nlm.nih.gov/BLAST).

Accession number	Strain	Identity to SLC66^T (%)[a]	Identity to Y002^T (%)[b]	Origin of isolation	Location	Reference
KU508623	Huett2	99	94	Acid mine drainage	Germany	Schopf *et al.* (2017)
MF163175	S^0AB	93	96	granular zero-valent sulfur	United Kingdom	Unpublished
GU167996	BGR 73	99	94	heap soil	Kazakhstan	Breuker *et al.* (2009)
KX815988	BOR5	99	94	tailings	Serbia	Unpublished
FN870328	FeSo-D4-20-CH	93	92	acidic coal mine lake	Germany	Lu *et al.* (2010)
FN870336	iFeo-D4-31-CH	93	92	acidic coal mine lake	Germany	Lu *et al.* (2010)
DQ533683	SDE2	93	96	abandoned copper mine tailings	Portugal	Bryan *et al.* (2006)
KX448798	A06	91	93	acid mine drainage sediments	North Lima, Ohio	Unpublished
DQ355185	CH2	92	93	La Andina copper mine tailings impoundment Piuquenes	Chile	Diaby *et al.* (2007)

[a] Similarity (in percentage) based on 16S rRNA gene sequence to "*Ab. ferrooxidans*" SLC66^T; [b] Similarity (in percentage) based on 16S rRNA gene sequence to *Ab. sulfurooxidans*" Y002^T.

Appendix 3C. List of closely related clones of "*Acidibacillus*" type strains obtained with BLAST analysis at the NCBI database (http://ncbi.nlm.nih.gov/BLAST).

Accession number	Clone	Identity to SLC66^T (%)	Identity to Y002^T (%)	Origin of isolation	Location	Reference
DQ364429	G26	98	95	processed gold ore	Beijing, China.	Unpublished
JF766473	Y1-13	93	93	soil from wastelands at a copper mine	Tongling, China.	Unpublished
HM745452	1200m_h6	93	94	acid mine drainage	La Zarza-Perrunal, Spain.	Gonzalez-Toril *et al.* (2011)
AB552385	OY07-C100	94	93	volcanic ash deposit	Island of Miyake, Japan.	Fujimura *et al.* (2012)
JN982081	VA2-bac_a9	93	94	acidic river	Argentina.	Urbieta *et al.* (2012)
EF612377	K6-C31	94	93	mine tailings	Klondyke, Arizona, USA.	Mendez *et al.* (2008)
FN391819	CG-18	93	94	sediments from acid mine drainage	Carnoules mine, France.	Bertin *et al.* (2011)
JF766474	Y1-14	93	93	soil from wastelands at a copper mine	Tongling, China.	Unpublished
EU419142	K17bXIb27	93	97	acidic volcanic soil	Mount hood, Oregon, USA.	Unpublished
HQ730616	SN108_6	94	97	extreme acid environment	Tinto River, Spain.	Sanchez-Andrea *et al.* (2011)
DQ464146	D1-23	94	97	acid mine drainage	Dongxiang copper mine and Yinshan lead-zinc mine, China.	He *et al.* (2008)
FM872847	FB03F10	92	93	floor dust	Finland.	Taubel *et al.* (2009)
EF409835	YTW-27-06	91	93	acid mine drainage	Dexing Copper Mine, China.	Yin *et al.* (2008)
KF287767	aw26	91	93	drainage from a copper mine	Daye, Hubei, China.	Unpublished
HQ730649	JL22_2009	93	93	extreme acid environment	Tinto River, Spain.	Sanchez-Andrea *et al.* (2011)
AY911435	NEC02064	92	93	rock interior	Yellowstone National Park, USA.	Walker *et al.* (2005)
GU229856	S3BC3	92	93	acid streamer at a sulfur mine	Cae Coch, North Wales, UK.	Kimura *et al.* (2011)
EU376022	CEM_Eug_h7	93	94	biofilm in a highly acidic river	Tinto River, Spain.	Unpublished

[a] Similarity (in percentage) based on 16S rRNA gene sequence to "*A. ferrooxidans*" SLC66^T; [b] Similarity (in percentage) based on 16S rRNA gene sequence to *A. sulfurooxidans*" Y002^T.

Appendix 3D. One-way analysis of variance (ANOVA) with post-hoc test comparing concentrations of Fe^{2+} oxidised during test with or without an enhanced CO_2 atmosphere in Fe medium amended with 0.005% (w/v) yeast extract. "Y002 (+)" refers to "*Ab. sulfuroxidans*" cultures under enhanced CO_2 atmosphere and "Y002 (-)" refers cultures incubated without enhanced CO_2 atmosphere. "Sb (+)" refers to *Sb. thermosulfidooxidans*^{T} under enhanced CO_2 atmosphere and "Sb (-)" refers cultures incubated without enhanced CO_2 atmosphere.

Descriptives

VAR00001

	N	Mean	Std. Deviation	Std. Error	90% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Y002 (+)	2	18.49	0.56	0.40	13.47	23.50	18.09	18.88
Y002 (-)	2	16.19	3.93	2.78	-19.13	51.51	13.41	18.97
Sb (+)	2	20.18	0.54	0.38	15.35	25.01	19.80	20.56
Sb (-)	2	17.76	1.26	0.89	6.45	29.07	16.87	18.65
Total	8	18.15	2.21	0.78	16.31	20.00	13.41	20.56

Test of Homogeneity of Variances

VAR00001

Levene Statistic	df1	df2	Sig.
5826785345389620.0	3	4	0.000

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.45	3	5.48	1.24	0.40
Within Groups	17.64	4	4.41		
Total	34.10	7			

Multiple Comparisons

Dependent Variable: VAR00001

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	95% Confidence Interval
					Lower Bound	Upper Bound
Y002 (+)	Y002 (-)	2.295	2.100	0.712	-6.254	10.844
	Sb (+)	-1.695	2.100	0.849	-10.244	6.854
	Sb (-)	0.725	2.100	0.984	-7.824	9.274
Y002 (-)	Y002 (+)	-2.295	2.100	0.712	-10.844	6.254
	Sb (+)	-3.990	2.100	0.354	-12.539	4.559
	Sb (-)	-1.570	2.100	0.874	-10.119	6.979

Tukey HSD[a]

VAR00002	N	Subset for alpha = 0.05
		1
Y002 (-)	2	16.1900
Sb (-)	2	17.7600
Y002 (+)	2	18.4850
Sb (+)	2	20.1800
Sig.		0.354

Means for groups in homogeneous subsets are displayed;
[a]Uses Harmonic. Mean Sample Size = 2.000.

Appendix 4A. Characteristics of *Alicylobacillus* spp.

Strains: **1**, *Alb. tolerans* K1^T; **2**, *Alb. acidoterrestris* DSM 3922^T; **3**, *Alb. pomorum* DSM 14955^T; **4**, *Alb. acidiphilus* TA-67^T; **5**, *Alb. cycloheptanicus* DSM 4006^T; **6**, *Alb. disulfidooxidans* DSM 12067^T; **7**, *Alb. hesperidum* DSM 12489^T; **8**, *Alb. acidocaldarius* subsp. *acidocaldarius* DSM 446^T; **9**, *Alb. ferrooxydans* TC-34^T; **10**, *Alb. vucanalis* CsHg2^T; **11**, *Alb. macrosporangiidus* DSM17980^T; **12**, *Alb. contaminans* 3-A191^T; **13**, *Alb. sacchari* DSM 17974^T; **14**, *Alb. fastidious* DSM 17978^T; **15**, *Alb. sendaiensis* JCM 11817^T; **16**, *Alb. aeris* ZJ-6^T; **17**, *Alb. dauci* 4F^T; **18**, *Alb. fodiniaquatilis* G45-16^T; **19**, *Alb. tengchongensis* ACK006^T.

