DOCTOR OF PHILOSOPHY

A study of the biogeochemistry and molecular biology of a chalybeate iron-rich spa (North Wales)

Jones, Rose Mary

Award date:
2015

Awarding institution:
Bangor University

PRIFYSGOL
BANGOR
UNIVERSITY

A study of the biogeochemistry and molecular biology of a chalybeate iron-rich spa (North Wales)

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

by

Rose Mary Jones

M Env Sci (Hons)

School of Biological Sciences

Bangor University

Gwynedd, LL57 2UW

U.K.

2015

Abstract

Cae Coch ore body is responsible for acid mine drainage from the abandoned Cae Coch sulfur mine and Trefriw Wells Spa water. These waters are extremely acidic and contain elevated ferrous iron concentration and low concentrations of other transition metals. Trefriw Wells Spa water has been used as a "natural" remedy for iron-deficiency for over 100 years. The current study examined biogeochemical processes related to weathering of the pyrite ore responsible for this water, focusing on fundamental and applied areas of acidophile microbiology.

Concentration of ferrous iron in the spa water is declining over time, so a protocol for synthesising a concentrated analogue was established to possibly supplement the product in future years. This process used the same general processes as the natural product (oxidative dissolution of pyrite catalysed by chemolithotrophic acidophiles), resulting in liquor containing ~x10 the ferrous iron concentration of the original water. The viability of two neutrophilic bacteria in the acidic spa water (strains of *Dermacoccus nishinomiyaensis* and *Kocuria marina*), isolated from Spatone sachets was also investigated. Isolates exhibited rapid morbidity, with no viable bacteria isolated 24 hours after addition to the spa water.

Bacterial communities at the site were examined by a combined molecular and cultivation-based approach; some bacteria not previously characterised were identified and, for some, isolated from waters samples from the Trefriw Wells Spa. These included the proposed novel species *"Acidocella acidivorans"* which was studied in detail, validated novel species *Acidocella aromatica* and validated novel genus and species *Acidithrix ferrooxidans*. All of these heterotrophic acidophiles catalysed the dissimilatory reduction of ferric iron. *Atx. ferrooxidans* also catalysed the dissimilatory oxidation of ferrous iron, a key process in Trefriw water formation. This ability, and streamer-forming growth in flowing water was harnessed in a continuous flow bioreactor, proposed as a component module of a system to remediate acidic, iron-rich mine waters. Each characterised novel bacterial species has characteristics that are potentially useful in biotechnologies such as bioremediation.

Acknowledgements

I would like to thank the following people for their kind support and advice:

in particular, my supervisor Barrie Johnson,

also Barry Grail, Sabrina Hedrich, Katherine Cay, Carmen Fálagan and all other BART members and visitors who have helped me.

Chris Oldknow and the Spatone personnel for their assistance, and access to the site and data.

Faith Jones for her assistance with R programming.

The KESS scholarship scheme for providing funding.

Contents

List of Figures

List of Tables

Abbreviations

16S rRNA Small subunit of ribosomal ribonucleic acid

AMD Acid mine drainage

ARD Acid rock drainage

ATP Adenozine tri-phosphate

BART Bangor acidophile research team

BLAST Basic logical alignment search tool

bp Base pair

BSA Bovine serum albumen

°C Degrees celsius

CFU Colony forming units

CTAB Cetyl trimethylammonium bromide

DMSO Dimethylsulfoxide

DNA Dioxyneucleic acid

dNTP Deoxynucleic triphosphate

EDTA Ethylene-diamine-tetraacetic acid

E_H Redox

EPS Extra-cellular polymeric substance

FeTSBo/PFBC Heterotrophic solid medium with tryptone soy broth and isolate PFBC in the underlayer

FeSo Autotrophic solid medium plates with iron and sulfur

FeTo Solid medium for moderate acidophiles

Fig. Figure

FISH Fluorescent *in situ* hybridisation

FOB Ferrous iron-oxidising bioreactor

Fru Fructose solid medium

Fruo Fructose solid overlay medium

Fru/YE Fructose and yeast extract solid medium

Fru/Yeo Fructose and yeast extract solid overlay medium

g Gram

G+C% mol Percentage of guanine and cytosine content

g/L Gram per liter

Glu Glucose solid medium

Glu/YE Glucose and yeast extract solid medium

Glu/Yeo Glucose and yeast extract solid overlay medium

h Hour

iFeo Autotrophic solid overlay medium for iron oxidisers

ITS 16S-23S internal transcribed spacer

KESS Knowledge Economy Skills Scheme

L Liter

m Meter

M Molar

MDS Multi-dimensional scaling

mm Milimeter

min Minute

mg Miligram

mg/L Miligrams per liter

mL Milliliter

MLSA Multi-locus sequence analysis

mM Millimolar

mV Milivolt

NADPH The reduced form of nicotinamide adenine dinucleotide phosphate

ND Not determined

N/S Not subcultured

nm Nanometers

nt Neucleotide

OD_{600} Optical density measured at 600 nm

OFN Oxygen-free nitrogen

PCR Polymerase chain reaction

PMF Proton motive force

p.s.i. Pounds per square inch

r^2 Regression analysis

RISC Reduced inorganic sulfur compound

RNA Ribonucleic acid

RO Reverse osmosis

rpm Revolutions per minute

s Seconds

TCA Tri-carboxylic acid

T-RF Terminal restriction enzyme fragment

T-RFLP Terminal restriction enzyme fragment length polymorphism

V Volt

VBNC Viable but not culturable

YE3 Yeast extract solid medium pH 3

YE3o Yeast extract solid overlay medium pH 3

YE3o/PFBC Yeast extract solid overlay medium pH 3 with isolate PFBC in the underlay

YE4 Yeast extract solid medium pH 4

ZVI Zero-valent iron

µm Micro-molar

µg/mL Microgram per liter

UPGMA Unweighted pair group method with arithmetic mean

w/v Weight per volume

< Less than

x g Gravitational force

~ Approximately

ΔG Gibbs free energy (potential energy obtainable from a thermodynamic system)

Chapter 1. Introduction

1.1. Acidic environments and acidophilic microorganisms

1.1.1. Acidophilic habitats

Lewis acid theory classifies an acid as a compound that can accept an electron pair (Lewis, 1923). According to the Brønstead-Lowry theory, an acid is a substance that increases the concentration of H^+ (H_3O^+) ions in a solution by donating protons (Brønstead, 1923; Lowry, 1923). Acidity is measured in logarithmic scale of proton activity (often assumed to correlate with proton concentration), or the pH scale. An environment is acidic if it has a pH of 5 or below (Kristjánsson and Hreggvidsson, 1995). Moderately acidic environments are defined as those with a pH of 3-5, and extremely acidic environments as those with a pH of 3 or less. Environmental pH values as low as -3.6 have been reported in Iron Mine, California (Nordstrom *et al.*, 2000). The current study focuses on the microbiology and geochemistry of water bodies that are classified as extremely acidic, according to the definition above.

Extremely acidic conditions are formed by a combination of biotic and abiotic processes. Sulfur from a variety of sources reacts with air and water in a variety of oxidation states (S^0 and tetrathionate being the most stable at low pH; Baker and Banfield, 2003). Resultant sulfur compounds contribute to acidic conditions through oxidation and formation of H_2SO_4. Therefore, extremely acidic habitats often contain high concentrations of soluble sulfur species (Kishimoto *et al.*, 1990; Rawlings, 2005). Sources of sulfur include geothermic activity (H_2S and SO_2), where hydrogen sulfide can be directly oxidised to sulfuric acid by prokaryotic microorganisms, or react with SO_2 to form elemental sulfur (S^0), which is then biologically oxidised. Sulfur sources also include oxidation of reduced sulfide (and elemental sulfur) minerals such as pyrite (FeS_2), chalcopyrite ($CuFeS_2$) and sphalerite (ZnS). Sulfur (in pyrite, organic-S and sulfate) also occurs in coal deposits. Acidic water bodies generated by oxidation of pyritic minerals are known as acid rock drainage (ARD) or acid mine drainage (AMD) when they result from mining activity (Johnson and Hallberg, 2005). These habitats

are not necessarily aerobic, as the oxygen requirement for oxidising reactions is often more than groundwater can supply. Thus, the lower reaches of an acidic water body can often be anaerobic (Baker and Banfield, 2003; Falagan *et al.*, 2014). The final pH of such habitats is dependent upon acid genesis rates and the geochemical nature and buffering capacity of the surrounding environment.

Acidic environments also contain relatively high dissolved metal concentrations, mainly due to increased solubility of cationic metals at low pH (Pikuta and Hoover, 2007). Solubility enhances the bioavailability of potentially toxic metals. Exley and Birchall (1992) define bioavailability as the potential of a substance to interact with a biological system, and its ability to produce a response from that system. Dissolved metals are more accessible, so they are more available for use by acidophiles (Bird *et al.*, 2011), and more toxic (Steven *et al.*, 1999). This results in inorganic electron donors such as iron being more abundant in these habitats than carbon, making them important in the ecosystem.

Iron has three forms; Fe^0 (zero-valent iron; ZVI), Fe^{2+} (ferrous iron) and Fe^{3+} (ferric iron). It is only present in soluble form in extremely low pH or reducing conditions (Lovely, 1987). Other factors that affect iron solubility include temperature, ionic solution strength and the presence of chelating agents (Suter *et al.*, 1991). In environments above ~pH 3.5, iron is mainly found as oxy-hydroxide minerals (e.g. goethite and ferrihydrite) and as soluble Fe^{2+} in anaerobic environments (e.g. sediments and anaerobic soils). Amorphous ferric iron compounds form a significant portion of freshly deposited ferric hydroxides, with more defined structure resulting from age and dehydration (Schwertmann and Taylor, 1977). pH also has a strong influence on the mineral species formed. Below ~pH 3.5, ferrous iron begins to dissolve, and below approximately pH 2.3 ferric iron also begins to dissolve. At low pH, the Fe^{2+}/Fe^{3+} redox couple standard reduction potential (E_0 +0.720 V at pH 3) is close to that of O_2/H_2O at pH 7 (+ 0.82 V) (Johnson *et al.*, 2012). This is an important reaction in extremely acidic conditions (Fig. 1.1). Iron-oxidising bacteria such as the acidophilic

Acidithiobacillus (At.) ferrooxidans can catalyse the rate of iron cycling, increasing the rate of iron oxidation by a factor of ~10^6 (Baldi *et al.*, 1992).

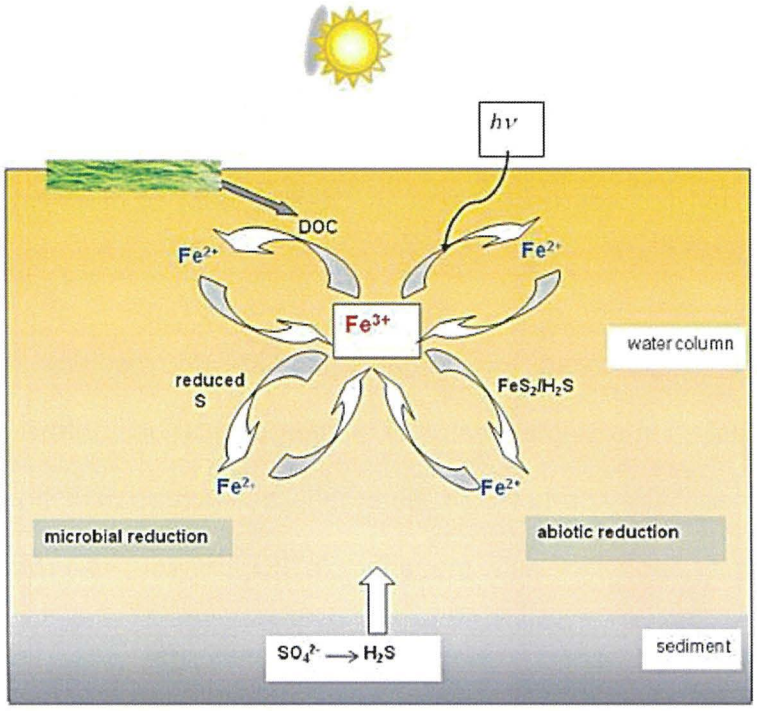


Figure 1.1. Schematic of redox transformations of iron in an extremely low pH (<3) water body (Johnson *et al.*, 2012).

Another feature of acidic environments is that they usually contain relatively small concentrations of dissolved organic carbon and soluble nitrogen. Acidic environments are classed as oligotrophic, as dissolved organic carbon is usually < 20 mg/L (Kimura *et al.*, 2011). Carbon can originate from external organic sources such as leaves and pit props, and carbon-fixing microbes such as chemo-lithotrophs and chemo-phototrophs. Ňanchueo and Johnson (2011) and Ňanchueo and Johnson (2013) showed that acidophilic heterotrophs in AMD can use exudates from both acidophilic chemoautotrophic bacteria and acidophilic algae. Nitrogen may derive from fixation of N_2 by diazotrophic species, with minimal addition from external sources. Heterotrophs play an important role as nutrient cyclers, often forming syntrophic relationships with autotrophic species (section 1.1.4).

1.1.2. General characteristics of acidophilic bacteria

Acidophiles are often differentiated according to whether their optimum pH coincides with threshold values of moderate or extreme acidic environments. They are mostly prokaryotic bacteria and archaea (Johnson, 1998), though many species of acidophilic eukaryotic microorganisms (algae, yeasts and fungi) have also been described (Baker and Banfield, 2003). This section focuses on bacteria. A brief account of other acidophiles is given in section 1.1.5.

Prokaryotes are single-celled microorganisms with no membrane-bound nuclei. The chromosome clusters in a single mass that is often circular, in contrast to eukaryotes where it is generally linear. They do not possess organelles such as mitochondria or chloroplasts, unlike eukaryotes. Table 1.1 lists the principle components of prokaryotic bacteria and archaea, and differences between Gram-positive and Gram-negative bacteria. Other differences between archaea and bacteria include genetic transcription and translation, and in cell biochemical structure.

Generally, Gram-negative bacteria are mesophilic, Gram-positive bacteria are moderately thermophilic and archaea are extreme thermophiles. Exceptions include *Acidicaldus organivorans*, a thermophilic Gram-negative genus (Johnson *et al.*, 2006) and *Ferroplasma acidiphilum* (Golyshina *et al.*, 2000).

Table 1.1. Some structures found within prokaryotic bacteria and archaea, showing differences between Gram-positive bacteria, Gram-negative bacteria, and archaea. + represents present, - represents absent and +/- represents variable presence (Madigan, 2014).

Location	Structure	Gram positive bacteria	Gram negative bacteria	Archaea	Function
Cytoplasm	Chromosome	+	+	+	Cellular DNA storage and replication
	Plasmid	+/-	+/-	+/-	Variable additional ring of DNA
	Ribosomes	+	+	+	Protein synthesis
Cell envelope	Cytoplasmic membrane	+	+	+	Selectively permeable lipid bi-layer with embedded proteins
	Periplasmic space	-	+	-	Space between outer membrane and cell wall, involved in biochemical processes.
	Cell wall	+/-	+	+/-	Rigid, permeable peptidoglycan structure for giving shape and protection
	Outer membrane	-	+	-	Second selectively permeable membrane
	S-layer	+/-	+/-	+/-	Single-molecule barrier layer.