Characteristics	**1**[a,b,d]	**2**	**3**[c]	**4**	**5**	**6**[a,b,d]	**7**	**8**	**9**[c]	**10**	**11**[c]	**12**[c]	**13**[c]	**14**[c]	**15**[c]	**16**[a, c]	**17**[c]	**18**[c]	**19**
Origin	lead–zinc ores	fruit juice	fruit juice	fruit juice	soil	waste water sludge	solfataric soil	acidic thermal environ.	solfataric soil	geother-mal pool	soil from crop field	soil from crop field	liquid sugar	juice	soil	copper mine	fruit juice	acid mine water	soil of a hot spring
G+C (mol%)[e]	48.7	52.2	53.1	54.1	54.0	53.0	53.3	60.3	48.6	62.0	62.5	60.6	56.6	53.9	62.3	51.2	49.6	51.3	53.7
pH range (optimum)	1.5-5.0 (2.0-2.7)	2-6 (4)	3-6 (4.0-4.5)	2.5-5.5 (3.0)	3-5 (4.5)	0.5 – 6 (1.5-2.5)	2-6 (4)	2-6 (4)	2-6 (3)	2-6 (4)	3.0-6.5 (4-4.5)	3-6 (4-4.5)	2-6.5 (4-4.5)	2-5.5 (4-4.5)	2.5-6.5	2-6 (3.5)	3-6 (4)	2.5-5.5 (3.5)	2-6 (3.2)
Temp (˚C) range (optimum)	20-55 (38-42)	35-55 (42-53)	30-60 (45-50)	20-55 (50)	40-53 (48)	4-40 (35)	35-60 (50)	45-70 (60)	17-40 (28)	35-65 (55)	35-60 (50-55)	35-60 (50-55)	30-55 (45-50)	20-55 (40-45)	40–65 (55)	25-35 (30)	20-50 (40)	20-45 (40)	30-50 (45)
2-5% NaCl[f]	ND	+	-	-	+	ND	+	-	+	+	+	+	-	-	+	+	ND	+	+
(Fe^{2+}, ZVS)[g]	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-
ω-alicyclic fatty acids[h]	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	+
Guaiacol[J]	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram[L]	+	+	+/-	+	+	+	+	+	+	+	+/-	+/-	+/-	+/-	-	+/-	v	v	+

[a]Autotrophic growth; [b]anaerobic growth using Fe^{3+} as electron acceptor; [c] strict aerobic; [d]requirement of yeast extract as growth factor; [e] guanine-cytosine content; [f] growth detected in medium containing 2-5% (w/v) NaCl; [g]dissimilatory oxidation of Fe^{2+}, sulfidic minerals and ZVS; [h] presence of ω-alicyclic compounds in the cellular fatty acids composition; [J]production of guaiacol during growth; [L]Gram staining: "+" refers to Gram-positive species, "-" Gram-negative, "+/-" Gram-positive and Gram-variable in old cultures; "v" Gram-variable; ND, not determined.

Appendix 4B. Full-length 16S rRNA gene sequence extracted from the draft genome sequence of isolate I2511.

TGATTTGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGCAAATTTCCGGTGCTTGCACTGG
AGAGATGAGCGGCGGACGGGTGAGTAACACGTGGGTAATCTACCTTTCAGACCGGAATAACGCCTGGAAACGGGTGCTAATGCCGGATAA
GGTGGCGGGGAGGCATCTTCCTGCCAAAAAAGGAGCTATTGCTCCACTGAGAGAGGAGCCCGCGGCGCATTAGCTAGTTGGCGGGGTAAA
GGCCCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGGACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGCGAGGAAGGCCTTCGGGTCGTAAAGCTCAGTCACT
CGGGAAGAGCGGTCCTGGGAGTGGAAAATCCAGGGCGAGACGGTACCGAGGGAGGAAGCCCCGGCAAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGGGCAAGCGTTGTCCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTTTGGCAAGTCTGGGGTGAAAGGCCATGGCTCA
ACCATGGTAATGCCTTGGAAACTGCTAGGCTTGAGTACTGGAGAGGCAAGGGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGA
GGAATACCAGTGGCGAAGGCGCCTTGCTGGACAGTGACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGGTCACTCCTCAGTGCCGAAGGTAACCCAATAAGCACTCCGCCTGGGGAGTAC
GGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGGCTTGACATCCCTCTGACGTCTCTAGAGATAGAGATTCCTTCGGGCAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTGTGTTACCAGCATGTAAAGATGGGGACTCACAGGTGACTGCCGGCGT
AAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTGATGTCCTGGGCCACACACGTGCTACAATGGGCGGAACAACGGGACG
CGAGACCGCGAGGTGGAGCCAAACCCTAAAAACCGTTCGTAGTTCGGATTGCAGGCTGCAACCCGCCTGCATGAAGCCGGAATTGCTAGTA
ATCGCGGATCAGCATGCCGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAACACCCGAAGTCGG
TGGGGTAACCCGCAAGGGAGCCAGCCGCCGAAGGTGGGGTCGATAATTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGG
CTGGATCACCTCCTTT

Appendix 4C. Partial 16S rRNA gene sequence of isolate I2511.

CAGTCGAGCGCAATTTCCGGTGCTTGCACCGGAGAGATGAGCGGCGGACGGGTGAGTAACACGTGGGTAATCTACCTTTCAGACCGGAATA
ACGCCTGGAAACGGGTGCTAATGCCGGATAAGGTGGCGGGGAGGCATCTTCCTGCCAAAAAAGGAGCTATTGCTCCACTGAGAGAGGAGC
CCGCGGCGCATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGGACGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGCGA
GGAAGGCCTTCGGGTCGTAAAGCTCAGTCACTCGGGAAGAGCGGTCCTGGGAGTGGAAAATCCAGGGCGAGACGGTACCGAGGGAGGAA
GCCCCGGCAAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGG
TTTGGCAAGTCTGGGGTGAAAGGCCATGGCTCAACCATGGTAATGCCTTGGAAACTGCTAGGCTTGAGTACTGGAGAGGCAAGGGGAATTC
CACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAATACCAGTGGCGAAGGCGCCTTGCTGGACAGTGACTGACGCTGAGGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGGTCACTCCTCAGTGCCGAA
GGTAACCCAATAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCTCTGACGTCTCTAGAGATAGAGATTCCTTCGGGCAGAGGAGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTGTGTTACCAGCATG
TAAAGATGGGGACTCACAGGTGACTGCCGGCGTAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTGATGTCCTGGGCCA
CACACGTGCTACAATGGGCGGAACAACGGGACGCGAGACCGCGAGGTGGAGCCAAACCCTAAAAACCGTTCGTAGTTCGGATTGCAGGCT
GCAACCCGCCTGCATGAAGCCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCG
TCACACCACGAGAGTTGGCAACACCCGGAAAGTCGGT

Appendix 5A. Data from experiment 1 of the priming phase operation of the hybrid sulfidogenic bioreactor.

Time (h)	pH[a]	Bacteria x10^7/ml[b]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[c]	Zn^{2+} mM				SO_4^{2-} mM			Net [SO_4^{2-}] reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h			H_2S produced from reduction of: (%)	
			in	out	Δ	out		in	out	Δ	%[d]	in	out	Δ	µmoles/L/h		in-line [ZnS]	off-line [CuS]	Σ	[SO_4^{2-}]	ZVS
0	3.7	1.46														21.9					
10	3.7	1.36	1.27	1.06	0.21	0.19	12	0.97	0.40	0.57	59	7.67	7.38	0.29	60	19.6	118	31	149	40	60
23	3.8	1.25	1.22	1.05	0.17	0.13	25	1.12	0.48	0.64	57	7.50	7.39	0.11	23	17.1	132	25	157	14	86
47	3.8	0.95	1.29	0.99	0.30	0.10	68	0.98	0.45	0.53	54	7.27	7.09	0.18	37	10.9	110	36	145	26	74
53	3.7	0.73	1.28	0.95	0.33	0.17	47	1.04	0.38	0.66	63	7.54	7.01	0.53	110	8.8	137	47	184	60	40

[a]bioreactor pH; [b]planktonic bacteria; [c]% of glycerol oxidised to CO_2; [d]% of Zn precipitated in the bioreactor vessel.

Appendix 5B. Data from experiment 2 of the priming phase operation of the hybrid sulfidogenic bioreactor.

Time (h)	pH[a]	Bacteria x10^7/ml[b]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[c]	Zn^{2+} mM				SO_4^{2-} mM			Net [SO_4^{2-}] reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h			H_2S produced from reduction of: (%)	
			in	out	Δ	out		in	out	Δ	%[d]	in	out	Δ	µmoles/L/h		in-line [ZnS]	off-line [CuS]	Σ	[SO_4^{2-}]	ZVS
0	3.76	0.56														23.9					
6	3.70	1.95	1.20	1.07	0.13	0.08	38	0.92	0.48	0.44	48	7.09	6.91	0.18	37	23.1	91	19	110	34	66
23	3.70	3.59	1.18	1.01	0.17	0.12	29	0.90	0.41	0.49	54	7.11	6.88	0.23	48	20.7	101	19	121	39	61
30	3.73	4.61	1.18	0.99	0.19	0.10	47	0.94	0.34	0.60	64	7.24	6.85	0.39	81	18.5	124	42	166	48	52
47	3.74	4.38	1.24	0.98	0.26	0.18	31	0.93	0.43	0.50	54	6.96	6.77	0.19	39	13.8	103	38	141	28	72
54	3.66	2.45	1.15	0.94	0.21	0.21	0	0.75	0.63	0.12	16	7.17	6.76	0.41	85	11.5	25	45	70	100	0
71	3.72	2.03	1.15	0.84	0.31	0.19	39	0.97	0.35	0.62	64	7.41	7.08	0.33	68	4.3	128	58	186	37	63
79	3.50	0.41	1.18	0.84	0.34	0.24	29	0.95	0.31	0.64	67	7.63	7.11	0.52	108	0.3	132	68	200	54	46

[a]bioreactor pH; [b]planktonic bacteria; [c]% of glycerol oxidised to CO_2; [d]% of Zn precipitated in the bioreactor vessel

Appendix 5C. Data from experiment 3 of the priming phase operation of the hybrid sulfidogenic bioreactor.

Time (h)	pH[a]	Bacteria x10^7/ml[b]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[c]	Zn^{2+} mM				SO_4^{2-} mM			Net [SO_4^{2-}] reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h			H_2S produced from reduction of: (%)	
			in	out	Δ	out		in	out	Δ	%[d]	in	out	Δ	µmoles/L/h		in-line [ZnS]	off-line [CuS]	Σ	[SO_4^{2-}]	ZVS
0	3.7	2.24														19.3					
7	3.4	1.67	2.20	1.82	0.38	0.30	21	0.92	0.20	0.72	78	7.48	7.32	0.16	33	18.0	149	24	173	19	81
23	3.4	0.83	2.26	1.77	0.49	0.33	33	0.79	0.21	0.58	73	8.10	7.71	0.39	81	9.75	120	71	191	42	58
27																47.8					
31	3.4	0.58	2.25	1.71	0.54	0.35	35	0.78	0.17	0.61	78	7.98	7.38	0.60	124	43.1	126	160	286	43	57
47	3.3	1.61	2.23	1.61	0.62	0.36	42	0.92	0.20	0.72	78	7.47	7.09	0.38	79	31.9	149	96	245	32	68
55	3.4	1.07	2.19	1.50	0.69	0.43	38	0.91	0.15	0.76	84	7.78	7.34	0.44	91	25.3	157	112	269	34	66
72	3.4	3.91	2.17	1.38	0.79	0.48	39	0.92	0.12	0.80	87	8.14	7.35	0.79	163	6.73	165	149	314	52	48
77	3.4	2.97	2.17	1.31	0.86	0.50	42	0.95	0.08	0.87	92	8.37	7.20	1.17	242	0.19	180	178	358	68	32

[a]bioreactor pH; [b]planktonic bacteria; [c]% of glycerol oxidised to CO_2; [d]% of Zn precipitated in the bioreactor vessel

Appendix 5D. Data from experiment 4 of the priming phase operation of the hybrid sulfidogenic bioreactor.

Time (h)	pH[a]	Bacteria x10^7/ml[b]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[c]	Zn^{2+} mM				SO_4^{2-} mM			Net [SO_4^{2-}] reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h			H_2S produced from reduction of: (%)	
			in	out	Δ	out		in	out	Δ	%[d]	in	out	Δ	µmoles/L/h		in-line [ZnS]	off-line [CuS]	Σ	[SO_4^{2-}]	ZVS
0	3.4															19.2					
9	3.4	2.58	2.19	1.76	0.43	0.34	21	0.96	0.27	0.69	72	7.16	6.72	0.44	91	17.5	143	25	168	54	46
24	3.4	2.03	2.13	1.42	0.71	0.41	42	0.97	0.20	0.77	79	7.40	6.67	0.73	151	15.3	159	20	179	84	16
33	3.5	1.33	2.12	1.09	1.03	0.06	94	0.95	0.11	0.84	88	7.38	6.50	0.88	182	12.6	174	41	215	85	15
49	3.3	3.59	2.1	0.95	1.15	0.44	62	0.96	0.07	0.89	93	6.90	6.30	0.60	124	4.18	184	72	256	49	51
51																22.8					
57	3.4	0.83	2.08	0.87	1.21	0.55	55	1.13	0.08	1.05	93	6.92	6.32	0.60	124	19.8	217	68	285	44	56
73	3.5	0.94	2.14	0.99	1.15	0.47	59	0.97	0.07	0.90	93	7.32	6.86	0.46	95	14.6	186	44	230	41	59
75																45.5					
79	3.4	2.34	2.02	0.95	1.07	0.43	60	1.07	0.06	1.01	94	7.49	6.71	0.78	161	42.8	209	92	300	54	46
98	3.4	1.80	2.06	1.07	0.99	0.43	57	0.97	0.08	0.89	92	7.82	7.29	0.53	110	40.3	184	18	202	54	46
106	3.5	1.56	2.04	1.08	0.96	0.48	50	0.85	0.06	0.79	93	7.48	7.03	0.45	93	37.9	163	41	205	45	55

[a]bioreactor pH; [b]planktonic bacteria; [c]% of glycerol oxidised to CO_2; [d]% of Zn precipitated in the bioreactor vessel

Appendix 5E. Data from experiment 5 of the priming phase operation of the hybrid sulfidogenic bioreactor.

Time (h)	pH[a]	Bacteria x10^7/ml[b]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[c]	Zn^{2+} mM				SO_4^{2-} mM			Net [SO_4^{2-}] reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h			H_2S produced from reduction of: (%)	
			in	out	Δ	out		in	out	Δ	%[d]	in	out	Δ	µmoles/L/h		in-line [ZnS]	off-line [CuS]	Σ	[SO_4^{2-}]	ZVS
0	4.4	4.84														42.8					
16	4.5	8.91	1.11	0.07	1.04	0.46	56	0.62	<0.01	0.62	>99	8.18	7.28	0.90	186	35.1	128	66	194	96	4
23	4.0	6.41	1.12	0.49	0.63	0.45	29	0.64	<0.01	0.64	>99	7.65	7.40	0.25	52	34.3	132	15	147	35	65
41	3.8	2.19	1.06	0.20	0.86	0.37	57	0.76	<0.01	0.76	>99	7.53	6.45	1.08	223	25.6	157	66	224	100	0
50	3.5	3.28	1.09	0.48	0.61	0.45	26	0.81	0.04	0.77	95	7.18	6.30	0.88	182	23.5	159	32	191	95	5
50																48.9					
66	4.1	7.81	1.10	0.19	0.91	0.20	78	0.91	<0.01	0.91	>99	7.18	6.32	0.86	178	45.9	188	26	214	83	17
69	4.1	6.25	1.09	0.17	0.92	0.19	79	0.81	<0.01	0.81	>99	6.97	6.45	0.52	108	41.7	168	188	356	30	70
88	4.3	4.53	1.13	0.17	0.96	0.52	46	0.79	<0.01	0.79	>99	7.22	6.36	0.86	178	25.9	163	113	277	64	36
88																56.5					
95	4.3	4.53	1.10	0.08	1.02	0.62	39	0.79	<0.01	0.79	>99	7.22	6.48	0.74	153	53.1	163	65	229	67	33
112	4.2	5.78	1.14	0.00	1.14	0.59	48	1.13	<0.01	1.13	>99	7.61	6.70	0.91	188	37.9	234	122	356	53	47
119	3.6	7.81	1.09	0.04	1.05	0.29	72	0.84	0.02	0.82	98	7.52	6.92	0.60	124	31.8	170	118	287	43	57
136	4.0	9.38	1.04	0.17	0.87	0.40	54	0.79	<0.01	0.79	>99	7.33	6.65	0.68	141	16.9	163	120	283	50	50
138																44.3					
144	4.5	5.47	0.99	0.14	0.85	0.41	52	0.79	<0.01	0.79	>99	6.88	6.67	0.21	43	43.2	163	25	189	23	77
160	6.1	3.91	0.82	0.00	0.82	0.46	44	0.84	<0.01	0.84	>99	7.60	6.56	1.04	215	27.8	174	131	304	71	29
168	6.1	10.94	1.08	0.05	1.03	0.50	51	0.79	<0.01	0.79	>99	7.10	6.15	0.95	196	20.9	163	119	282	70	30
184	6.0	5.70	0.91	0.00	0.91	0.48	47	0.81	<0.01	0.81	>99	7.07	6.40	0.67	139	4.17	168	142	310	45	55
185																43.8					
192	6.0	7.66	1.02	0.05	0.97	0.48	51	0.80	<0.01	0.80	>99	7.11	6.58	0.53	110	39.3	165	88	253	43	57

[a]bioreactor pH; [b]planktonic bacteria; [c]% of glycerol oxidised to CO_2; [d]% of Zn precipitated in the bioreactor vessel

Appendix 5F. Independent-samples t-test conducted with bioreactor pH of experiments 1 to 4 (group 1) and experiment 5 (group 2) of the priming phase operation of the hybrid sulfidogenic bioreactor.