					Gram-positive contains peptidoglycan. Gram-negative contains lipoplysaccharide
Cell exterior and appendages	Glycolax	+	+	+	Variable protective layer of extra-cellular polymeric substances (EPS), proteins and DNA
	Flagella	+/-	+/-	+/-	Variable long structures for mobility
	Fimbriae/pili	+/-	+/-	+/-	Variable hollow protein structures for adhesion and (pili) genetic exchange.

Acidophilic prokaryotes can also be differentiated by their oxygen requirement. Facultative or obligate aerobes require oxygen. Those that grow optimally under a reduced percentage of oxygen are microaerobic, and obligate or facultative anaerobes require no oxygen at all. Anaerobic respirators can use Fe^{3+} (Baker and Banfield, 2003), S^0 and SO_4^{2-} (Ohmura *et al.*, 2002) as terminal electron acceptors, coupled to an energy source (or electron donor). A large proportion of known acidophilic heterotrophs can use ferric iron as a terminal electron acceptor for growth under microaerobic conditions and, in some cases, in anoxic environments. There are no currently known extremely acidophilic bacterial or archaeal fermenters.

The cytoplasm of acidophilic bacteria is circum-neutral, though the pH of the periplasm between layers of cell membrane can be much lower (Norris and Ingledew, 1992). This difference in pH between cell cytoplasm and the external environment creates a large proton gradient, encouraging diffusion of H+ across the membrane. The energy potential generated by this is the proton motive force (PMF) ATP formation can be generated using this proton gradient; converting energy caused by the difference in charge potential into a chemical form the bacteria can use (Fig. 1.2). In bacteria that oxidise iron, electrons generated by that process are combined (via cytochromes) with protons to oxidise oxygen to water.

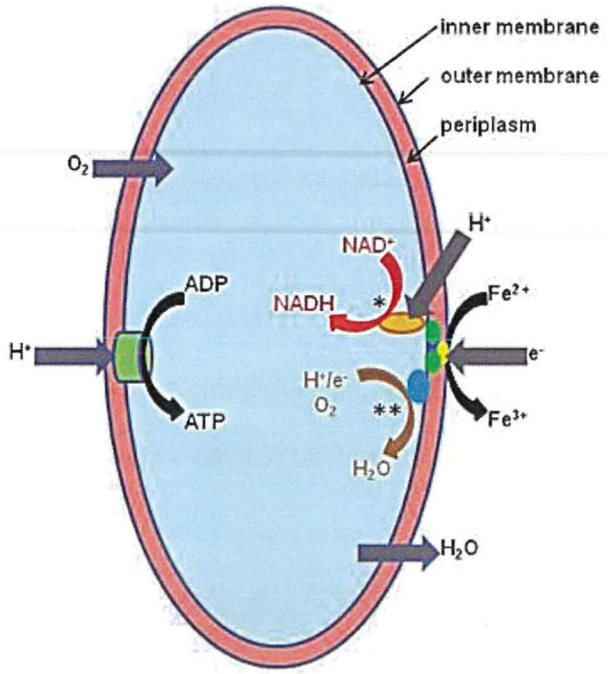


Figure 1.2. Diagrammatic representation of ferrous iron oxidation and energy generation by the Gram-negative acidophilic bacteria *At. ferrooxidans* (Johnson *et al.*, 2012).

The PMF can also drive flagella (for cell motility) and fuel active transport across cell membranes.

Other mechanisms used by extremely acidophilic bacteria in response to the low pH of their environment include maintenance of a positive charge of the cell membrane (Arsene-Ploetze *et al.*, 2012), and inclusion of hopenoids and other strengthening molecules in the cell membrane (Arsene-Ploetze *et al.*, 2012). Hopenoids are pentacyclic compounds that have roles in strengthening and reducing permeability of the bacterial plasma membrane (Kannenberg and Poralla, 1999; Welander *et al.*, 2009). Intracellular accumulation of cations (mostly K^+) maintains the positive charge, in contrast to neutrophiles, which have negatively charged membranes (Matin, 1999). This positive charge makes them more tolerant of cationic heavy metals than neutrophiles, though more sensitive to anionic metals (Norris and Ingledew, 1992).

Acidophilic bacteria may be either planktonic (free-swimming) or exist as embedded communities in gelatinous EPS matrices such as biofilms (thin layers), streamers (filaments) and 'snottites' or 'bio-tubes' (pendulous stalactite-like formations). The tendency to exist as one of these forms varies between genera, species and sometimes strains, and can be influenced by time, substrate type and substrate surface conditions (Ghauri *et al.*, 2007, Harneit *et al.*, 2006; Sand and Gherke, 2006). Most studies on this topic have focused on *At. ferrooxidans* strains (Wakao *et al.*, 1984, Kinzler *et al.*, 2001, Rohwerder *et al.*, 2003). Attachment initially involves electrostatic interactions followed by strengthening in some cases by EPS, though this is not always permanent (Ghauri *et al.*, 2007, Harneit *et al.*, 2006).

Acidophiles can use carbon for cellular biosynthesis from inorganic sources (autotrophy) or organic sources (heterotrophy). Chemo-organotrophy and chemo-lithotrophy refer to sourcing energy from organic or inorganic compounds. They may have obligate requirements for organic or inorganic carbon, or can use both (facultative autotrophs). Some acidophilic microorganisms can also use solar energy from light (acidophilic phototrophs). Obligately photosynthetic acidophiles appear to be exclusively eukaryotic algae (e.g. *Cyanidium* spp.); no extremely acidophilic cyanobacteria have yet been described. However, some acidophilic bacteria such as *Acidisphaera* spp. possess bacteriochlorophyll which may supplement their heterotrophic lifestyle (Hiraishi and Shimada, 2001; Hiraishi *et al.*, 2000). Hydrogen as an energy source is also used by some acidophilic bacteria, coupled to the reduction of oxygen or ferric iron (Hedrich and Johnson, 2013), or the reduction of sulfate such as by *Desulfosporosinus acididurans* (Sanchez-Andrea *et al.*, 2015).

In addition to oxidising iron (section 1.1.1), some acidophilic bacteria and archaea can also reduce iron. This can be assimilatory, dissimilatory or non-enzymatic. Assimilatory iron reduction involves uptake and intracellular reduction of free or complexed Fe^{3+} for use as a micronutrient. In dissimilatory reduction, Fe^{3+} is used as an external electron acceptor

(Jones, 1986). This is affected by pH, dissolved oxygen concentrations, iron availability and other factors.

1.1.3. Autotrophic acidophilic bacteria

Strictly speaking, an autotrophic bacterium acquires all its carbon from inorganic sources, though they may have a requirement for trace amounts of an essential organic compound (e.g. vitamins). The inorganic carbon source is usually carbon dioxide, fixed via the Calvin-Benson-Bassham cycle. The most common example is the RuBisCO pathway. This pathway requires energy, ATP and reductants e.g. NADPH. Other identified carbon sources used by acidophilic autotrophs includes carbon disulfide (CS_2; Smith and Kelly, 1988; Henstra and Stams, 2004).

Sirinivasan *et al.* (2012) argued that the addition of vitamins, pH buffers and other incidental carbon sources to media has muddled the strict definition of what constitutes an autotroph. This echoes Rittenberg (1971), who also declared the term 'obligate autotrophy' obsolete. However, this strict definition may be irrelevant as natural environments, including oligotrophic ones, are rarely completely free of organic carbon. In any case, it retains its usefulness as a concept, as its common use shows.

1.1.3.1. Acidophiles in the phylum *Nitrospira*

Phylum *Nitrospira* is a deeply-branching group, consisting of Gram-negative bacteria. Molecular biology studies indicates that there is more diversity than currently isolated (Rappé and Giovanni, 2003).

(i) Genus *Leptospirillum (L.)*

There are two validly described species, one contested species and two tentatively named isolates belonging to this Gram-negative genus; *L. ferrooxidans* (Hippe, 2000), *L. ferriphilum* (Coram and Rawlings, 2002), "*L. ferrodiazotrophum*" (Tyson *et al.*, 2005) and "*L. rubarum*"

which is known only by its genome sequence (Goltsman *et al.*, 2009). *L. thermoferrooxidans* (Golovacheva *et al.*, 1992) is no longer considered valid, as it was lost before a 16S rRNA gene sequence was obtained. Its physiological description is very similar to that of some strains of *L. ferriphilum*, and current consensus is that it was a strain of this later-described and validated *Leptospirillum* sp.. Strains and environmental clones of these species, along with many isolates known only by their 16S rRNA gene sequence, are distributed among four groups according to their phylogeny (Bond *et* al., 2000; Bond and Banfield, 2001; Coram and Rawlings, 2002; Tyson *et al.*, 2005; Goltsman *et al.*, 2013).

Leptospirillum spp. are often present in much higher abundance than *Acidithiobacillus* spp. in aerobic iron-rich extremely acidophilic environments, though members of both genus can oxidise iron (Sans *et al.*, 1992). *Leptospirillum* spp. are more acidophilic, have a higher affinity for ferrous iron and a higher tolerance of ferric iron than *Acidithiobacillus* spp., meaning they can better exploit aerobic extremely acidic environments that contain small concentrations of ferrous iron and large concentrations of ferric iron (Rawlings *et al.*, 1999; Norris *et al.*, 1988). Mesophilic and moderately thermophilic strains of *Leptospirillum* have been described (Coram and Rawlings, 2002), and have been found in liquors with pH as low as 0.83 (Tyson *et al.*, 2004). *L. ferrooxidans* and "*L. ferrodiazotrophum*" also fix nitrogen, making them an important source of these macronutrients in environments such as underground AMD sites (Tyson *et al.*, 2004).

1.1.3.2. Acidophiles of the phylum *Proteobacteria*, class *Betaproteobacteria*

Proteobacteria is a large phylum of Gram-negative bacteria, split into classes *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, and *Epsilon-proteobacteria* and the recently delineated *Acidithiobacillia*.

(i) Genus *"Ferrovum" (Fv.)*

"*Ferrovum*" currently contains only one described species; "*Ferrovum myxofaciens*" (Johnson *et al.*, 2014). It is the only known acidophile within the *Betaproteobacteria* class,

with isolates obtained from iron-rich mine waters from Germany (Tischler *et al.*, 2013), USA (Jones *et al.*, 2014), and Wales (Kay *et al.*, 2012; Kimura *et al.*, 2011). Clones of closely related (99% 16S rRNA gene sequence similarity) have been isolated from mine waters throughout the world (Johnson *et al.*, 2014). These studies often identified "*Ferrovum*"-like bacteria as numerically dominant in those communities, including some sites in which it was absent in the community profiles of AMD sources and surrounding environments (Tischler *et al.*, 2013).

"Fv. myxofaciens" is obligately aerobic and psychro-tolerant (growth at 4°C). This species generates large amounts of EPS in which cells are embedded, forming macroscopic flocs and streamers in continuous flow systems (Hedrich and Johnson, 2012). Ferrous iron is the only identified electron donor for this species (Johnson *et al.*, 2014). Analysis of its genome sequence identified genes coding for glycolysis and tri-carboxylic acid (TCA) pathways suggesting that it might be able to use some organic carbon compounds (Moya-Beltrán *et al.*, 2014), though extensive laboratory tests have failed to confirm this (D.B. Johnson, personal communication).

1.1.3.3. Acidophiles of the phylum *Proteobacteria,* class *Gammaproteobacteria*

(i) Genus *Acidiferrobacter (Af.)*

Acidiferrobacter thiooxydans is currently the only described species in this genus (Hallberg *et al.*, 2011). Other isolates have been detected globally in acid mine drainage sites (Mitchell *et al.*, 2004; He *et al.*, 2007; Mendez *et al.*, 2008) and also as a minor component of a brackish lake community (Zaitseva *et al.*, 2014). *Af. thiooxydans* was previously identified as "*Thiobacillus ferrooxidans"* strain m1, despite physiological differences in comparison with other *Thiobacilli* (Harrison, 1984; Rawlings and Kusano, 1994). *Af. thiooxydans* is thermo-tolerant (up to approximately 47°C), facultatively anaerobic and can grow using ferrous iron, S^0 and RISCs (reduced inorganic sulfur compounds). It is also a moderate osmophile, requiring an osmotic potential of 2.3 bar to grow optimally (Hallberg *et al.*, 2011). Bellenberg

et al. (2013) reported that a consortium of *Af. thiooxydans* and *L. ferrooxidans* oxidised pyrite far less effectively than pure cultures of these bacteria, an example of microbial amensalism (a member of one species is inhibited or destroyed by a member of another species).

1.1.3.4. Acidophiles of the class *Acidithiobacillia*

Acidithiobacillia is a recently proposed class, containing *Acidithiobacillus* spp. only (Williams and Kelly, 2013). Prior to this, genus *Acidithiobacillus* was included in the class *Gammaproteobacteria*.

(i) Genus *Acidithiobacillus (At.)*

A strain of *Acidithiobacillus thiooxidans* (then "*Thiobacillus thiooxydans*") was the first identified extreme acidophile. Similar extreme acidophiles were reclassified as *At. thiooxidans* by Kelly and Wood (2000). These were: "*Thiobacillus thermitanus*"; "*Thiobacillus lobatus*"; "*Thiobacillus crenatus*"; "*Thiobacillus umbonatus*" and "*Thiobacillus concretivorus*". Kelly and Wood (2000) also reclassified a number of other *Thiobacilli* spp. into the genus *Acidithiobacillus*. These include *Acidithiobacillus caldus* (Hallberg and Lindström, 1994), *Acidithiobacillus ferrooxidans* (*Ferrobacillus ferrooxidans*; *Ferrobacillus sulfooxidans* and *Thiobacillus ferrooxidans*), and *Acidithiobacillus albertensis* (originally *Thiobacillus albertis*; Kelly and Wood, 2000). There was still taxonomic uncertainty concerning *Acidithiobacillus* species, as many strains showed differing 16S rRNA gene sequences, phenotypic traits and chemotaxonomic characteristics (Stackebrandt and Goebel, 1994; Hallberg *et al.*, 2010; Kelly and Wood, 2000; Ni *et al.*, 2008). Ni *et al.*, (2008) assigned this variability to the large range of habitats where *Acidithiobacillus*. spp. occur. Amouric *et al.* (2011) and Hedrich and Johnson (2013) stated that this was due to a past tendency to assign any rod-shaped iron- and sulfur-oxidising acidophiles as strains of *At. ferrooxidans*, despite the differences previously mentioned.

This uncertainty led to further studies on *At. ferrooxidans* taxonomy, which found four groupings of strains based on multi-locus sequence analysis (MLSA; Amouric *et al.*, 2011). Each group displayed distinct phylogenetic and genetic differences, with the type strain of *At. ferrooxidans* (ATCC 23270) as the representative of Group I (Amouric *et al.*, 2011). Hallberg *et al.* (2010) reclassified some strains as the novel species *At. ferrivorans*, corresponding to the Group III iron-oxidising *Acidithiobacilli* described by Amouric *et al.* (2011). Hedrich and Johnson (2013) did the same for the Group II strains, naming the new species *At. ferridurans*. Currently, Group IV of the iron-oxidising A*cidithiobacilli* are also being characterised and classified as a novel species (Falagan and Johnson, personal communication). There is still uncertainty concerning the taxonomic position of *At. albertensis*. Kelly and Wood (2000) suggested its inclusion as a separate species of *Acidithiobacillus* on a tentative basis, as other traits indicated a greater distance between it and the other species (at the time of publication, no 16S rRNA gene sequence was available). 16S rRNA gene sequence analysis has since confirmed this species as closely related to *At. thiooxidans* (Xia *et al.*, 2007). Ni *et al.*, (2008) showed that ITS gene sequence comparisons for *At. albertensis* and some *At. thiooxidans* strains were identical (bootstrap value of 1000), and suggested that the former may be a sub-species or strain of the latter.