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
Group 1	27	3.53	0.16	0.03
Group 2	16	4.56	0.92	0.23

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	35.55	0.00	-5.69	41	0.00	-1.03	0.18	-1.39	-0.66
	Equal variances not assumed			-4.41	15	0.00	-1.03	0.23	-1.53	-0.53

Appendix 5G. Data from operating the hybrid sulfidogenic bioreactor at pH 3.5 in batch mode.

Time h	Bioreactor pH	Glycerol mM	Acetic acid mM	SO_4^{2-} mM	SO_4^{2-} reduced mM	SO_4^{2-} reduced µmoles	Off-line Cu^{2+} mM	H_2S produced µmoles
0	3.5	5.3	2.4	11.1	0.0	0	25.7	0
3	3.3	4.8	2.0	10.8	0.3	660	23.3	720
18	3.5	4.3	2.8	10.0	1.1	2420	19.5	1860
22	3.5	4.0	2.0	10.0	1.1	2420	17.5	2460
25	3.4	4.0	2.8	9.9	1.2	2640	16.5	2760
27	3.4	3.8	3.0	9.7	1.4	3080	16.3	2820
42	3.5	2.8	3.3	9.1	2.0	4400	11.2	4350
45	3.5	2.6	3.4	9.0	2.1	4620	9.9	4740
45							23.8	
48	3.5	2.3	3.5	9.0	2.1	4620	22.4	5160
54	3.5	1.8	3.6	8.8	2.3	5060	20.2	6240
66	3.3	1.3	3.7	8.0	3.1	6820	16.2	8520

Appendix 5H. Data from operating the hybrid sulfidogenic bioreactor at pH 3.0 in batch mode.

Time h	Bioreactor pH	Glycerol mM	Acetic acid mM	SO_4^{2-} mM	SO_4^{2-} reduced mM	SO_4^{2-} reduced µmoles	Off-line Cu^{2+} mM	H_2S produced µmoles
0	3.2	5.5	1.2	12.6	0.0	0.0	19.4	0
5	3.2	5.0	1.2	12.1	0.5	1100	15.3	1235
21	3.2	4.0	1.9	11.3	1.3	2860	6.4	3898
25	3.0	3.8	1.9	11.1	1.5	3300	4.4	4502
29	3.0	3.5	1.9	10.8	1.8	3960	3.5	4765
29							21.2	
44	3.0	2.4	2.3	10.1	2.5	5500	13.5	7075
49	2.9	2.1	2.3	9.7	2.9	6380	10.7	10225
52	2.9	1.9	2.4	9.5	3.1	6820	8.7	13975
68	3.0	1.0	2.4	8.4	4.2	9240	1.1	20005
68							20.3	
72	3.1	0.8	2.4	8.1	4.5	9900	19.0	20403
77	3.0	0.6	2.3	7.8	4.8	10560	18.4	20980

Appendix 5J. Data from operating the hybrid sulfidogenic bioreactor at pH 2.5 in batch mode.

Time h	Bioreactor pH	Glycerol mM	Acetic acid mM	SO_4^{2-} mM	SO_4^{2-} reduced mM	SO_4^{2-} reduced µmoles	Off-line Cu^{2+} mM	H_2S produced µmoles
0	2.5	5.9	0.5	10.8	0.0	0.00	21.7	0
15	2.6	5.6	0.7	10.6	0.2	440	16.9	1454
19	2.6	5.5	0.7	10.3	0.5	1100	15.6	1852
23	2.5	5.5	0.8	10.3	0.5	1100	14.8	2072
38	2.6	5.0	0.9	10.1	0.7	1540	12.1	2895
43	2.6	5.0	1.0	10.0	0.8	1760	11.8	2977
47	2.5	4.8	1.0	9.8	1.0	2200	11.7	3005
62	2.5	4.3	1.2	9.4	1.4	3080	10.1	3481
66	2.5	4.1	1.3	9.2	1.6	3520	9.5	3680
86	2.5	2.5	1.6	8.7	2.1	4620	7.2	4366

Appendix 5K. Data from the hybrid sulfidogenic bioreactor operated in continuous flow mode and fed with pH 2.5 influent liquors.

Time (h)	pH[a]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[b]	SO_4^{2-} mM			Net $[SO_4^{2-}]$ reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h	H_2S produced from reduction of: (%)	
		in	out	Δ	out		in	out	Δ	µmoles/L/h		off-line [CuS]	$[SO_4^{2-}]$	ZVS
0	2.6										17.6			
6	2.6	1.01	0.92	0.09	0.22	0	7.85	7.74	0.11	23	16.1	36	64	36
26	2.6	1.01	0.76	0.25	0.19	24	7.85	7.69	0.16	33	9.82	43	78	22
32											7.64	50		
32											17.4			
48											12.6	40		
56	2.6	1.26	0.93	0.33	0.15	55	7.79	7.59	0.20	41	9.85	48	87	13
72	2.6	0.70	0.51	0.19	0.07	63	7.88	7.71	0.17	35	4.75	43	81	19
											4.12	22		

[a]bioreactor pH; [b]% of glycerol oxidised to CO_2;

Appendix 5L. Data from the hybrid sulfidogenic bioreactor operated in continuous flow mode and fed with pH 2.0 influent liquors.

Time (h)	pH[a]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[b]	SO_4^{2-} mM			Off-line Cu^{2+} mM	H_2S µmoles/L/h
		in	out	Δ	out		in	out	Δ		off-line [CuS]
0										16.2	
5										14.6	43
5										16.8	
23										10.4	49
25	2.02	1.05	1.09	-0.04	0.15	0	8.31	8.35	-0.04	9.46	63
31										7.37	47
47	2.03	1.05	1.01	0.04	0.31	0	8.35	8.43	-0.08	1.37	51
77										19.1	
95	1.98	1.05	1.09	-0.04	0.16	0	9.01	8.91	0.10	12.9	46
102	1.98	1.03	1.05	-0.02	0.07	0	8.77	9.12	-0.35	11.3	33
116	2.05	1.07	1.09	-0.02	0.15	0	8.75	8.86	-0.11	7.01	41

[a]bioreactor pH; [b]% of glycerol oxidised to CO_2;

Appendix 5M. Data from independent-samples t-test conducted with rates of H_2S production in experiments of the hybrid sulfidogenic bioreactor operated in continuous flow mode and fed with pH 2.5 and pH 2.0 influent liquors. Outliers were excluded from the analysis.

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
Rates of H_2S pH 2.0	7	44.3	6.0	2.3
Rates of H_2S pH 2.5	6	43.3	5.1	2.1

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	.17	.69	.30	11	.77	.95	3.13	-5.94	7.85
	Equal variances not assumed			.31	11	.76	.95	3.09	-5.85	7.75

Appendix 5N. One-way analysis of variance (ANOVA) with post-hoc test comparing rates of H_2S production of the priming phase operation of the hybrid sulfidogenic bioreactor, when bioreactor pH liquors was at pH 3.4, 3.7, 4.3 and 6.0. The outlier data point was included in the analysis.