Currently there are currently six validated acidophilic *Acidithiobacillus* species. *At. thiooxidans, At. albertensis* (Kelly and Wood, 2000) and *At. caldus* (Hallberg and Lindström, 1994; Kelly and Wood, 2000) which oxidise sulfur but not iron, and *At. ferrooxidans, At. ferrivorans* (Hallberg *et al.*, 2010) and *At. ferridurans* (Hedrich and Johnson, 2013), which oxidise both ferrous iron and reduced sulfur. Genomes of *At. ferrooxidans*^{T} and other *At. ferrooxidans* strains, and draft genomes of *At. caldus*, *At. thiooxidans* and *At. ferrivorans* strains (Liljeqvist *et al.*, 2011; Valdés *et al.*, 2008; Valdés *et al.*, 2011; You *et al.,* 2011) have allowed comparisons of these species at the genetic level.

Acidithiobacillus spp. show many characteristics that are useful for biotechnological applications. They are obligately acidophilic, and oxidise sulfur (S^0 and RISCS from metal

sulfides) to fix CO_2 and generate energy (Kelly and Wood, 2005). Smeulders *et al.* (2013) enriched a number of *Acidithiobacillus* strains that could fix carbon from CS_2, confirming an observation of Smith and Kelly (1988) that *At. caldus* could do so. *Acidithiobacillus* strain TJ330 is the only other bacterium so far confirmed in this ability (Hartikainen *et al.*, 2000). Valdes *et al.* (2008) reported that *At. ferrooxidans* possessed genes coding for the sulfur quinone oxidoreductase pathway for sulfur oxidation as opposed to the genes coding for the sulfur oxidase pathway found in *At. caldus* and *At. thiooxidans*. *At. ferrooxidans*, *At. caldus* and *At. ferridurans* can use hydrogen as an electron donor (Hallberg *et al.*, 2010, Hedrich and Johnson, 2013; Drobner *et al.*, 1990). *At. ferrooxidans* and *At. ferrivorans* also use iron as an electron donor (Hallberg *et al.*, 2010). *At. ferrooxidans* oxidises iron through the rusticyanin pathway (RusA genes), while *At. ferrivorans* possesses RusB genes that code for a homologous protein (Hallberg *et al.*, 2010). *At. ferrooxidans* and *At. ferrivorans* can also fix nitrogen, and are considered important primary producers of this nutrient in extremely acidic environments (Hallberg *et al.*, 2010; Jones *et al.*, 2012; Valdés *et al.*, 2008). *At. caldus* growth on sulfur has been reported to be enhanced by including yeast extract in its growth medium (Hallberg and Lindström, 1994).

Acidithiobacillus spp. are found globally in natural and anthropogenic extremely acidic environments. They are used extensively in metal recovery from ores and wastes in laboratory and commercial operations (Rowherder *et al.*, 2003; Kelly and Wood, 2014). This is due to their ability to solubilise metals by degrading sulfur-containing minerals, and for some species, oxidising ferrous iron-containing minerals in extremely acid environments (pH 0.5 - 5.5; Kelly and Wood, 2014). *At. ferrooxidans* in particular has been examined as potential analogues of ancient life (Dietrich *et al.*, 2006), extra-terrestrial life (Gonzáles-Toril *et al.*, 2005), as a microbial fuel cell organism (Sulonen *et al.*, 2015) and for biomining and bioremediation applications. Extensive study of this bacterium has enabled an understanding of functions such as sulfur- and iron-oxidation (Osorio *et al.*, 2013; Bond and Banfield, 2003). For some such mechanisms, it is the only species studied (Rowherder *et al.*, 2003). Despite

this, many of the adaptations that allow it to grow in such extreme conditions have yet to be fully characterised (Valdés *et al.*, 2008). Due to the changing taxonomy of the genus, studied strains attributed to *At. ferrooxidans* may now be reclassified as another *Acidithiobacillus* spp.

1.1.4. Heterotrophic acidophilic bacteria

Heterotrophic acidophiles use organic compounds as a carbon source. They can be divided into obligate heterotrophs that do not fix inorganic carbon, and facultative heterotrophs (or facultative autotrophs) that preferentially use organic carbon, but which can also fix inorganic carbon. The term 'mixotroph' is sometimes used interchangeably with 'facultative heterotroph', though it can also refer to an organism that uses inorganic electron donors and organic carbon sources (Bacelar-Nicolau and Johnson, 1999; Rittenberg, 1969).

Carbon sources are generally monomeric low-weight molecules such as glucose and some amino acids, though some species can use aromatic compounds. Alternative metabolic pathways that supplement their heterotrophic lifestyle, such as iron oxidation and reduction, photosynthesis and sulfur oxidation are common. This is unsurprising, given the oligotrophic nature of their habitat. These pathways directly affect acidic conditions through nutrient cycling and pH balance (Hiraishi *et al.,* 1998; Coupland and Johnson, 2008).

Another important function of heterotrophic bacteria in acidic communities is their use of waste compounds and other organic acids as growth substrates. This removes substances that inhibit other acidophiles, allowing them to continue past the point where they would otherwise kill themselves by their own exudates (Johnson, 2012). This mutualistic relationship creates more stable communities, and allows the exploitation of resources not otherwise available (Bacelar-Nicolau and Johnson, 1999; Kimura *et al.*, 2006; Ñanchucheo and Johnson, 2010).

1.1.4.1. Acidophiles of the phylum *Actinobacteria*

Actinobacteria are Gram-positive bacteria that contain relatively high relative percentages of the bases guanine and cytosine (G + C) in their chromosomal DNA. This phylum was proposed by Stackenbrandt *et al.*, (1997) on the basis of 16S rRNA gene sequence comparison, and is widely accepted (Ludwig *et al.*, 2012) despite being considered illegitimate at that time on the grounds of no class type according to Euzéby and Tindall (2001). All known acidophilic *Actinobacteria* species are currently placed in the order *Acidimicrobiaceae*.

(i) Genus *Acidimicrobium (Am.)*

Acidimicrobium ferrooxidans is the only described species of this genus. The type strain (ICP) was isolated from an Icelandic geothermal site (Clark and Norris, 1996), though another strain (TH3) had previously been isolated from a copper leaching operation (Brierley, 1978). *Am. ferrooxidans* is rod-shaped and motile, though strain TH3 was reported to grow filamentously (Clark and Norris, 1996). It can grow autotrophically, is capable of both oxidising and reducing iron (but does not oxidise sulfur), is moderately thermophilic (optimum 45-50°C) and extremely acidophilic (pH optimum ~2). A draft genome of this species was published by Clum *et al.* (2009).

(ii) Genus *Ferrimicrobium (Fm.)*

Several strains of *Ferrimicrobium acidiphilum* have been reported from AMD (Bacelar-Nicolau and Johnson, 1999; Mazuelos *et al.*, 2012). However, *Fm. acidiphilum,* from the abandoned Cae Coch mine in North Wales, is the only species yet described (Johnson *et al.*, 2009). Uncultured clones of this genus, identified through 16S rRNA gene sequencing, appear to have widespread global distribution (Johnson and Roberto, 1997; García-Moyano *et al.*, 2012).

Fm. acidophilum is extremely acidic (minimum pH 1.4), mesophilic (growth optimum 32°C), and tolerates relatively high concentrations of metals, e.g. 200 mM ferric and ferrous iron and 150 mM copper (Johnson *et al.*, 2009). *Fm. acidophilum* also oxidises iron, contributing to iron cycling in extremely acidic environments where it is found. It requires a carbon source for growth, which may come from chemolithotrophic bacteria such as *At. thiooxidans* or from extraneous sources (Bacelar-Nicolau and Johnson, 1999; Johnson *et al.*, 2009).

(iii) Genus *Ferrithrix (Fx.)*

Currently the only species of this genus is *Ferrithrix thermotolerans* (Johnson *et al.*, 2009). A small number of closely related bacteria have been identified by 16S rRNA gene sequencing, such as in an AMD lake in Anhui province, China (Hao *et al.*, 2012) and in a solfatara crater, Italy (Glamoclija *et al.*, 2004).

The type strain of *Fx. thermotolerans* was isolated from Yellowstone National Park, Wyoming. It grows as entangled filaments, is extremely acidophilic (optimum pH 1.8) and a moderate thermophile (optimum growth temperature 43°C). *Fx. thermotolerans* tolerates elevated concentrations of some high metal concentrations, such as 200 mM of copper, zinc and ferrous iron, though is inhibited by 100 mM ferric iron. Like *Fm. acidophilum, Fx. thermotolerans* grows and oxidises iron in the presence of yeast extract (Johnson *et al.*, 2009).

(iv) Genus *Acidiferrimonas (Atn.)*

Acidiferrimonas ferrireducens is the single species in this genus, isolated from a solfatara field in Hakone, Japan (Itoh *et al.*, 2011). It is moderately acidophilic (optimum pH 3), moderately thermophilic (optimum 50°C) and facultatively heterotrophic. *Atn ferrireducens* also catalyses the dissimilatory reduction of iron under anaerobic conditions using organic carbon or hydrogen as electron donors. However, unlike other extremely acidophilic *Actinobacteria*, it does not oxidise ferrous iron (Itoh *et al.*, 2011). There are no published data on metal tolerance of this species.

Itoh *et al.* (2011) noted that, due to distances of relationship with other *Actinobacteria*, *Atn. ferrireducens* may represent a new family. However, more information is required to confirm this.

(v) Genus *Acidithrix* (Atx.)

This recently described genus currently contains a single species, *Atx. ferrooxidans*. It is described in detail in chapter 8.

1.1.4.2. Acidophiles of the phylum *Firmicutes*

The bacterial phylum *Firmicutes* is a large genus of Gram-positive prokaryotes. It consists of low G+C, endospore forming bacteria. This is in contrast to the *Actinobacteria* (the other phylum of Gram-positive bacteria), which have high G+C content and do not form endospores (section 1.1.4.1).

(i) Genus *Alicyclobacillus (Alb.)*

There are currently 19 validly described species, 1 sub-species and 2 genomic species of this genus. These are here split into two groups, based on site origin characteristics. The first group were isolated from soils and from beverages such as fruit juice. The second, and currently smaller group were isolated from mineral-rich acidic environments. While the major focus of research involving species of this genus has concerned their effects on spoilage of fruit juices, identification of species from geothermal habitats has shown that *Alicyclobacillus* spp. have a role in acidic environments, the importance of which is probably currently underestimated (Watling *et al.*, 2010).

The taxonomy of the species within this genus is somewhat complex. *Alicyclobacillus acidocaldarius* subsp. *rittmannii* was declared non-valid in 2011, until cultures of this sub-species could be deposited in separate culture collections (De Voss and Trüper, 2000; Euzéby and Tindall, 2004). Wisotzkey *et al.* (1992) proposed a 92% 16S rRNA gene sequence similarity threshold for inclusion into this genus, which is less than the currently

recommended 95% 16S rRNA gene sequence similarity threshold for a genus (Tindall *et al.*, 2010). Consequently, some species of this genus are more distantly related to each other than is usual in a genus. The proposed species *Alicyclobacillus cellulosilyticus* has a 91% 16S rRNA gene sequence similarity to the closest relative *Alicyclobacillus macrosporangiidus* (Kusube *et al.*, 2014).

Alicyclobacillus acidocaldarius subsp. *acidocaldarius* (formerly *Bacillus acidocaldarius*), *Alb. ferrooxydans* and *Alicyclobacillus hesperidium* were first isolated from sofatara soils (Albuquerque *et al.*, 2000; Darland and Brock, 1971; Jiang *et* al., 2008; Nicolaus *et al.*, 1998; Wisotzkey *et al.*, 1992). *Alicyclobacillus aeris* was isolated from AMD, along with *Alicyclobacillus disulfidooxidans* (formerly *Sulfobacillus disulfidooxidans*) and *Alicyclobacillus tolerans* (formerly *Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans* (Guo *et al.*, 2009; Karavaiko *et al.*, 2005). *Alicyclobacillus vulcanalis* was isolated from an extremely acidic (pH 1.7) geothermic (78°C) pool, though its pH minima is described as pH 2.0, and its temperature range as 35-65°C (Simbahan *et al.*, 2004). Albuquerque *et al.*, 2000, and Goto *et al.*, 2003 identified strains from sofatara soils, which were physiologically indistinguishable from *Alicyclobacillus acidocaldarius*, but whose 16S rRNA sequencing identified as a separate species. These are classified as *Alicyclobacillus* genomic species 1 and 2.

The other species are moderately acidophilic or acid tolerant, and are mentioned briefly here as they are not within the scope of this study (i.e. they are not extreme acidophiles). *Alicyclobacillus acidoterrestris* (formerly *Bacillus acidoterrestris*), *Alicyclobacillus cycloheptanicus* (formerly *Bacillus cycloheptanicus*), *Alicyclobacillus kakegawensis*, *Alicyclobacillus contaminans, Alb. macrosporangiidus, Alicyclobacillus shizuokensis* and *Alicyclobacillus sendaiensis* were isolated from non-geothermal, mainly arable soils (Goto *et al.*, 2007; Tsuroka *et al.,* 2003; Wisotzkey *et al.*, 1992). *Alb. cellulosilyticus* was isolated from steamed cedar chips (Kusube *et al.*, 2014). *Alb. pomorum, Alicyclobacillus acidiphilus, Alicyclobacillus fastidiosus, Alicyclobacillus sacchari* and *Alicyclobacillus herbarius* were originally isolated from spoiled acidic beverages, liquid sugar and hibiscus tea (Goto *et al.*,

2002; Goto *et al.*, 2003; Goto *et al.*, 2007; Matsuraba *et al.*, 2002), though their primary habitat is believed to be soil (Matsuraba *et al.*, 2002). Along with *Alb. acidoterrestris* and *Alb. contaminans,* they are responsible for spoiling the beverages by the production of guaiacol (Goto *et al.*, 2007; Matsuraba *et al.*, 2002). They are particularly problematic as contaminants because they can survive usual pasteurisation processes (Smitt *et al.*, 2011).

Alicyclobacillus spp. have been found in a wide range of habitats and show a wide range of physiological characteristics. Different species may be mesophilic or moderately thermophilic, obligate or facultative aerobes and obligately heterotrophic or facultatively autotrophic. The temperature range across the genus is 17-65°C, and the pH ranges from extremely acidophilic to acid tolerant (pH range 1.5-7.5). *Alb. tolerans*, *Alb. disulfidooxidans*, *Alb. ferrooxidans* and *Alb. aeris* (Guo *et al.*, 2009; Jiang *et al.*, 2008; Karavaiko *et al.*, 2005) have the ability to oxidise ferrous iron and reduced sulfur, making them facultative mixotrophs. ω-alicyclic fatty acids were thought to be a distinguishing feature of *Alicyclobacillus* spp. (Albuquerque *et al.*, 2000) until the discovery of *Alb. pomorum*, which has none (Goto *et al.*, 2003). *Alicyclobacillus* spp. are Gram-positive, but give variable results with a Gram-stain test (Guo *et al.*, 2009).