Descriptives

VAR00001

	N	Mean	Std. Deviation	Std. Error	90% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
pH 3.4±0.1	18	237.06	54.93	12.95	209.74	264.37	168.00	358.00
pH 3.7±0.1	12	161.67	55.85	16.12	126.18	197.15	70.00	287.00
pH 4.3±0.2	9	249.44	73.73	24.58	192.77	306.12	147.00	356.00
pH 6.0±0.1	4	287.25	25.81	12.91	246.18	328.32	253.00	310.00
Total	43	223.28	69.45	10.59	201.90	244.65	70.00	358.00

Test of Homogeneity of Variances

VAR00001

Levene Statistic	df1	df2	Sig.
1.750	3	39	0.173

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	71500.068	3.000	23833.356	7.090	0.001
Within Groups	131102.583	39.000	3361.605		
Total	202602.651	42.000			

Multiple Comparisons

Dependent Variable: VAR00001

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	95% Confidence Interval
					Lower Bound	Upper Bound
pH 3.4±0.1	pH 3.7+-0.1	**75.389***	21.608	0.006	17.408	133.370
	pH 4.3+-0.2	-12.389	23.670	0.953	-75.904	51.126
	pH 6.0+-0.1	-50.194	32.049	0.409	-136.194	35.806
pH 3.7±0.1	pH 3.4+-0.1	**-75.389***	21.608	0.006	-133.370	-17.408
	pH 4.3+-0.2	**-87.778***	25.566	0.007	-156.382	-19.174
	pH 6.0+-0.1	**-125.583***	33.474	0.003	-215.407	-35.759
pH 4.3±0.2	pH 3.4+-0.1	12.389	23.670	0.953	-51.126	75.904
	pH 3.7+-0.1	**87.778***	25.566	0.007	19.174	156.382
	pH 6.0+-0.1	-37.806	34.841	0.701	-131.297	55.686
pH 6.0±0.1	pH 3.4+-0.1	50.194	32.049	0.409	-35.806	136.194
	pH 3.7+-0.1	**125.583***	33.474	0.003	35.759	215.407
	pH 4.3+-0.2	37.806	34.841	0.701	-55.686	131.297

*The mean difference is significant at the 0.05 level.

Appendix 5P. One-way analysis of variance (ANOVA) with post-hoc test comparing rates of H_2S production of the priming phase operation of the hybrid sulfidogenic bioreactor, when bioreactor pH liquors was at pH 3.4, 3.7, 4.3 and 6.0. The outlier data point was included in the analysis

Homogeneous Subsets

VAR00001

Tukey HSD[a,b]

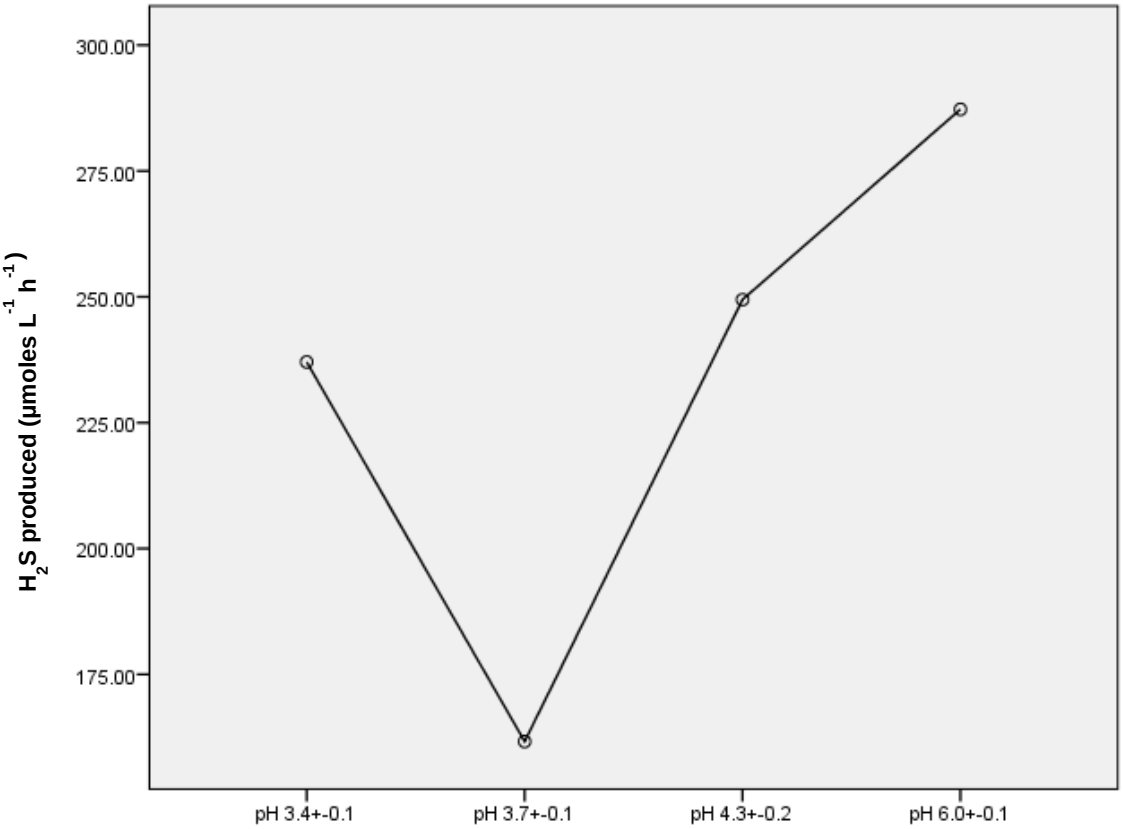
VAR00002	N	Subset for alpha = 0.05	
		1	2
pH 3.7+-0.1	12	161.667	
pH 3.4+-0.1	18	237.056	237.056
pH 4.3+-0.2	9		249.444
pH 6.0+-0.1	4		287.250
Sig.		0.060	0.322

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Means Plots



Appendix 5Q. Data from the HSB as a sulfur-only reducing bioreactor operated in continuous flow mode and fed with pH 4.0 influent liquor.

Time (h)	pH[a]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[b]	Off-line Cu^{2+} mM	H_2S µmoles/L/h
		in	out	Δ	out			
0							20.6	
7	4.8	1.1	0.2	0.9	0.7	25	17.7	56
25							6.42	85
25							24.3	
30	4.1	1.0	0.2	0.8	0.8	7	23.2	31
33							21.0	98
48							12.8	74
51							10.3	115
51							25.7	
54	4.8	1.1	0.1	1.0	0.7	27	24.9	36
58	4.8	1.1	0.1	1.0	0.8	23	21.5	117
73	4.7	1.1	0.2	1.0	0.8	21	11.9	86
76							10.4	72
76							28.4	
97							19.0	61
100	4.6	1.3	0.2	1.1	0.8	33	17.8	56
103							14.9	127

[a]bioreactor pH; [b]% of glycerol oxidised to CO_2;

Appendix 5R. Data from the HSB as a sulfur-only reducing bioreactor operated in continuous flow mode and fed with pH 3.0 influent liquors.

Time (h)	pH[a]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[b]	Off-line Cu^{2+} mM	H_2S µmoles/L/h
		in	out	Δ	out			
0							7.45	
4							6.98	16
5	3.15	1.08	0.87	0.21	0.21	0	6.79	27
5							23.2	
6							22.7	67
26							20.1	18
31	3.17	1.08	0.97	0.11	0.21	0	19.1	33
48							15.0	31
53	3.12	1.05	0.93	0.12	0.20	0	12.5	69
56							11.7	35
56							22.5	
73							15.6	59
77	3.00	1.02	0.89	0.13	0.28	0	14.4	35

[a]bioreactor pH; [b]% of glycerol oxidised to CO_2;

Appendix 5S. Data from independent-samples t-test conducted with values of glycerol consumption of the HSB as a sulfur-only reducing bioreactor operated in continuous flow mode and fed with pH 4.0 and pH 3.0 influent liquors.

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
glycerol oxidised pH 4.0	6	0.97	0.10	0.040
glycerol oxidised pH 3.0	4	0.14	0.05	0.023

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	1.406	0.270	15.641	8.000	0.000	0.829	0.053	0.707	0.951
	Equal variances not assumed			18.041	7.481	0.000	0.829	0.046	0.722	0.936

Appendix 5T. Data from independent-samples t-test conducted with values of acetic acid production of the HSB as a sulfur-only reducing bioreactor operated in continuous flow mode and fed with pH 4.0 and pH 3.0 influent liquors.

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
Acetic acid pH 4.0	6	0.758	0.050	0.020
Acetic acid pH 3.0	4	0.225	0.037	0.018

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	1.310	0.285	18.258	8	0.000	0.533	0.029	0.466	0.601
	Equal variances not assumed			19.460	7.788	0.000	0.533	0.027	0.470	0.597

Appendix 5U. Data from independent-samples t-test conducted with rates of H_2S production of the HSB as a sulfur-only reducing bioreactor operated in continuous flow mode and fed with pH 4.0 and pH 3.0 influent liquors.