(ii) Genus *Sulfobacillus (Sb.)*

This genus currently comprises five species: *Sulfobacillus thermosulfidooxidans* (Golovacheva and Karavaiko, 1991), *Sulfobacillus acidophilus* (Norris *et al.*, 1996), *Sulfobacillus benefaciens* (Johnson *et al.,* 2008), *Sulfobacillus sibiricus* (Melamud *et al.*, 2003) and *Sulfobacillus thermotolerans* (Bogdanova *et al.*, 2006). Other, undescribed and uncultured, isolates show this genus to have a wide global distribution (Watling *et al.*, 2008).

Species of this genus oxidise ferrous iron and reduced sulfur, and are facultative heterotrophs with versatile metabolisms (with the exception of *Sb. sibiricus*; Johnson *et al.,* 2008). They are facultative anaerobes that use ferric iron as an alternative terminal electron acceptor to oxygen, are moderately thermophilic and can tolerate down to pH 0.7 (Johnson

et al., 2008). *Sulfobacillus* spp. also attach to sulfide minerals, possibly to enhance oxidation and other functions (Watling *et al.*, 2008). They are often important members of mineral bioleaching operations, with factors such as pH, temperature and flow being important factors in the establishment of dominance of species (Johnson *et al.,* 2008).

1.1.4.3. Acidophiles of the phylum *Acidobacteria*

Prokaryotes of the phylum *Acidobacterium* are Gram-negative bacteria. Molecular biology studies suggest that they are widespread in the environment and physiologically diverse (Barns *et al.*, 1999), though they have proved generally difficult to isolate (Pankratov, 2012).

(i) Genus *Acidobacterium (Abm.)*

Acidobacterium capsulatum (Kishimoto *et al.*, 1991) is the only bacterium so far described in this genus, though there are other undescribed isolates and uncultured clones known.

Abm. capsulatum is aerobic, moderately thermophilic, acidophilic and rod-shaped, generating a polysaccharide capsule and producing an insoluble orange pigment (Inagaki *et al.*, 2001; Kishimoto *et al.*, 1991). It also reduces iron, an ability that is apparently common among acidophilic heterotrophic bacteria (Coupland and Johnson, 2008).

1.1.4.4. Acidophiles of *Proteobacteria,* sub-class *Alpha-proteobacteria*

(i) Genus *Acidiphilium (A.)*

The genus *Acidiphilium* currently consists of; *Acidiphilium cryptum* (Harrison *et al.*, 1981), *Acidiphilium angustum* (Wichlacz *et al.*, 1986), *Acidiphilium organovorum* (Lobos *et al,*. 1986), *Acidiphilium rubrum* (Wichlacz *et al.*, 1986), *Acidiphilium multivorum* (Wakao *et al.*, 1994), *Acidiphilium symbioticum* (Bhattacharyya *at al.*, 1991), *Acidiphilium iwatense* (Okamura *et al.*, 2015) and *Acidiphilium acidophilum* (formerly *Thiobacillus acidophilus;* Hiraishi *et al.*, 1998). These are separated into two groups by 16S rRNA gene sequence analysis, with the closely related *A. rubrum* and *A. angustum* in one (99.9% 16S rRNA gene

sequence similarity; Wakao *et al.*, 1994), and the rest in another. Possibly this grouping relates to production of bacteriochlorophyll pigments (Hiraishi *et al.*, 1998; Hiraishi and Shimada, 2001).

Acidiphilium spp. have been found in acidic mine environments, or as contaminants of *At. ferrooxidans* (Bhattacharyya *at al.*, 1991; Harrison *et al*, 1981; Lobos *et al.* 1986; Peccia *et al.*, 2000; Wakao *et al.*, 1994; Wichlacz *et al.*, 1986). They have also been identified as major species in geothermal springs (Burton and Norris, 2000).

Acidiphilium spp. are Gram-negative facultative anaerobes that grow between pH 1.5 and 5.9, and are mesophilic (Harrison *et al.*,1981; Hiraishi and Shimada, 2001). They have the ability to accumulate some metals (Itoh *et al.*, 1998; Matsuzawa *et al.*, 2000), with *A. rubrum* reported to generate magnetite-like minerals (Matsuzawa *et al.*, 2000). *Acidiphilium* spp. are inhibited by some organic acids, including (for various species) peptone, acetate and lactate (Harrison, 1981; Kishimoto *et al.*, 1990; Wakao *et al.*, 1994).

Members of this genus possess bacteriochlorophyll with a zinc substitution for magnesium (Hiraishi and Shimada, 2001). This is responsible for the pink colouration of many species (Wichlacz *et al.*, 1986). Hiraishi and Shimada (2001) postulated that harvesting solar energy could supplement available carbon in the oligotrophic conditions that prevail in acidic environments. However, only *A. rubrum* and *A. angustum* synthesise bacteriochlorophyll in significant amounts (Hiraishi and Shimada, 2001; Wakao *et al.*, 1994). Although there is no firm evidence for light-stimulated growth, harvesting solar energy allows some *Acidiphilium* spp. to survive for short periods under starvation conditions (Hiraishi and Shimada, 2001). Zinc bacteriochlorophyll is more stable than magnesium bacteriochlorophyll under acidic conditions, which Wakao *et al.*, (1996) suggested as a reason for its substitution.

A. acidophilum can oxidise sulfur and fix CO_2, making it a facultative heterotroph (Harrison *et al.*, 1981; Hiraishi *et al.,* 1998; Pronk *et al.*, 1990), an ability that led to its initial placement within *Thiobacillus,* a genus of neutrophilic sulfur oxidisers (Hiraishi *et al.,* 1998). Other

Acidiphilium strains such as strains of *A. cryptum* and *A. rubrum* also show evidence of sulfur oxidation, but they have not shown an ability to fix CO_2 (Rohwerder and Sand, 2007). *A. multivorum* can oxidise arsenite to arsenate, an ability which is plasmid mediated (Suzuki *et al.*, 1997; Suzuki *et al.*, 1998; Wakao *et al.*, 1994). *Acidiphilium* isolate 3.2 sup 5 has been shown to use electrodes in an electrochemical cell as electron donors (Malki *et al.*, 2008).

(ii) Genus *Acidocella (Ac.)*

There are currently four described species in this genus: *Acidocella facilis* (Wichlacz *et al.*, 1986; Kishimoto *et al.*, 1995), *Acidocella aminolytica* (Kishimoto *et al.*, 1993), *Acidocella aluminiidurans* (Kimoto *et al.*, 2010) and *Acidocella aromatica* (Jones *et al.*, 2013).

Acidocella spp. are obligate aerobes, though *Ac. aromatica* strain PFBC has been reported to grow anaerobically by a syntrophic pathway. It was reported to do this by oxidising acetic acid to carbon dioxide and hydrogen, but only when grown in co-culture with a hydrogen-metabolising (sulfidogenic) acidophile (Kimura *et al.*, 2006; Johnson *et al.*, 2006b; Rowe, 2007). This type of syntrophic relationship is common among strictly anaerobic organisms, with methanogens and sulfur reducers as the usual H_2-metabolising partners (Kamagata and Tamaki, 2005). *Ac. aromatica* also uses some aromatic compounds and aliphatic acids ac carbon sources in preference to yeast extract and other more commonly used substrates (Gemmel and Knowles, 2000; Hallberg *et al.*, 1999; Kimura *et al.*, 2006; Jones *et al.*, 2013). Previous studies had observed that other *Acidocella* spp. were more able to tolerate and metabolise these substrates than *Acidiphilium* spp. (Kishimoto et al., 1993; Kishimoto et al., 1995).

Acidocella spp. often display elevated (though variable) tolerance to transition metals and aluminium such as a 500 mM aluminium tolerance reported for *Ac. aluminiidurans* (Kimoto *et al.*, 2010) and 175 mM zinc by *Ac. aminolytica* (Kishimoto *et al.*, 1993). Copper, in contrast, appears to be far more toxic, the highest level of tolerance reported being 15 mM by *Ac. aminolytica (*Kishimoto *et al.*, 1993).

(iii) Genus *Acidicaldus (Acd.)*

Acidicaldus organivorans was isolated from a geothermal area within Yellowstone National Park, USA (Johnson *et al.*, 2006), and is currently the only species of this genus. Related isolates include MK6, also isolated from Yellowstone (Kozubal *et al.*, 2012), and T163 from New Zealand soil (Stott *et al.*, 2008). Its closest classified relative is *Acidisphaera rubrifaciens* (Johnson *et al.*, 2006).

Acd. organivorans is facultatively aerobic and moderately thermophilic (Johnson *et al.*, 2006). It is extremely acidophilic (growth minimum pH 1.75, optimum pH 3.0), and oxidises sulfur aerobically in the presence of a carbon source (Johnson *et al.*, 2006). It does not use organic acids as growth substrates, but does oxidise phenol (below concentrations of 5 mM), one of the few acidophiles to do so. It is facultatively anaerobic, being capable of anaerobic growth by iron reduction (Johnson *et al.*, 2006).

1.1.4.5. Acidophiles of *Proteobacteria,* class *Gammaproteobacteria*

(i) Genus *Acidibacter (Ab.)*

Acidibacter ferrireducens is currently the only species in this genus, isolated from an AMD lake, Spain (Falagán and Johnson, 2014). Closely-related bacteria have been detected in clone libraries from other AMD sites in Spain, Germany and China (but not reported in studies) (Sánchez-Andrea *et al.* 2011; Santofimia *et al.* 2013; Lu *et al.* 2010; Yin *et al.* 2008).

Ab. ferrireducens is an obligate heterotroph with the ability to reduce iron under microaerobic conditions, and is moderately acidophilic and mesophilic. It is also highly tolerant to arsenic (V), having been observed growing in the presence of 50 mM arsenic (V) (Falagán and Johnson, 2014).

1.1.5. Acidophilic microbial communities

Extremely acidic environments usually contain relatively limited microbial diversity, though indigenous microorganisms may be from many different phyla distributed throughout the tree of life (Baker and Banfield, 2003; Denef *et al.*, 2010; Johnson, 2012). These communities include archaea and eukaryotes as well as bacteria. Archaea and eukaryotes are mentioned briefly here, as they are not within the scope of the current study.

Eukaryotes found in extremely acidic environments include algae, yeasts, protists, amoeba, fungi and plants. These may be acidophilic or acid-tolerant (Aguilera, 2013). There are currently relatively few classified acidophilic species, and they can be challenging to isolate and cultivate *in vitro* (Amaral-Zettler, 2012). There are also problems with molecular biology techniques, such as PCR-bias due to extreme rRNA gene length variation, variability in rRNA gene copy numbers and inconsistency in database reference sequences (Amaral-Zettler, 2012).

Archaea are found in some of the most extreme environments known, including extremely acidic habitats. This group are considered by some to be the closest living relatives of ancient life forms (Offre *et al.*, 2013). Baker *et al.* (2010) identified a deeply branching uncultured phylum with genes associated with both bacteria and crenarchaeotes. Most known archaeal diversity is from 16S rRNA sequences of uncultivated archaea, with a fair proportion of the remainder from archaea isolated from extreme environments. Like bacteria, various species of archaea use a wide range of substrates such as iron, sulfur and organic carbon, and contribute to nutrient and metal cycling in extremely acidic habitats (Bini, 2010; Offre *et al.*, 2013).

Members of acidophilic microbial communities interact with each other in positive and negative ways (Denef *et al.*, 2010). Photosynthetic eukaryotes such as micro-algae provide a carbon source for heterotrophs (Ňanchueo and Johnson, 2012; Aguilera, 2013). These heterotrophs can use small molecular weight carbon substances that are often toxic to

chemolithotrophic acidophiles (Hallberg *et al.*, 1999; Kimura *et al.*, 2006). Prokaryotic community members may oxidise and reduce iron and sulfur, providing potential electron donors and acceptors for other members of a consortium. Various members may also compete with, and predate upon, each other (Hao *et al.*, 2010).

Combined geochemical and microbiological/molecular studies found that community structure is greatly affected by pH, and becomes less diverse as pH decreases (Amaral-Zettler, 2012; Lauber *et al.*, 2009; Li *et al.*, 2014; Liu *et al.*, 2014). Other influential factors included temperature, and concentrations of iron, other metals, and sulfate (Amaral-Zettler, 2012; Li *et al.*, 2014; Liu *et al.*, 2014).

Until recently, there had been few reports published on effect of geochemistry on bacterial communities (Baker and Banfield, 2003; Lauber *et al.*, 2009; Liu *et al.*, 2014). Evidence such as habitat specific genes has prompted a reassessment of the importance of environmental factors in microbe biochemistry (Baker and Banfield, 2003; Martiny *et al.*, 2006). Geochemical variation among soil prokaryotes at community level has been identified at continental (Fierer and Jackson, 2006) and country scale (Griffiths *et al.*, 2011). The same was found of marine community distributions (Galand *et al.,* 2010; Zinger *et al.*, 2011) and of extremophiles. Rosselló-Mora *et al.* (2008) identified three strains of the extreme halophile *Salinibacter ruber* on a geographical basis through ultrahigh resolution mass spectrometry analysis of their metabolic products. Arsene-Ploetze *et al.* (2010), in their study of *Thiomonas* (moderate acidophiles) genomes, concluded that differences between the strains studied were a response to the differing conditions of their original habitat. This kind of geochemical variation can be difficult to define, however. DNA-based techniques do not often identify phenotypic variation, as expression of genes can depend on environmental circumstances (Cavalier-Smith, 2007; Green *et al.*, 2008).

1.2. The continuing effort to identify and characterise microbial diversity

Classified species represent only a fraction of the bacterial diversity known to exist. Others include cultivated isolates that are yet to be fully characterised and validated (such as the genus *'Acidithiomicrobium';* Norris *et al.*, 2011), those that have been identified *in situ* (mostly from molecular analysis) but not isolated (e.g. *Gallionella*-like bacteria; Hedrich *et al.*, 2011; Tischler *et al.*, 2013; Jones *et al.*, 2014), and those that are currently unknown. Only 1-10% of all microbial species are thought to be isolatable and cultivable using traditional methods (Cardenas and Tiedje, 2008; Amann *et al.*, 1995; Hugenholtz *et al.*, 1998), the so-called 'great plate anomaly' (Staley and Konopka, 1985; Fig. 1.3).