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
Rates of H_2S pH 4.0	13	78.00	30.39	8.43
Rates of H_2S pH 3.0	10	39.00	19.24	6.08

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	2.350	0.140	3.539	21	0.002	39	11.021	16.081	61.919
	Equal variances not assumed			3.752	20	0.001	39	10.395	17.342	60.658

Appendix 6A. Data from in-line remediation of synthetic Minsterley Brook waste water supplemented directly with glycerol and yeast extract.

Time (h)	pH[a]	Bact [b]	Glycerol mM				Acetic acid mM	Gly → CO_2 (%)[d]	Zn^{2+} mM				SO_4^{2-} mM			Net $[SO_4^{2-}]$ reduced	Off-line Zn^{2+} mM[f]	Off-line Cu^{2+} mM	H_2S µmoles/L/h				H_2S produced from reduction of: (%)	
			in	out	Δ	%[c]	out		in	out	Δ	%[e]	in	out	Δ	µmoles/L/h			in-line [ZnS]	off-line [ZnS]	off-line [CuS]	Σ	$[SO_4^{2-}]$	ZVS
0																		43.5						
1	3.8	10.31	1.02	0.05	0.97	95	0.34	65	1.04	0.10	0.94	90	7.46	6.44	1.02	211		41.6	194	0	259	454	46	54
18	3.3	6.88	1.00	0.16	0.84	84	0.24	71	1.14	0.13	1.01	89	7.56	6.65	0.91	188		33.5	209	0	65	274	69	31
25	3.3	7.50	1.01	0.15	0.86	85	0.25	71	1.02	0.04	0.98	96	7.43	6.58	0.85	176		29.4	203	0	79	282	62	38
25																	1.46	22.9						
42	3.3	10.94	1.21	0.16	1.05	87	0.21	80	1.28	0.06	1.22	95	7.43	7.25	0.18	37	0.55	16.9	252	188	48	489	8	92
45																		29.0						
49	3.3	9.06	0.98	0.16	0.82	84	0.20	76	1.03	0.02	1.01	98	7.61	6.22	1.39	287	0.51	25.6	209	8	116	333	86	14
64	3.2	8.91	0.96	0.11	0.85	89	0.18	79	1.12	0.05	1.07	96	8.08	7.10	0.98	203	0.43	14.3	221	17	103	340	60	40
69																		44.1						
73	3.3	9.69	0.95	0.10	0.85	89	0.18	79	1.20	0.05	1.15	96	8.41	7.45	0.96	199	0.40	40.6	238	6	117	361	55	45
92	3.4	14.22	0.94	0.08	0.86	91	0.16	81	1.07	0.03	1.04	97	7.63	7.44	0.19	39	0.40	28.9	215	0	84	299	13	87
97	3.3	6.17	0.92	0.08	0.84	91	0.16	81	1.09	0.03	1.06	97	7.60	7.09	0.51	105	0.36	26.3	219	8	74	301	35	**65**

[a]bioreactor pH; [b]planktonic bacteria, values in $x10^7$ mL^{-1} ; [c]% of glycerol oxidised; [d]% of glycerol oxidised to CO_2; [e]% of Zn precipitated in the bioreactor vessel; [f]Zn concentration in off-line treatment of synthetic waste water.

Appendix 6B. Data from in-line remediation of synthetic Minsterley Brook waste water, with glycerol and yeast extract supplied in a separate feed.

Time (h)	pH[a]	Bact x10^7/ml[b]	GYE feed flow rate[c] (mL/h)	YE[d] mg/L	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[f]	Zn^{2+} mM				SO_4^{2-} mM			Net [SO_4^{2-}] reduced	Off-line Cu^{2+} mM	H_2S µmoles/L/h			H_2S produced from reduction of: (%)	
					in[e]	out	Δ	out		in	out	Δ	%[g]	in	out	Δ	µmoles/L/h		in-line [ZnS]	off-line [CuS]	Σ	[SO_4^{2-}]	ZVS
0	3.3	9.2																47.9					
5	3.3	23.4	3.6	39	1.62	0.51	1.11	0.50	56	1.05	0.18	0.87	83	6.97	6.79	0.18	37	47.8	180	3	183	20	80
25	3.3	11.9	3.2	35	1.45	0.16	1.29	0.46	65	0.98	0.06	0.92	94	7.47	6.53	0.94	194	47.4	190	3	193	100	0
31	3.4	8.3	3.5	38	1.56	0.23	1.33	0.52	61	0.96	0.06	0.90	94	7.94	6.71	1.23	254	43.5	186	88	274	93	7
52	3.5	8.8	3.6	39	1.62	0.14	1.48	0.55	64	1.23	0.02	1.21	98	7.98	6.87	1.11	230	40.5	250	19	269	85	15
56	3.4	11.6	3.6	39	1.62	0.03	1.59	0.42	74	1.19	0.04	1.15	97	7.90	7.45	0.45	93	40.1	238	16	253	37	63
77	3.5	8.8	4.7	51	2.09	0.57	1.52	0.73	53	1.43	0.03	1.40	98	7.60	6.83	0.77	159	38.2	290	12	302	53	47
80	3.6	15.6	4.7	51	2.09	0.55	1.54	0.73	54	1.15	0.02	1.13	98	7.79	6.78	1.01	209	37.7	234	25	259	81	19

[a]bioreactor pH; [b]planktonic bacteria; [c]flow rate of the glycerol/yeast extract feed solution; calculated concentrations of [d]yeast extract and [e]glycerol in the combined synthetic mine water and glycerol/yeast extract influent solutions; [f]% of glycerol oxidised to CO_2; [g]% of Zn precipitated in the bioreactor vessel.

Appendix 6C. Independent-samples t-test conducted with relative amounts of H_2S generated *via* sulfate reduction when glycerol and yeast extract were supplied with a separate feed and when supplemented directly to the Minsterley Brook synthetic waste water

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
separate feed	7	67.00	30.52	11.54
directly supplemented	9	48.22	25.67	8.56

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	0.76	0.40	1.34	14	0.20	18.78	14.04	-11.33	48.88
	Equal variances not assumed			1.31	12	0.22	18.78	14.36	-12.59	50.15

Appendix 6D. Data from in-line remediation of actual Minsterley Brook waste water, with glycerol and yeast extract supplied in a separate feed.

Time (h)	pH[a]	Gly/YE feed Flow rate[b] (mL/h)	YE[c] mg/L	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[e]	Zn^{2+} mM				SO_4^{2-} mM			Net $[SO_4^{2-}]$ reduced	H_2S µmoles/L/h	H_2S produced from reduction of: (%)	
				In[d]	out	Δ	out		in	out	Δ	%[f]	in	out	Δ	µmoles/L/h	in-line [ZnS]	$[SO_4^{2-}]$	ZVS
0	6.7	3.70	40																
6	6.8	3.79	41	1.71	0.79	0.92	0.61	34	0.88	0.01	0.87	99	8.00	7.83	0.17	36	180	20	80
24	6.8	3.77	41	1.70	0.67	1.03	0.66	37	0.88	0.01	0.87	99	8.00	7.66	0.33	68	180	38	62
29	6.7	3.84	42	1.73	0.77	0.96	0.61	37	0.88	0.01	0.87	98	8.00	7.56	0.44	92	179	51	49
48	6.7	3.89	42	1.75	0.67	1.09	0.50	55	0.88	0.01	0.87	99	8.00	7.80	0.20	41	180	23	77
56	6.8	3.75	41	1.69	0.69	1.00	0.41	59	0.88	0.02	0.86	98	8.00	7.57	0.43	90	179	50	50
73	6.9	3.74	41	1.69	0.44	1.25	0.36	71	0.88	0.06	0.82	93	8.00	7.70	0.30	61	169	36	64
78	7.0	3.82	42	1.72	0.13	1.59	0.48	70	0.88	<0.01	0.88	>99	8.00	7.30	0.70	145	182	80	20
95	6.9	3.56	39	1.61	0.14	1.46	0.39	73	0.88	0.14	0.74	84	8.00	7.65	0.35	72	152	47	53
102	6.9	3.95	43	1.78	0.06	1.72	0.38	78	0.88	<0.01	0.88	>99	8.00	7.80	0.20	42	182	23	77
119	6.7	3.85	42	1.74	0.20	1.54	0.69	55	0.88	0.02	0.85	97	8.00	7.53	0.47	98	177	55	45
123	6.8	3.90	42	1.76	0.13	1.63	0.70	57	0.88	0.02	0.86	97	8.00	7.74	0.26	54	177	31	69

[a]bioreactor pH; [b]flow rate of the glycerol/yeast extract feed solution; calculated concentrations of [c]yeast extract and [d]glycerol in the combined actual mine water and glycerol/yeast extract influent solutions; [e]% of glycerol oxidised to CO_2; [f]% of Zn precipitated in the bioreactor vessel.