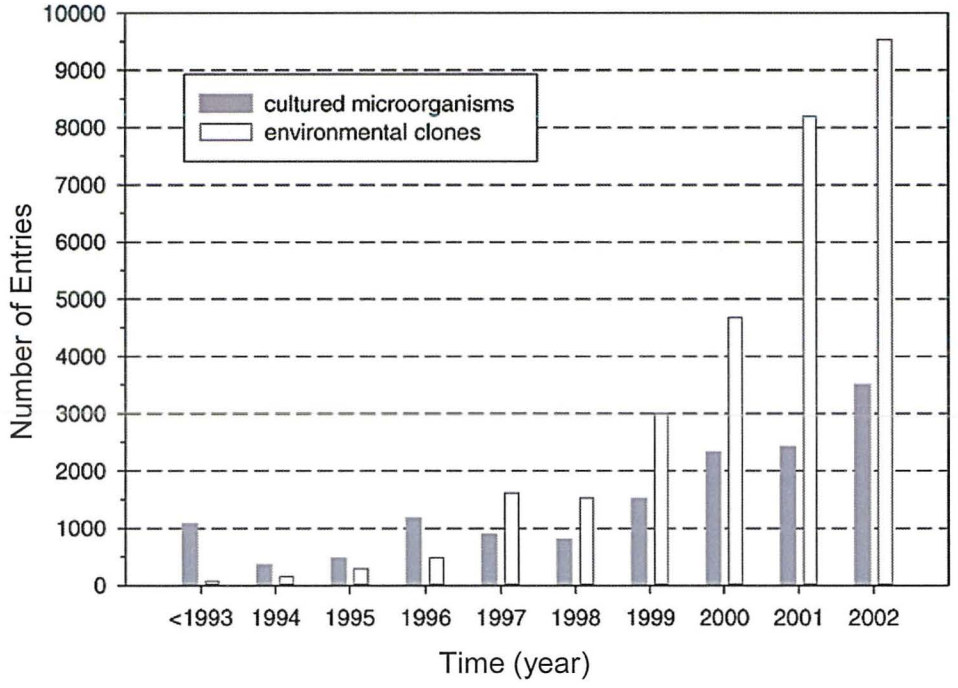


Figure 1.3. Proportions of cultured and uncultured 16S rRNA gene sequences submitted to Gen-Bank (After Rappé and Giovanni, 2003).

Specific environmental requirements, the presence or absence of specific growth factors and signalling molecules, and reliance on partner organisms have all been proposed as reasons for failing to cultivate the majority of prokaryotic microorganisms *in vitro* (Vartoukian *et al.*, 2010).

Novel cultivation methods include modification of growth media and conditions, community and co-cultivation, single-cell isolation using optical tweezers, gel encapsulation and micromanipulators, use of membranes and sterile environmental samples as liquid media have been used to isolate and cultivate previously 'uncultivable' prokaryotes (Cardenas and Tiedje, 2008; Pham and Kim, 2012; Vartoukian *et al.*, 2010; Zengler *et al.*, 2002). Replacing agar with agarose (a more pure gelling agent) has facilitated the isolation of some acidophilic bacteria that are more sensitive to organic acids, which are released in greater amounts during hydrolysis of agar (Harrison *et al*, 1981; Lobos *et al.*, 1986). Some reasons suggested for problems encountered with cultivating some acidophiles are that the media used to attempt to isolate extremely acidophilic microorganisms lack an essential unknown growth factor, or there is one or more substances present that inhibit their growth (Tyson *et al.*, 2005; Zengler *et al.*, 2002). The latter was the basis of the "overlay plate" technique (Johnson and McGuiness, 1992; Johnson and Hallberg, 2007), where heterotrophic acidophilic bacteria are included in the lower layer of a double layer gel specifically to remove small molecular weight organic compounds released via acid hydrolysis of agar an its derivatives. This has proved successful in isolating organic-sensitive acidophilic bacteria and archaea (Johnson and Hallberg, 2007).

Molecular techniques such as 16S rRNA gene sequencing, FISH (fluorescent *in situ* hybridisation), identification and amplification of specific genes, transcriptomics, metagenomics and whole genome sequencing can provide culture-independent data. These analyses are free of the problems mentioned above, though they may introduce biases of their own (described below). Information from these techniques can be used to identify and quantify microbial communities, and also the genes responsible for individual operations, including those species which have proved recalcitrant to isolation and cultivation (Bond and Banfield, 2001; Denef *et al.*, 2010; Tyson *et al.*, 2005).

Molecular methods rely on the assumption that methods of DNA extraction and processing work on all bacteria concerned. This is not always the case, as there are bacteria that

appear resistant to commonly-used DNA extraction methods and are currently only found through successful isolation (Auld *et al.*, 2013; Delavat *et al.,* 2012; Shade *et al.*, 2012). Other errors include primer bias, sequencing errors and chimeras (Haas *et al.*, 2011; Schloss *et al.*, 2011). Kunin *et al.* (2010) estimated that sequencing errors could cause an overestimation of bacterial diversity by as much as two orders of magnitude. In addition, these techniques can only give detailed information on bacterial identity, population structure and metabolic information where there is some previous information on the subjects concerned (Denef *et al.*, 2010; Rappé and Giovannoni, 2003). Studies that compared both methods of bacterial population estimation concluded that an approach that combined molecular biology and cultivation techniques was likely to be the most successful in assessing environmental microbial diversity (Auld *et al.*, 2013; Shade *et al.*, 2012).

1.3. The Trefriw Wells Spa site

1.3.1. Site description

The Cae Coch pyrite ore body (277562°E, 365262°N) occurs in a hillside near the village of Trefriw in North wales. It is responsible for two extremely acidic water bodies. The first of these is Trefriw Wells Spa (OS Grid ref. SH775653, approximately 270 m due east of the Cae Coch ore body). It consists of a grotto set into the hillside, containing two passages that end at two wells. These are well 1 (enclosed in a metal tank), well 2 (images can be seen in Fig. 1.4) and a pool directly opposite the entrance (images in Fig. 1.5). Section 5.2, Fig. 5.1 contains a diagram of the cave layout. The cave is completely dark apart from occasional short periods of low artificial light for ease of access, though there have been periods in history when the grotto was lit for part of the time (section 1.3.2).

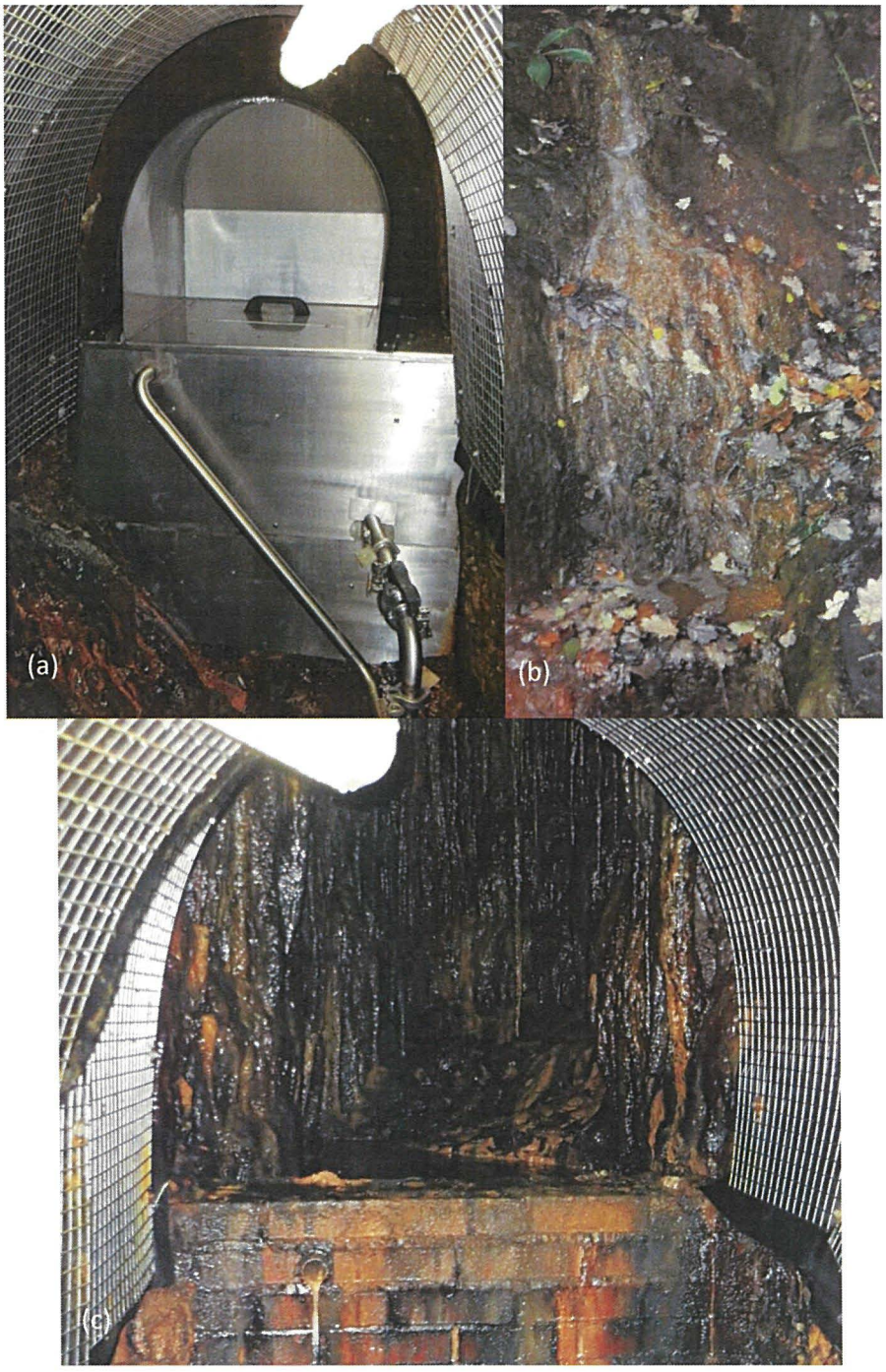


Figure 1.4. (a) image of well 1 outlet, (b) outflow stream below the bath house, (c) image of well 2 pool.

Well 2 is a raised pool approximately 1 m deep, 1.5 m in diameter. Overflow water is channelled through shallow open drains in the floor into a second drain outside near a bathhouse (built in 1863; Mather, 2013), a little way down the hill (Fig. 1.4 (b)). Acidic water also drips from the ceiling and walls, especially during wet weather.

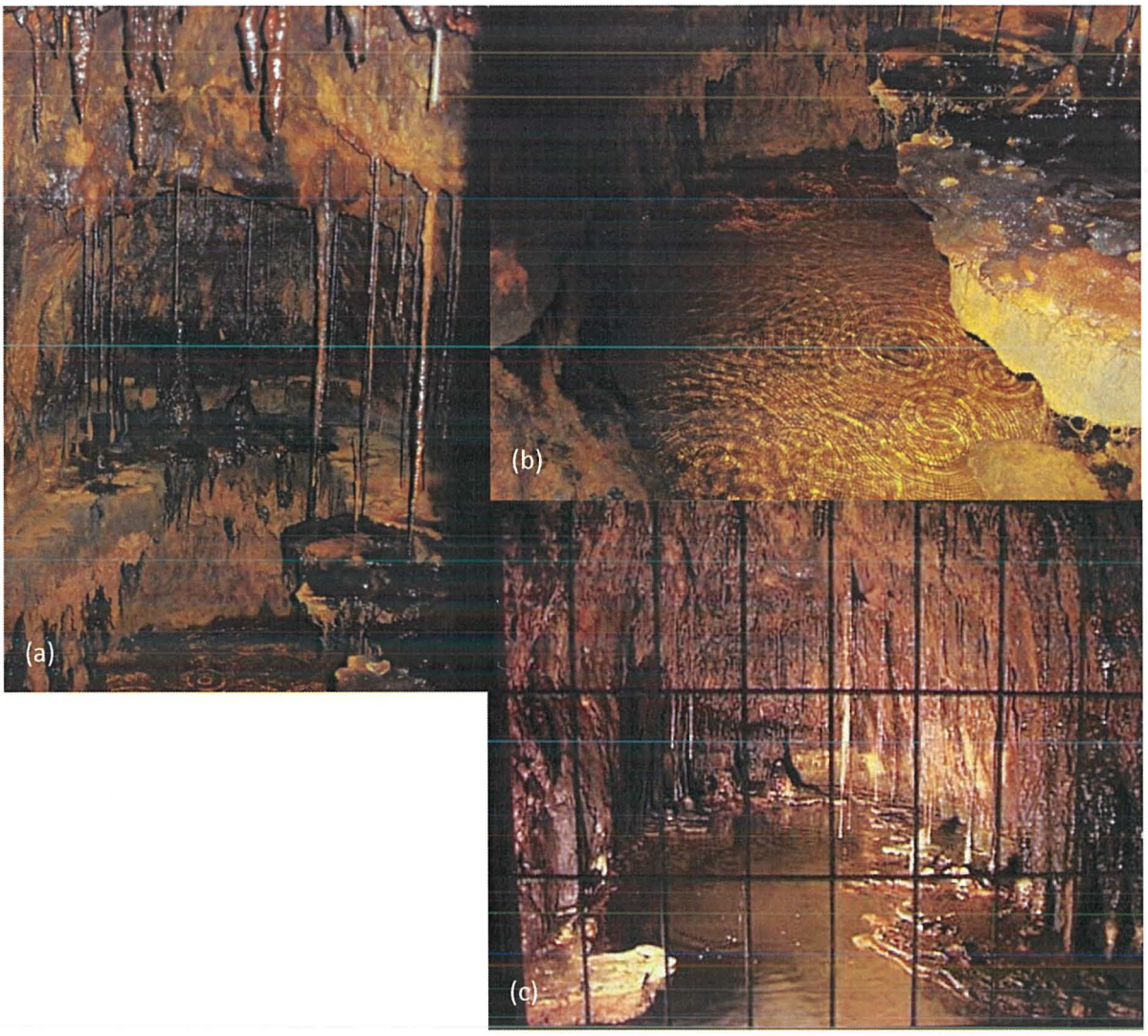


Figure 1.5. (a) Image of pool 11/11/14, (b) Image illustrating new water level (11/11/14)– shelf shows previous water level (c) Image of well pool, 2004 (image source http://www.rhylphotosoc.co.uk/GALLERIES/digital/Trefriw/pages/Trefriw%2020e.htm).

The second water body is that associated with the abandoned Cae Coch sulfur mine Outflow adits for this mine exit the hill above the Trefriw Spa site. Kimura *et al.* (2011) carried out an extensive geochemical and microbiological study of the abandoned mine site. It was worked on a modest scale from 1817 until 1919, partially working out the Cae Coch pyrite ore (Bennett and Vernon, 1997; Pierce and Kemp, 1994). The Cae Coch mine is relatively dry until the lowest levels, and it is thought unlikely that the two sites (the abandoned mine and

the Spa) are directly connected by water flow (Secretary of the Great Orme Exploratory Society, personal communication).

1.3.2. Site History

The spa site is commonly held as being first discovered by the XXth Roman Legion, while they were mineral prospecting in the region. Romans are known to have been in the area; the fort of Kanovium (Caerhun) is 6 km distant, and the Roman road of Sarn Helen passes nearby. The exact course of the road is unknown, but the most likely route passes the Spa (Cantrell and Rylence, 1992). Other evidence of their presence at Trefriw is, at best, tenuous. Mather (2013) asserts that parts of the site suggest ancient baths, and Bennett and Vernon (1997) infer the presence of early workings from historical site descriptions. One of these historic descriptions is that of Hayward (1881), who records an eyewitness account of the discovery of previously worked caves in 1833 at Trefriw, previously buried under debris from the Cae Coch mine uphill. Interestingly, there is no mention of Roman presence connected with the Spa in reports by Hayward (1881) and Jones (1878), despite both making a point of documenting local oral tradition concerning the area. Therefore, it is possible that Romans could have stopped there but did not leave any archaeological evidence.