Appendix 6E. Data from in-line remediation of synthetic Force Crag mine water supplemented with glycerol and yeast extract.

Time (h)	pH[a]	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[b]	Cu^{2+} mM				Zn^{2+} mM				SO_4^{2-} mM			Off-line Cu^{2+} mM	H_2S µmoles/L/h			
		in	out	Δ	out		in	out	Δ	%[c]	in	out	Δ	%[d]	in	out	Δ		in-line [ZnS]	in-line [CuS]	off-line [CuS]	Σ
0																		13.18				
18	3.6	0.042	0.0	0.04	0.036	14	0.055	0	0.055	>99	0.050	0.014	0.036	72	0.32	0.47	-0.15	12.91	7	11	2	9
26	3.6	0.039	0.0	0.04	0.032	18	0.057	0	0.057	>99	0.091	0.009	0.082	90	0.41	0.53	-0.12	12.74	17	12	3	20
42	3.6	0.038	0.0	0.04	0.048	0	0.057	0	0.057	>99	0.046	0.008	0.038	83	0.34	0.50	-0.16	11.63	8	12	9	17
49	3.6	0.035	0.0	0.04	0.027	23	0.060	0	0.060	>99	0.079	0.009	0.070	89	0.40	0.51	-0.11	11.49	14	12	3	17
66	3.6	0.041	0.0	0.04	0.036	12	0.060	0	0.060	>99	0.045	0.005	0.040	89	0.33	0.50	-0.17	11.24	8	12	2	10

[a]bioreactor pH; [b]% of glycerol oxidised to CO_2; [c]% of Cu precipitated in the bioreactor vessel; [d]% of Zn precipitated in the bioreactor vessel.

Appendix 6F. Data from in-line remediation of actual Force Crag waste water, with glycerol and yeast extract supplied in a separate feed.

Time (h)	pH[a]	Mine water Flow rate[b] (mL/h)	Gly/YE feed Flow rate[c] (mL/h)	YE[d] mg/L	Glycerol mM			Acetic acid mM	Gly → CO_2 (%)[f]	Zn^{2+} mM				SO_4^{2-} mM			Off-line Cu^{2+} mM	H_2S µmoles/L/h		
					In[e]	out	Δ	out		in	out	Δ	%[g]	in	out	Δ		in-line [ZnS]	off-line [CuS]	Σ
0																	14.94			
19	5.8	455	6	33	0.26	<0.01	0.26	0.53	0	0.049	0.005	0.044	90	0.38	0.46	-0.09	13.81	9	8	17
21	6.0	455	6	33	0.26	<0.01	0.26	0.45	0	0.049	0.005	0.044	90	0.38	0.46	-0.09	13.71	9	8	17
38	6.3	455	6	33	0.26	<0.01	0.26	0.44	0	0.052	<0.001	0.052	>99	0.37	0.44	-0.07	13.10	11	5	16
42	6.3	455	6	33	0.26	<0.01	0.26	0.35	0	0.052	<0.001	0.052	>99	0.37	0.44	-0.07	12.74	11	13	23
47	6.0	840	6	18	0.14	<0.01	0.14	0.42	0	0.052	<0.001	0.052	>99	0.37	0.44	-0.07	12.61	20	3	23
61	6.4	1520	6	10	0.08	<0.01	0.08	0.08	0	0.052	0.019	0.033	63	0.37	0.43	-0.06	12.61	23	<0.1	23
63	6.5	1180	7	15	0.29	0.03	0.26	0.10	62	0.052	0.014	0.038	73	0.37	0.44	-0.07	12.61	20	<0.1	20
67	6.3	1180	7	15	0.29	0.04	0.26	0.18	30	0.052	<0.001	0.052	>99	0.38	0.45	-0.08	12.61	28	<0.1	28
69	6.3	1180	7	15	0.29	0.03	0.26	0.17	36	0.052	<0.001	0.052	>99	0.38	0.45	-0.08	12.61	28	<0.1	28
71	6.3	1320	14	25	0.51	0.07	0.43	0.17	60	0.052	<0.001	0.052	>99	0.38	0.46	-0.09	12.61	31	<0.1	31
74	6.6	1520	11	17	0.35	0.08	0.27	0.09	65	0.047	<0.001	0.047	>99	0.38	0.44	-0.07	12.61	32	<0.1	32
78	6.3	1520	11	17	0.35	0.15	0.20	0.21	0	0.047	<0.001	0.047	>99	0.38	0.45	-0.08	12.61	32	<0.1	32

[a]bioreactor pH; [b]Mine water flow rate; [c]flow rate of the glycerol/yeast extract feed solution calculated concentrations of [d]yeast extract and [e]glycerol in the combined actual mine water and glycerol/yeast extract influent solutions; [f]% of glycerol oxidised to CO_2; [g]% of Zn precipitated in the bioreactor vessel.

Appendix 6G. Independent-samples t-test conducted with rates of H_2S generated during in-line treatment of Force Crag mine water

Group Statistics				
Variable	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
Synthetic mine water	5	14.6	4.8	2.2
Actual mine water	12	24.1	6.1	1.8

Independent Samples Test										
		Levene's Test for Equality of Variances		**t-test for Equality of Means**						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Variable	Equal variances assumed	0.53	0.48	-3.09	15	0.007	-9.48	3.07	-16.0	-2.94
	Equal variances not assumed			-3.41	9.50	0.007	-9.48	2.78	-15.7	-3.24

Appendix 6H. One-way analysis of variance (ANOVA) with post-hoc test comparing percentages of H_2S production *via* sulfate reduction during in-line treatment of Minsterley Brook Catchment waste water. “Actual separate feed” to waste water collected at Snailbeach Farm with glycerol and yeast extract supplied as a separate feed. “Synthetic separate feed” refers to glycerol and yeast extract also supplied as a separate feed. “Synthetic direct” refers to synthetic waste water supplemented directly with glycerol and yeast extract

Descriptives

VAR00001

	N	Mean	Std. Deviation	Std. Error	90% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Actual separate feed	12	42.42	17.40	5.02	31.36	53.47	20.00	80.00
Synthetic separate feed	7	67.00	30.52	11.54	38.77	95.23	20.00	100.00
Synthetic direct	9	48.22	25.67	8.56	28.49	67.95	8.00	86.00
Total	28	50.43	25.04	4.73	40.72	60.14	8.00	100.00

Test of Homogeneity of Variances

VAR00001

Levene Statistic	df1	df2	Sig.
2.404	2	25	0.111

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2736.4	2	1368.2	2.41	0.11
Within Groups	14190.5	25	567.6		
Total	16926.9	27			

Multiple Comparisons

Dependent Variable: VAR00001

Tukey HSD

(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	95% Confidence Interval
					Lower Bound	Upper Bound
Actual separate feed	Synthetic separate feed	-24.58	11.33	0.10	-52.81	3.64
	Synthetic direct	-5.81	10.51	0.85	-31.97	20.36
Synthetic separate feed	Actual separate feed	24.58	11.33	0.10	-3.64	52.81
	Synthetic direct	18.78	12.01	0.28	-11.13	48.68
Synthetic direct	Actual separate feed	5.81	10.51	0.85	-20.36	31.97
	Synthetic separate feed	-18.78	12.01	0.28	-48.68	11.13

Appendix 6J. One-way analysis of variance (ANOVA) with post-hoc test comparing percentages of H_2S production *via* sulfate reduction during in-line treatment of Minsterley Brook Catchment waste water. “Actual separate feed” to waste water collected at Snailbeach Farm with glycerol and yeast extract supplied as a separate feed. “Synthetic direct” refers to synthetic waste water supplemented directly with glycerol and yeast extract. “Synthetic separate feed” refers to glycerol and yeast extract also supplied as a separate feed.