The earliest documented evidence for iron-rich water is a letter from Sir Hugh Myddleton to the then landowner Sir John Wynn in 1607, in which the latter is requested to send a bottle of water from a mineral rich source on his land (Bennett and Vernon, 1997). However, this may refer to waters from Cae Coch mine. The site began its development as a health spa in the mid-1700's (Bennett and Vernon, 1997), though this date probably refers to the waters from Cae Coch mine rather than the Trefriw caves (undiscovered until ~1833; Howard, 1881). Jones (1878) describes tourists coming to take the waters directly from a pool and caves in a field, with a hotel present nearby, indicating that the site was well established by

then. The site reached its heyday in the late 1800s under the name Cae Coch (Hayward, 1881; Jones, 1878; Mather, 2013). Its popularity as a health resort declined after the First World War, though attempts were made in the 1930's to reinvigorate the site under the name *Belle Vue Spa*, including the addition of a new guest house (Mather, 2013). It closed in 1957, and reopened as a tourist attraction in 1972 as Trefriw Roman Spa, before closing to the public in early 2000 (Mather, 2013).

1.3.3. Current use of the Trefriw Wells Spa site

The chalybeate spring water at Trefriw is marketed as Spatone natural iron supplement. The site owners, Spatone Ltd. (a subsidiary of Nelsons UK) collect, filter-sterilise and package the water, sometimes amended with apple juice concentrate. They then sell it in many different countries throughout the world as "*Spatone*", "*Ferrotone*" and "*Pur-Adsorb*" as a natural iron supplement. Halksworth *et al.* (2003) and McKenna *et al.* (2003) reported the water as an acceptable alternative to iron tablets, due to the high concentration of soluble ferrous iron content of the water. Ferrous iron is the species required in haemoglobin, creating a protein that reversibly binds with oxygen. If Fe^{3+} is incorporated instead, it creates methaemoglobin that bonds irreversibly to oxygen (Quinteros *et al.*, 2001). Dissolved Fe^{2+} is also fat-soluble and so is easier for the body to absorb (Quinteros *et al.*, 2001). Iron is also a co-factor in many cytochromes and iron-sulfur proteins, such as ferredoxin (Johnson *et al.*, 2005).

Chalybeate spring water formation is subject to variation in iron concentration and water volume, as it is dependent on environmental factors. Iron concentrations in Spatone water are also decreasing over time, as the pyrite in the ore body becomes increasingly depleted. Therefore, it is important that the processes involved in the formation of the spa is understood so that forward planning is possible for developments at Trefriw Wells Spa.

1.3.4. Site geology

The Cae Coch pyrite ore body sits on a dolerite sill included into the Llanruchwyn slates (Fig. 1.6 (a)). These are Upper Ordovician in origin, and described as bituminous shale rich in pyrites (Botterell and Morton 1992; Mather, 2013) or cleaved pyritic mudstone (Ball and Bland. 1985; Pearce and Kemp, 1994). The difference in classification is likely due to imprecise definition of the rock types, which are very similar in nature (Merriman *et al.*, 2003). The pyrite deposit is a stratiform pyrite body of mean 1.8 - 2.2 m thickness, and is estimated at approximately 20,000 tonnes (present and worked; Ball and Bland, 1985), which occurs in two discrete bodies (Fig. 1.6 (b)) (Ball and Bland, 1985; Botterell and Morton, 1992). The pyrite contained is fine-grained and relatively free of other mineral sulfides, with some associated silicates (including quartz) and calcite. It weathers relatively easily because it is fine-grained, (Nordstrom, 2011). Botterell and Morton (1992) proposed that the deposit was formed by anoxic marine biological sulfate reduction sediment collecting in a basin formed of the surrounding Dolgarrog Volcanic Formation, which is mainly basaltic.

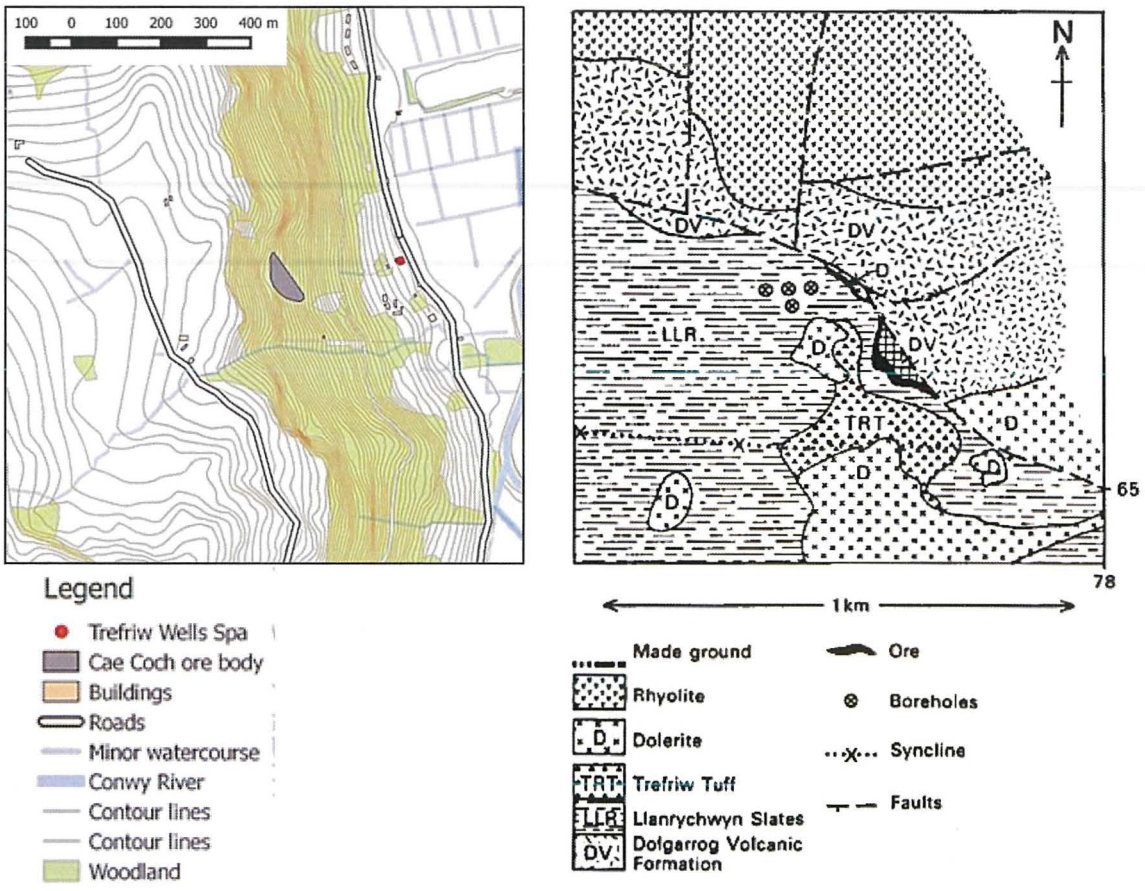


Figure 1.6. (a) Map of Cae Coch ore body in relation to Trefriw spa. Data ©crown copyright Ordinance survey and British Geological society. (b) Sketch map of Cae Coch pyrite deposit and surrounding geology (after Ball and Bland, 1985).

1.3.5. Geochemistry of the Trefriw Wells Spa and Cae Coch site

Pyrite (FeS_2) is an iron disulfide mineral, and the most abundant sulfide mineral in the lithosphere. It can be oxidised by molecular oxygen and ferric iron, releasing ferrous iron and more oxidised sulfur (e.g. thiosulfate or sulfate) in an abiotic reaction. In circum-neutral pH environments, ferric iron is insoluble and pyrite is oxidised by molecular oxygen in the presence of water (equation [1.1]):

$$FeS_2 + 3.5\ O_2 + H_2O \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+ \qquad [1.1]$$

At this pH, ferrous iron oxidises spontaneously in the presence of oxygen, producing ferric iron. The ferric iron hydrolyses rapidly to form ferric hydroxide (equation [1.2]) or ferrhydrite ($5Fe_2O_3.9H_2O$):

$$Fe^{2+} + 0.5\ O_2 + 2.5\ H_2O \rightarrow Fe(OH)_3 + 2\ H^+ \qquad [1.2]$$

The insolubility of ferric iron at circum-neutral pH means that it is ineffective at oxidising pyrite. However, in acidic liquors the solubility of ferric iron is far greater, and the oxidation of ferrous iron proceeds as in equation [1.3]:

$$Fe^{2+} + 0.25\ O_2 + H^+ \rightarrow Fe^{3+} + 0.5\ H_2O \quad [1.3]$$

Ferric iron is a more powerful oxidising agent than molecular oxygen, and oxidises pyrite forming ferrous iron and thiosulfate as end products (equation [1.4]; Schippers *et al.*, 2006):

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

For the reaction to continue, ferric iron has to be regenerated (Fig. 1.7). At low pH (<3.5) spontaneous abiotic oxidation of ferrous iron is very slow, and the reaction is catalysed by species of acidophilic iron-oxidising bacteria and archaea described in sections 1.1.3-1.1.5 via the proton-consuming reaction [1.3].

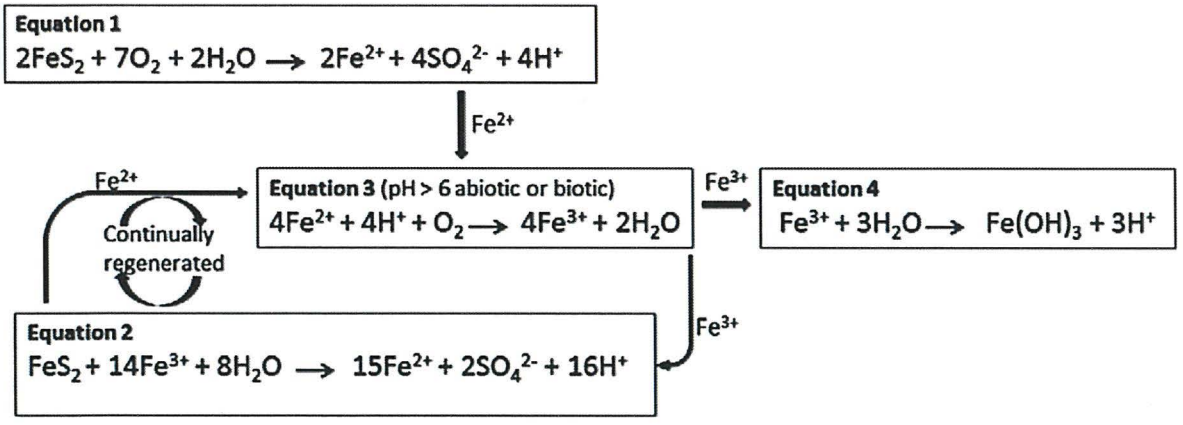


Figure 1.7. Schematic representation of the iron reactions involved in the oxidation of pyrite (Streten-Joyce *et al.*, 2013).

Thiosulfate anions generated in reaction [1.4] are oxidised, ultimately to sulfate, by sulfur-oxidising prokaryotes (equation [1.5]):

$$S_2O_3^{2-} + 2\ O_2 + H_2O \rightarrow 2\ SO_4^{2-} + 2\ H^+ \qquad [1.5].$$

However, thiosulfate is also acid-labile, and is readily oxidised by ferric iron in acidic liquors, forming elemental sulfur and a variety of other sulfur oxyanions (Schippers *et al.*, 1996). These are also metabolised by sulfur-oxidising prokaryotes (e.g. equation [1.6]):

$$S^0 + 1.5\ O_2 + H_2O \rightarrow 2\ H^+ + SO_4^{2-} \qquad [1.6]$$

Oxidation of elemental sulfur and sulfur oxyanions is an acid-generating process, as shown in equations [1.5] and [1.6]. This counterbalances the protons consumed during ferrous iron oxidation in acidic liquors (equation [1.3]), so that complete oxidation of pyrite is net acid-generating. When the pH is sufficiently low to maintain ferric iron in solution, the complete oxidation of pyrite can be represented by equation [1.7]:

$$FeS_2 + 3.5\ O_2 + H_2O \rightarrow Fe^{3+} + 2\ SO_4^{2-} + 2\ H^+ \qquad [1.7]$$

However, at pH >2.5, hydrolysis of ferric iron becomes increasingly significant, forming (typically) schwertmannite ($Fe_8O_8(SO_4)(OH)_6$) as a secondary mineral phase and generating significantly more proton acidity (3.75 moles per mole pyrite oxidised; equation [1.8], rather than the 1.0 generated in equation [1.7]):

$$8\ FeS_2 + 30\ O_2 + 18\ H_2O \rightarrow Fe_8O_8(OH)_6SO_4 + 30\ H^+ + 15\ SO_4^{2-} \qquad [1.8].$$

The chemistry of the water within the Trefriw Wells Spa was reported by Hallberg *et al.* (2006). Their data (Table 1.2) showed that water entering the Spa was acidic, rich in soluble iron (mostly ferrous) and sulfate, and essentially anoxic. This is typical of water sampled at the point close to where it is discharged from a sulfidic ore body or sulfide-rich mineral tailings. Table 1.2 also lists data from the Hallberg *et al.* (2006) reporting the geochemistry of

water draining the Mynydd Parys copper mine, at its (then) point of discharge (the Afon Goch east).

Table 1.2. Physico-chemistry of Trefriw Spa pool water (March 2004) and water draining the abandoned Mynydd Parys copper mine, Anglesey (February 2002) and water draining the Cae Coch sulfur mine (October 2003) (Hallberg *et al.*, (2006)). All concentrations shown are mg/L.

Analyte	Trefriw Wells Spa (pool)	Mynydd Parys	Cae Coch
pH	2.6	2.7	2.25
Temp (°C)	9.8	8.8	ND
E_H (mV)	+670	+420	+725
Conductivity (µS/cm)	1,520	2,994	3.2
Dissolved oxygen	0.9	0.55	3.4
Dissolved organic carbon	4.3	6.0	6.0
Fe^{2+}	193	473	220
Fe_{total}	259	514	1560
Mn	1.1	5.0	5.0
Cu	<1	35	0.64
Zn	<1	50	1.3

Acidic iron-rich waters form as surface water percolates through cracks and fissures in the pyrite ore body above both water body sites, initiating the reactions described above. Initially, dissolved oxygen is likely to be the more important mineral oxidant (equation [1.1]), given the pH of the modified rainwater. Oxygen in the percolating water is consumed during oxidation of ferrous iron and reduced sulfur (equations [1.2], [1.3], [1.5] and [1.6]) and is not readily replenished in the confined subterranean environment, causing the water to become increasingly depleted of dissolved oxygen and enriched with ferrous rather than ferric iron. As the water becomes more acidic and oxygen depleted, ferric iron would become the more significant oxidant (equation [1.4]). Fig. 1.8 illustrates the process by which Trefriw Spa water is thought to form. The water from the Cae Coch mine forms in the same way, though

individual water bodies within the mine vary in chemical and species composition (Kimura *et al.*, 2011).

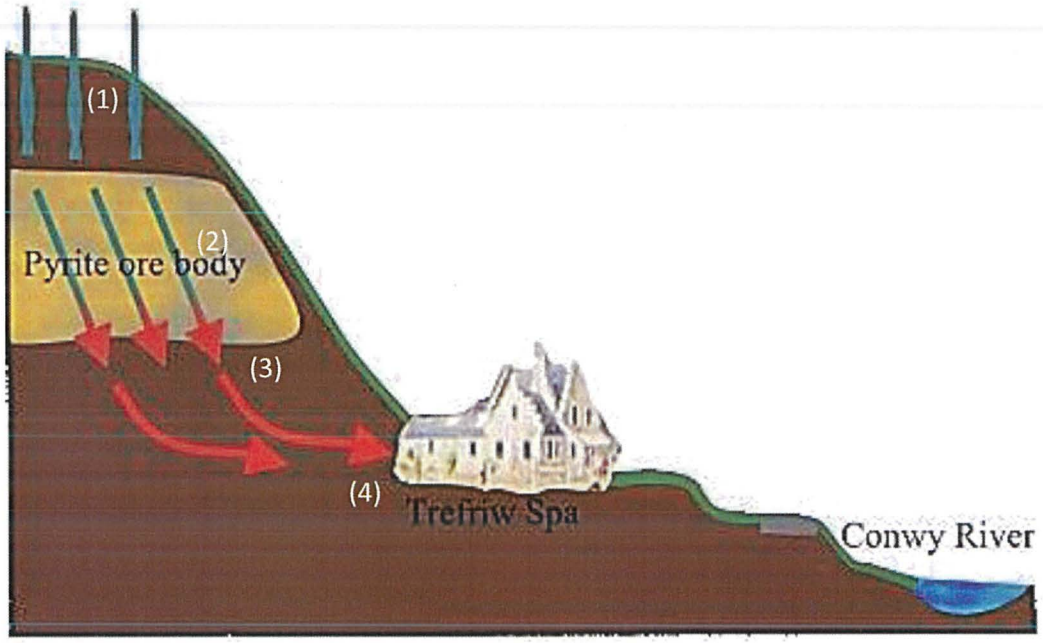


Figure 1.8. Diagram of the proposed process of Spatone water formation. Blue represents aerobic conditions and red lines/arrows represent anaerobic conditions. Water flows down the hillside, entering fissures in the pyrite ore body (1). Pyrite is oxidised by molecular oxygen and ferric iron, generating acidity (2). Oxygen is consumed, chiefly due to microbial oxidation of ferrous iron and reduced sulfur (3). Water entering the Trefriw Spa grotto is therefore extremely acidic, anaerobic, and the iron mostly present as ferrous (4).

1.4. Project Rationale

The project described was funded jointly by the European Union (under the *Knowledge Economy Skills Scheme*; KESS) and Nelsons Ltd. (parent company of Spatone), to examine in detail the biogeochemical processes that occur in the Cae Coch pyrite ore body, which result in the low pH, iron-rich water found at the Trefriw Wells Spa site and Cae Coch sulfur mine. The owners of Spatone are keen to extend their knowledge of the genesis of the iron-rich water at the Spa, and the roles of microorganisms in this process, and whether this knowledge can be used to predict future trends in Spa water chemistry and/or provide alternative scenarios for enhancing or modifying the product. Also, the mean concentration

of iron (as ferrous) in the spa waters is decreasing, meaning that it will drop below the 5 mg/L ferrous iron per 20 mL sachet required by Spatone Ltd in the near future. The same biogeochemical processes that result in the spa water are also responsible for the iron-rich acidic water draining from Cae Coch mine, which is a source of pollution. Therefore, this study also considered a possible amelioration strategy for Cae Coch AMD.

1.4.1. Aims and Objectives

The aim of this project was to increase understanding of the formation of the acidic chalybeate waters associated with the Cae Coch pyrite ore body, principally those of the less reported Trefriw Wells Spa, through a study of the biogeochemical reactions and interactions. The study also included studying some species of hitherto uncharacterised bacteria that are indigenous to the Spa water and, in the case of one isolate, its potential application in remediating Cae Coch mine water. Finally, the work included preparing a synthetic and concentrated version of Trefriw Spa water, which could be used to supplement the "natural" Spa water in future years in order to maintain its ferrous iron content.

Chapter 2. Materials and Methods

Materials and methods described in this chapter are used routinely throughout the study. Modifications to these methods and specific procedures used for individual experiments are described in detail in respective chapters. All chemicals and reagents are supplied by Fisher Scientific (U.S.A.) or Sigma-Aldrich (U.S.A.) and molecular biology reagents are from GenomeLab and Promega. All water is reverse osmosis (RO) grade, unless otherwise stated.

2.1. Microorganisms

Table 2.1 lists the microorganisms used in this study.

Table 2.1. List of acidophilic bacteria used in this study, their culture collection numbers and source.

Name (strain)	Culture collection number(s)	Culture source
At. ferrooxidans$^{T.}$		Bangor University Acidophile Culture Collection (Bangor, Wales)
Sb. thermosulfidooxidans$^{T.}$		Bangor University Acidophile Culture Collection (Bangor, Wales)
L. ferriphilum$^{T.}$		Bangor University Acidophile Culture Collection (Bangor, Wales)
Ac. aromatica$^{T.}$	NCCB 100456; DSM 27026	Bangor University Acidophile Culture Collection (Bangor, Wales)
Ac. aromatica (WJB-3)	NICMB 13670	National Collections of Industrial and Marine Bacteria (Aberdeen, Scotland)
Ac. aromatica (LGS-3)	NICMB 13671	National Collections of Industrial and Marine Bacteria (Aberdeen, Scotland)
Ac. facilis$^{T.}$	NCCB 86038	*Netherlands Culture Collection of Bacteria* (Utrecht, The Netherlands)

Ac. aminolytica T.	DSM 11237	*Deutsche Sammlung von Mikroorganismen und Zellkulturen* (Braunschweig, Germany)
Ac. aluminiidurans T.	NBRC 10430	*National Collection of Technology and Evaluation* (Tokyo, Japan)
"Acidocella organivorans"	DSM 100119; NCCB 100557	Trefriw Wells Spa, North Wales.
Atx. ferrooxidans T.	DSM 28176; JCM 19728	Bangor University Acidophile Culture Collection (Bangor, Wales)
Am. ferrooxidans	DSM 10331	*Deutsche Sammlung von Mikroorganismen und Zellkulturen* (Braunschweig, Germany)
Kocuria marina strain 16-57		Contaminated Spatone, Trefriw, North Wales.
Dermacoccus nishinomiyaensis T.		Contaminated Spatone, Trefriw, North Wales.
RF7		Trefriw Wells Spa, North Wales.
RW2		Trefriw Wells Spa, North Wales.
RW1		Trefriw Wells Spa, North Wales.
RF6		Trefriw Wells Spa, North Wales.
RF13a		Trefriw Wells Spa, North Wales.
RF3		Trefriw Wells Spa, North Wales.
RF13		Trefriw Wells Spa, North Wales.
FE1		Trefriw Wells Spa, North Wales.
FE3		Trefriw Wells Spa, North Wales.

2.2. Cultivation techniques

2.2.1. Sterilization

Heat-sterilization of liquids was carried out by autoclaving at 120°C for 20 minutes. Any heat-labile or volatile solutions were filter-sterilised using 0.2 µm (pore size) nitro-cellulose

membranes (Whatman, UK). Glassware and heat-stable solid materials were sterilised at 160°C for >8 hours. Elemental sulfur was sterilised at 110°C for 1 hour.

2.2.2. Microbial growth media and culturing conditions

2.2.2.1. Basal salts solutions and trace elements mixture

Different liquid media for cultivating acidophilic bacteria were prepared using one of three different basal salts formulation listed in Table 2.2.

Table 2.2. Compositions of basal salts mixtures used in the present study. All salts concentrations shown are in g/L.

Basal salts # 1(50x concentrate)	Basal salts # 2 (10x concentrate)	Basal salts # 3 (10x concentrate)
$Na_2SO_4.10H_2O$ (7.5) $(NH_4)_2SO_4$ (22.5) KCl (2.5) $MgSO_4.7H_2O$ (2.5) KH_2PO_4 (2.5) $Ca(NO_3)_2.4H_2O$ (0.7)	$MgSO_4.7H_2O$ (5.0) $(NH_4)_2SO_4$ (1.5) KCl (1.0) $Ca(NO_3)_2$ (0.1)	$Na_2SO_4.H_2O$ (32) $(NH_4)_2SO_4$ (30) KCl (1.0) $MgSO_4.7H_2O$ (5) KH_2PO_4 (0.5) $Ca(NO_3)_2.4H_2O$ (0.14)

Liquid and solid media that did not contain yeast extract were amended with trace elements, prepared as a 1000x concentrate (Table 2.3).

Table 2.3. Composition of the trace elements mixture used, diluted 1000-fold, in growth media.

Analyte (g/L)	
$ZnSO_4.7H_2O$; 10.0	$Na_2MoO_4.2H_2O$; 0.5
$CuSO_4.5H_2O$; 1.0	$NiSO_4.6H_2O$; 1.0
$MnSO_4.4H_2O$; 1.0	$NaSeO_4.10H_2O$; 1.0
$CoSO_4.7H_2O$; 1.9	$NaSeO_4.10H_2O$; 1.0
$Cr_2(SO_4)_3.15H_2O$; 0.5	$NaVO_3$; 0.1
H_3BO_3; 0.6	(H_2SO_4; 10 mM)

2.2.2.2. Liquid media

Table 2.4 lists the liquid media used routinely in this study. These were heat-sterilized and the media stored at room temperature. Ferrous iron (from a 1 M ferrous sulfate stock solution, adjusted to pH 2 with sulfuric acid) and any other heat-labile solutions were added immediately prior to inoculation.

Table 2.4. List of liquid media used in this investigation.

Medium	Constituents	pH
Ferrous iron medium	$FeSO_4$ (10 mM); basal salts#1 (25 mM); trace elements (1 mM)	2.5
Sulfur medium	1% (w/v) elemental S; 50 µM $FeSO_4$; basal salts#3 (80 mM); trace elements (0.4 mM)	3.0
Fructose medium	5 mM fructose; 100 µM $FeSO_4$; (25 mM); trace elements (1 mM)	3.0
Glucose medium	5 mM glucose; 100 µM $FeSO_4$; (25 mM); trace elements (1 mM)	3.0
Glucose/yeast extract medium	5 mM glucose; 0.01% (w/v) yeast extract; 100 µM $FeSO_4$; basal salts#1(25 mM)	3.0
Minimal salts medium	100 µM $FeSO_4$; (25 mM); trace elements (1 mM)	3.0

2.2.2.3. Solid media

(i) Non-overlay solid media

A variety of non-overlay solid media were prepared, gelled with agarose (Type 1; Sigma) at a final concentration of 0.5% w/v).

Acidic growth media and agarose solutions were heat sterilised separately and combined after cooling to avoid acid-hydrolysis of polysaccharide agarose. Various growth media solutions ("solutions A" in Table 2.5) were prepared (300 mL of each) and 100 mL of 2% agarose solution (solution B). After autoclaving, solution A was cooled to about 40°C, while solution B was placed in a water bath at 55°C to avoid gel formation. Filter-sterilised 1 M filter-sterilised ferrous sulfate solution was added to each solution A to give a ferrous iron concentration of 100 µM. Solution B was then added, the suspension mixed thoroughly then poured into Petri plates. The final pH of the solid media were generally ~ 0.5 pH unit higher

than the solution A on which it was based. Once set, plates were stored at 4°C for up to one month.

Table 2.5. Non-overlay solid media used in the present study.

Designation	Solution A contents	pH
YE4	yeast extract (0.02%*); basal salts#1	4.0
YE3	yeast extract (0.02%*); basal salts#1	3.0
Glu/YE	glucose (5 mM*); yeast extract (0.02%*); basal salts#1	3.0
Fru/YE	fructose (5 mM*); yeast extract (0.02%*); basal salts#1	3.0
Glu	glucose (5 mM*); basal salts#1; trace elements	3.0
Fru	fructose (5 mM*); basal salts#1; trace elements	3.0
Minimal salts	basal salts#1; trace elements	3.0

* final concentration in the combined solid medium

(ii) Overlay solid media

Overlay solid media were similar in some ways to the non-overlay variants. The main difference between them was that the gels were doubled-layered in the overlay solid media, with the lower layer inoculated with an acidophilic heterotroph (*Acidiphilium* SJH or *Ac. aromatica* PFBC).

Agarose and the main media components were heat-sterilised separately and combined when cooled. Table 2.6 lists the components of solutions A used for overlay media, and which heterotroph was inoculated into the gel underlayers. Ferrous iron was added at various concentrations to the combined media, which was then split (~60:40, by volume) and 2.0 mL of heterotrophic culture added to the smaller volume (~150 mL). This was poured into Petri plates to form a thin underlayer. Once these had set, the still molten (non-

inoculated) overlays were poured on top. Overlay plates were allowed a day to "mature" before used, and remained usable for up to 2 months by storing at 4°C.

Table 2.6. Solutions and microorganisms used in overlay plates used in the present study.

Designation	Solution A	Fe^{2+} (mM)	pH	Underlay organism
YE3o	yeast extract (0.02%*); basal salts #1	0.1	3.0	*Acidiphilium* SJH
YE3o/ PFBC	yeast extract (0.02%*); basal salts#1	0.1	3.0	*Ac. aromatica* PFBC
FeTSBo/ PFBC	tryptone soy broth (0.025%*); basal salts #2	10	3.0	*Ac. aromatica* PFBC
iFeo	basal salts #1; trace elements	20	2.5	*Acidiphilium* SJH
FeSo	potassium tetrathionate (2.5 mM*); tryptone soy broth (0.025%*); basal salts #2	20	2.5	*Acidiphilium* SJH
FeTo	sodium thiosulfate (2.5 mM*); tryptone soy broth (0.025%*); basal salts #2	10	6.5	*Acidiphilium* SJH
Glu/YEo	glucose (5 mM); yeast extract (0.02%*); basal salts #1	0.1	3.0	*Ac. aromatica* PFBC
Fru/YEo	fructose (5 mM); yeast extract (0.02%*); basal salts #1	0.1	3.0	*Ac. aromatica* PFBC
Fruo	fructose (5 mM); basal salts #1; trace elements	0.1	3.0	*Ac. aromatica* PFBC

* final concentration in the combined solid medium

2.2.2.4. Growth of bacteria on hydrogen

Minimal salts liquid medium (or iFeo plates) were inoculated and placed in a 2.5 L AnaeroGen jar (Oxoid Ltd., UK). A hydrogen/carbon dioxide enriched atmosphere was created inside the jar by mixing together (in a 25 mL universal bottle) 1.3 g $NaHCO_3$, 1.0 g

sodium citrate trihydrate and 0.3 g sodium borohydride, and adding 10 mL water. The jar was immediately sealed and incubated at 30°C without shaking, with the hydrogen-generating mix was replaced as required (usually every 7 days).

2.2.3. Incubation under different oxygen status

Cultures occasionally required incubation under microaerobic or anaerobic conditions. To generate a microaerobic atmosphere, inoculated liquid or solid media cultures were placed in a 2.5L sealable jar (Oxoid Ltd., UK) containing a Campy*Gen*™ CN25 sachet (Oxoid, UK). This lowered the oxygen concentration to ~6% (by volume) conditions. To generate anaerobic conditions, inoculated liquid and solid media were placed in 2.5 L sealable jars (Oxoid Ltd., UK.) containing Anaero*Gen*™ AN25 anaerobic atmosphere generating sachets. In this system, oxygen was reduced to CO_2.