Homogeneous Subsets

Tukey HSD[a,b]

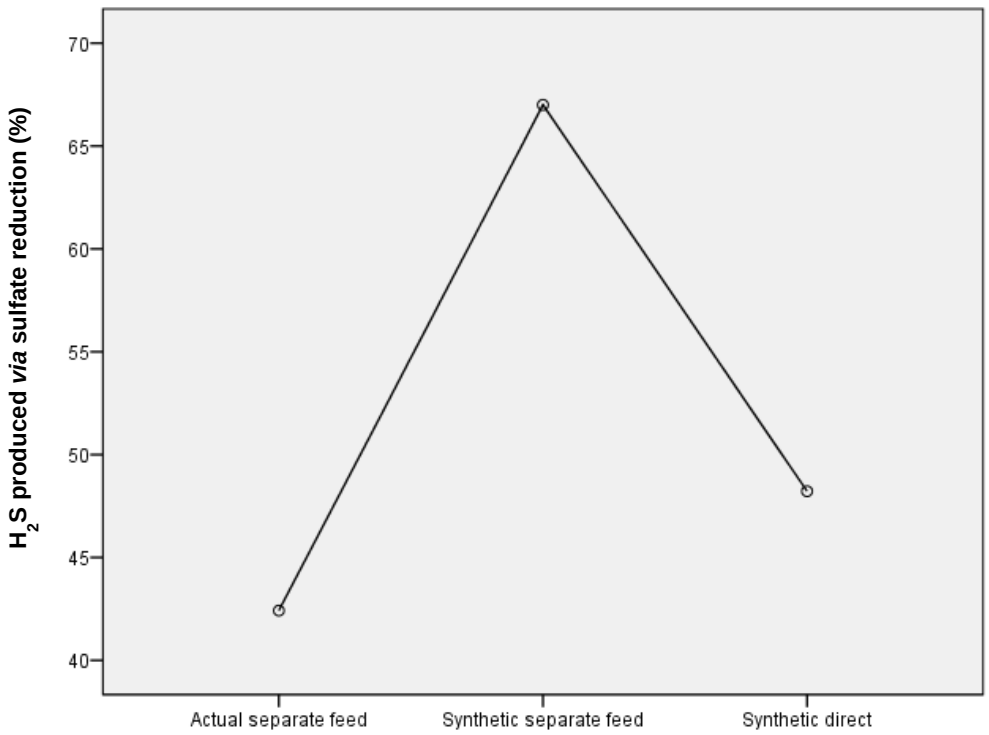
VAR00002	N	Subset for alpha = 0.05
		1
Actual separate feed	12	42.42
Synthetic direct	9	48.22
Synthetic separate feed	7	67.00
Sig.		0.095

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.894.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Means Plots



Appendix 7A. Data from experiment 1.

Time h	H_2SO_4 mL[a]	H_3O^+ mmoles[b]	Off-line Cu^{2+} mM	H_2S produced mmoles
0	0	0	283	0
20	3	4	283	0
44	31	39	283	0
116	77	98	283	0
141	83	105	283	0
171	179	227	279	1.8
188	181	230	276	3.0
212	183	232	271	4.8

[a]volume of sulfuric acid consumed; [b]H_3O^+ consumption equivalent to volume of acid consumed

Appendix 7B. Data from experiment 2.

Time h	H_2SO_4 mL[a]	H_3O^+ mmoles[b]	Off-line Cu^{2+} mM	H_2S produced mmoles
0	0	0	283	0
19	31	39	275	3.4
22	37	47	275	3.4
24	37	47	250	13.4
28	38	48	250	13.4
46	41	52	250	13.4
47	41	52	250	13.4
69	44	56	250	13.4
91	45	57	250	13.4

[a]volume of sulfuric acid consumed; [b]H_3O^+ consumption equivalent to volume of acid consumed

Appendix 7C. Data from experiment 3.

Time h	H_2SO_4 mL[a]	H_3O^+ mmoles[b]	Fe^{2+} mmoles[c]	Off-line Cu^{2+} mM	H_2S produced mmoles
0	0	0		245	0
2	3	4	0	245	0
4	23	32	1	245	0
6	69	95	19	245	0
8	69	95	20	245	0
23	70	96	43	245	0
25	70	96	39	245	0
27	70	96	41	245	0
29	70	96	42	245	0
32	70	96	42	245	0
47	70	96	46	245	0
51	70	96	46	245	0
74	70	96	52	245	0

[a]volume of sulfuric acid consumed; [b]H_3O^+ consumption equivalent to volume of acid consumed; [c]ferrous iron generated during experiment.

Appendix 7D. Data from experiment 4

Time h	H_2SO_4 mL[a]	H_3O^+ mmoles[b]	Fe^{2+} mmoles[c]	Off-line Cu^{2+} mM	H_2S produced mmoles	H_2S produced µmoles L^{-1} h^{-1} [f]
0.0	0	0	0	234	0.0	
4.3	0	0	6	234	0.0	
24.8	0	0	10	234	0.0	
27.5	4	7	11	231	1.0	
44.3	14	23	17	220	5.5	
48.3	16	26	22	220	5.5	
52.1	20	33	26	220	5.5	
116.3	46	76	50	198	14.1	
116.3				10.1		
122.8	50	83	53	1.0	17.8	
140.3	58	96		17.4		
140.8	58	96	59	15.3	18.6	
147.8	63	104	64	9.7	20.8	
164.3	72	119	75	2.0	23.9	
168.5	76	125	75	0.1	24.7	
168.5				15.7		
189.1	82	139	95	10.7	26.7	
196.1	87	148	91	7.8	27.8	
212.3	94	160	98	0.7	30.7	145
212.8				17.2		
218.3[d]	103	170	103	13.3	32.2	
263.8	132	218	129	0.0	37.6	
263.8				18.9		
283.8	152	251	146	11.5	40.5	117
290.1[e]	162	267	155	10.9	40.7	
291.3				17.5		
291.8	174	287	166	7.3	44.8	
308.3	196	323	177	7.3	44.8	
308.3				18.6		
308.8	202	333	193	2.7	51.1	
315.8	214	353	193	2.7	51.1	
315.8				20.0		
316.3	220	363	194	10.5	54.9	
340.3	254	419	216	10.0	55.1	
340.3				19.8		
340.5	260	429	229	6.3	60.5	
358.4	290	479	249	6.0	60.6	
358.4	290	479		50.4		
358.8	294	485	252	40.8	64.5	346

[a]volume of sulfuric acid consumed; [b]H_3O^+ consumption equivalent to volume of acid consumed; [c]ferrous iron generated during experiment; [d]first sample with temperature of 35 °C; [e]first sample with temperature of 45 °C; [f]overall rate of H_2S produced at 25°C, 35 °C and 45 °C.

Appendix 7E. Data from experiment 5

Time h	H_2SO_4 mL[a]	H_3O^+ mmoles[b]	Fe^{2+} mmoles[c]	Off-line Cu^{2+} mM	H_2S produced mmoles	H_2S produced µmoles L^{-1} h^{-1} [f]
0.0	0		0	19	0	
2.7	0	0	1	19	0	
18.1	2	3	1	19	0	
18.2	4	7	7	17	1	
27.7	4	7	7	17	1	
27.8	6	10	13	12	3	
45.2	7	12	13	12	3	
45.3	9	15	21	5	6	
50.9	9	15	21	5	6	
51.0	9	15		50		
51.1	12	20	27	46	7	
66.2	12	20	26	45	7	
66.4	14	23	32	42	9	
75.1	14	23	33	41	9	
75.3	16	26	37	37	11	
91.0	16	26	37	37	11	
91.1	18	30	44	32	13	
99.1	18	30	44	31	13	
99.3	20	33	48	28	14	
162.1	24	40	53	28	14	
162.3	28	46	62	21	17	106
170.5[d]	28	46	68	21	17	
170.7	34	56	79	10	21	
186.4	34	56	80	10	21	
186.6	42	69	98	0	25	
191.6	42	69		47		
194.7	42	69	97	47	25	
194.9	47	78	110	36	30	
211.0	48	79	112	36	30	
211.1	52	86	126	25	34	317
218.8[e]	54	89	126	25	34	
218.8	54	89		48		
219.0	60	99	138	38	38	
234.5	60	99	146	38	38	
234.5	60	99		47		
234.6	70	116	152	35	43	
242.6	70	116	164	35	43	
242.6	70	116		47		
242.9	80	132	180	32	49	
259.7	80	132	191	32	49	
259.9	102	168	208	17	55	
266.5	102	168	202	15	55	
266.5	102	168		46		
266.6	113	186	240	22	65	639

[a]volume of sulfuric acid consumed; [b]H_3O^+ consumption equivalent to volume of acid consumed; [c]ferrous iron generated during experiment; [d]first sample with temperature of 35 °C; [e]first sample with temperature of 45 °C; [f]overall rate of H_2S produced at 25°C, 35 °C and 45 °C.

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