2.3. Long term cryogenic storage of cultures

Either 50% glycine butane or 7% dimethylsulfoxide (DMSO) were added as cryo-protectants to triplicate 1 mL tubes containing active cultures in liquid media. These were stored at -80°C. To confirm viability, after 7 days one culture containing each cryoprotectant was defrosted and a small amount added to solid and liquid media suitable for the bacterial culture being stored.

2.4. Microscopy

2.4.1. Stereo-scan microscopy

Colonies grown on solid media were observed and enumerated with a stereo-scan microscope (Leitz-Wild M32, Switzerland) at between x50-x400 magnification. Images were captured with a GXCAM-5 (GT Vison Ltd.) digital camera attached to the microscope.

2.4.2. Phase-contrast microscopy

Visualisation of individual microbial cells was carried out with a Leitz Labolux phase contrast microscope at x400 magnification. Phase contrast images were captured with an ECLIPSE E600 microscope (Nikon, Japan) at x1000 magnification and a COOLPIX digital camera (Nikon, Japan).

2.4.3. Cell counts

Microbial cells were dispersed and approximately 20 µL of culture placed on a Thoma bacterial counting chamber with coverslip (Hawksley, U.K). A Leitz Labolux phase contrast microscope and x40 objective lens (x400 magnification) was used to count cells in triplicate. Once a total of at least 50 cells was reached, the number of cells in 1 mL was calculated with equation [2.1] (assuming 1 mL (=10^{12} µm^3/5x10^4)

$$[2.1] \quad \text{Cell concentration} = \frac{\text{Total mean cells counted} \times (2 \times 10^7)}{\text{Number of squares}}$$

2.5. Preparation of biomass

2.5.1. Cell harvesting

For culture volumes of 400 mL or more, equally weighted centrifuge bottles were spun in a refrigerated centrifuge at 32480 x g for 20 min at 4°C (Avanti J-26-XP and JLA 16.250 rotor). Pellets were re-suspended in sterile basal salts water pH 2 (8 mL of basal salts#1 per 1 L of water, adjusted to pH 2 with 1 M H_2SO_4), before being transferred to 1.5 mL Eppendorf tubes and re-centrifuged (Eppendorf centrifuge 5415 D) for 3 min at 16,000 x g and the supernatant removed. For culture volumes of less than 400 mL, equally weighted sterile 50 mL Falcon tubes were spun in a refrigerated centrifuge (Sigma 3-16PK and 11150 rotor) at 3450 x g for 20 min at 5°C. The method used was subsequently as previously described. Pellets were stored at -20°C until required.

2.5.2. Freeze drying

Harvested biomass was washed by Tris buffer (pH 7) until its pH approached neutral (supernatant sample colour changed to blue-green using 1:1 bromoethymol blue indicator). Samples were then given a final wash with sterile water, frozen using liquid nitrogen and dried using a lypophiliser.

2.6. Analytical Methods

2.6.1. Measurement of pH and redox potentials (E_H)

A pHase combination glass electrode (VWR) calibrated between 1.00 and 7.000, coupled to an Accumet 50 pH meter was used to measure pH. E_H was measured using a combined platinum-Ag/AgCl redox electrode (InLab® 501; Mettler Toledo, U.S.A) coupled to the above

meter, and calibrated against a standard (+440 +/- 5 mV; ZoBell's solution: 4.22 g $K_4Fe(CN)_6$ and 4.65 g $K_3Fe(CN)_6$ dissolved in water and made up to 100 mL).

2.6.2. Determination of ferrous and total iron concentrations

Ferrous iron concentration was determined by Ferrozine assay (Stookey, 1970). Ferrozine reagent was prepared as follows: 50 mL HEPES Buffer (11.915 g N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid, adjusted to pH 7 with 2 M KOH) and 0.5 g Ferrozine (3-(2-pyridyl)-5,6-bis (phenyl-sulfonic acid)-1,2,4-triazine) were dissolved in 500 mL distilled water. This was stored in darkness at room temperature. Colour strength when 50 µL of sample containing ferrous iron was added to 950 µL of Ferrozine reagent was quantified using a spectrophotometer (Cecil CE 1011) at 562.0 nm, with a reagent blank. An optical density of 2.000 or higher approached the upper limit of the calibration curve so the sample was diluted appropriately with distilled water. Optical density was converted to Fe^{2+} mM using an equation from a calibration curve. This calibration curve was generated by measuring the optical density of a range of $FeSO_4$ standards of known Fe^{2+} concentration (Stock solution 50 mM: 1.39 g ferrous sulfate in 100 mL distilled water, adjusted to pH 1.7 with 1 M H_2SO_4 and diluted to 10 mM before use as standards).

For total iron concentration, an excess of L-ascorbic acid was added to solution to reduce Fe^{3+} to Fe^{2+}. The L-ascorbic acid powder was completely dissolved in the sample before performing a Ferrozine analysis. Ferric iron concentrations were determined as the difference between total iron and ferrous iron concentrations.

2.6.3. Determination of protein

Protein concentrations were determined by the Bradford assay (Bradford, 1976). A calibration curve was prepared by preparing standards containing 0-100 µg/mL bovine serum albumen (BSA) (0.05 g BSA powder stored at 4°C, in 5 mL distilled water for a 10 mg/L stock solution, which was stored at -20°C), made up to 100 µL with 0.5 M NaOH. 1 mL of Bradford reagent (100 mg/L Coomassie Brilliant Blue G-250 dissolved in water containing 5% (v/v) ethanol, 10% (v/v) phosphoric acid in water, and stored in darkness room temperature) was added to this, and incubated in the dark for 2 min. Optical density was measured at 595 nm, with standard 0 as blank. Biomass was harvested by refrigerated centrifugion (section 2.4.4) and re-suspended in 0.5 mL 0.5 M NaOH. Biomass was then incubated at room temperature (~22°C) for 15 min, and treated the same as BSA calibration samples.

2.6.4. Ion chromatography (IC)

Concentrations of glucose, fructose and metal cations were determined by ion chromatography. Approximately 10 mL of samples were filtered (through Whatman 0.2µm nitro-cellulose membranes) and stored at 4°C until required. Aliquots of sample were appropriately diluted with ultra-pure water to give an estimated substance concentration of less than 2 mM with distilled water in IC vials. Standards were 0.1, 0.5 and 1 mM concentration, and run once at the beginning and end of a program. Occasional Ultra-pure water samples were also included as part of the program. Data was analysed by Chromelion software (Version 6.40 SP2 Build 731; Dionex, U.S.A).

Glucose and fructose were analysed using a Dionex AP autosampler with ICS-3000 pump and ED50 electrochemical detector, ED 40 amperometric detector and a Carbo Pac™ Pa10 column. Column temperature was maintained at approximately 30°C, sample injection was

250 µl and flow rate was 1 mL/min. Column pressure was maintained at approximately 2.500 p.s.i. (minimum 200 p.s.i., maximum 3,000 p.s.i.). Eluent (KOH) was 18 mM and pH of sample/eluent mix was maintained between pH 10 and 13.

Concentrations of metal cations were detected with an Ion Pac^{R} CS5A column, AS50 autosampler, LC25 chromatography oven, AD25 absorbance detector (520 nm) and IP25 isocratic pump. Flow rate was 1.2 mL/min, column pressure was 1600 p.s.i. and sample injection was 250 µL. The eluent contained 7 mM pyridine-2,6-dicarboxylic acid (PDCA), 66 mM KOH, 5.6 mM K_2SO_4 and 74 mM formic acid at pH 4.2 +/- 0.1. The post column reagent contained 1 M 2-dimethylaminoethanol; 0.5 M ammonium hydroxide; 0.3 M sodium bicarbonate and 0.12 g/L 4-(2-pyridylazo) resorcinol (PAR) at pH 10.4+/- 0.2.

2.6.5. Determination of sulfate concentrations

Sulfate concentrations were determined by precipitating sulfate as insoluble barium sulfate and measuring turbidity (Kolmert *et al.*, 2005). 400 µL conditioning reagent (100 mL glycerol; 60 mL concentrated HCL; 200 mL 95% ethanol and 150g NaCl in RO water) was added to 5 mL sample (and potassium sulfate standards for a calibration curve) and vortexed, then an excess of crushed barium chloride crystals added (~60 mg). This was vortexed for 20 s and left to settle for 5 min, and then absorbance measured at 420 nm (Cecil 1100E spectrophotometer) against a sulfate-free blank.

2.7. Biomolecular methods

2.7.1. Preparation of template DNA

Three different approaches were used to prepare template DNA. All product was stored at -20°C.

2.7.1.1. CTAB (cetyl trimethylammonium bromide) method

Biomass was washed in 50 mM Tris buffer pH 7 (1.14 g Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) dissolved in 800 mL water, adjusted to pH 7 with 2 M KCl, made up 1 L and stored at room temperature) to remove acid, re-suspended in a mix containing 567 µL TE buffer (10 mM Tris buffer pH 7 and 1 mM 0.5 M EDTA (ethylene-diamine-tetraacetic acid) in ultra-pure water), 5 µL RNAase A, 15 µL 10% SDS and 4 µL proteinase K (18 mg/ mL) and incubated at 37°C for 1 h. Next, 100 µL 5 M NaCl was added and mixed well (otherwise CTAB formed a precipitate with the nucleic acid, rather than assisting in removing unwanted cell contents). 80 µL of CTAB/NaCl (10% w/v; 0.7 M) solution was then added, and the mix incubated for 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (1:1) was added, the mix centrifuged (5 min at 16,000 x g), and the lower viscous supernatant fraction removed. 1 volume of isopropanol was added, removed by centrifugion (5 min at 16,000 x g), the resulting pellet washed with 70% ethanol and re-suspended in 50 µL Tris buffer pH 7.

2.7.1.2. Lysis method

1.5 mL of culture was centrifuged in 1.5 mL Eppendorf tubes at 16,000 x g for 10 minutes. The supernatant was drawn off, and pellets containing iron-oxidising microbes were washed by re-suspension and centrifugation (16,000 x g for 10 minutes) in 0.5 mL 100 mM oxalic acid to remove ferric iron precipitates. Pellets were also washed in ultra-pure water then re-suspended in 20 µL PCR lysis buffer (9.25% SOS, 0.05 M NaOH). This was transferred to 0.5 mL PCR tubes and run through a "lysis program" (Table 2.9) in a thermo-cycler (Techgene 12.02). 180 µL of ultra-sterile water was added, and the tubes centrifuged at 16,000 x g for a further 10 minutes. Appropriate dilutions (usually 1:5 or 1:10) were made by diluting the supernatant in ultra-pure water.

2.7.1.3. Lysosyme method

20 µL of lysosyme suspension (10 mg/mL) and 80 µL 50 mM EDTA was added to washed (to remove acid) and pelleted biomass, and incubated at 27°C for 15 min. This was then centrifuged at 16,000 x g for 10 min, and 5 µL of the liquid lysed as for 2.7.1.2.

2.7.2. PCR (polymerase chain reaction) and gel electrophoresis

1 µL of extracted DNA was added to 19 µL of PCR reagent (Tables 2.7 and 2.8), with a positive and negative bacterial control. These were centrifuged (on short program) for 4 seconds, and put through a thermo-cycler machine (Techne® TC-312; Midwest Scientific, USA). Table 2.9 lists the PCR programs used. Digests were stored at -20°C for up to 1 week.

Table 2.7. Table of reagents used for various PCR reactions, and their specific roles.

Reagent	Role	16S/18S rRNA gene reaction mix1(µL)	*NifH* reaction mix x1(µL)	*cbbL* reaction mix x1(µL)	*cbbM* reaction mix x1(µL)
Ultra-pure water (Sigma)	Eluent	9.6	5.6	9.6	9.2
Go taq buffer x5	Maintain optimal chemical conditions for Taq enzyme	4.0	4.0	4.0	4.0
dNTPs (2 mM)*	Provides deoxynucleic triphosphates for DNA synthesis	2.0	2.0	6.0	6.0
$MgCl_2$ (25 mM)	Mg^{2+} orientates dNTPs to DNA for elongation.	2.0	1.5	0.38	1.6
Forward primer (10 pmol/ µL)	Complementary to 3' ends of required gene region, allowing sequencing of only that region.	0.4	3.5	1.0	1.0
Reverse primer (10 pmol/ µL)		0.4	3.5	1.0	1.0
DMSO	Binds to cytosine residue, changing the structure to make DNA more heat labile.	0.4	-	-	-
Go-Taq polymerase	Assembles DNA from nucleotides. Taq polymerase enzyme from *Thermus aquaticus*.	0.2	0.2	0.6	0.6
Total volume		19.0	19.0	19.0	19.0

*2 mM dATP, dCTP, dTTP and dGTP.

Table 2.8. Primers used for PCR in the present study.

Primer	Sequence (5'-> 3')	Action
27FC (labelled)	AGAGTTTGATC(A/C)TGGTCAG	Bacterial 16S T-RFLP forward primer labelled with D4-phosphoramadite (ResGen, U.K.)
27FC (unlabelled)	AGAGTTTGATC(A/C)TGGTCAG	Bacterial 16S forward primer
13872R	GGGCGG(A/T)GTGTACAAGGC	Bacterial 16S reverse primer
Nifu 19F	ATGTGGGATTATTCGGAAAA	*Nif* forward primer
Nifu 4072	TCAGCCTCCATCTGCCGTGGG	*Nif* reverse primer
cbbL F	CTTCGAGTTCGTGGGC	RuBisCO I forward primer
cbbL R	GCACGCTCGTACATCT	RuBisCO I reverse primer
cbbM F	CAGGCCAAGATTTTTCTCTGC	RuBisCO II forward primer
cbbM R	TACCTGCACTACCATCGTGC	RuBisCO II reverse primer
EukF	ACCTGGTTGATCCTGCCAGT	Eukaryote 18S forward primer
EukR	TGATCCTTCYGCAGGTTCAC	Eukaryote 18S reverse primer
Arch 20F	TTCCGGTTGATCCYGCCRG	Archaeal 16S forward primer
Arch 915R	GTGCTCCCCCGCCAATTC	Archaeal 16S forward primer

Table 2.9. List of thermo-cycler PCR programs used in the present study.

Program name	No. cycles	Cycle details		
		Description	Temperature	Duration
Lysis	1	Warm up		4 min
		Denaturation	95°C	15 min
		Hold	4°C	Indefinite
Bacterial 16S rRNA gene		Warm up		
		Denaturation	95°C	5 min
	35	Segment	95°C	30 s
		Segment	55°C	30 s
		Segment	72°C	1 min 30 s
		Finish	72°C	10 min
		Hold	4°C	Indefinite
NifH gene		Warm up		
		Denaturation	95°C	5 min
	40	Segment	95°C	30 s
		Segment	56°C	30 s
		Segment	72°C	1 min 30 s
		Finish	72°C	5 min
		Hold	4°C	Indefinite
cbbL gene		Warm up		
		Denaturation	95°C	5 min
	40	Segment	95°C	30 s
		Segment	57°C	30 s
		Segment	72°C	30 s
		Finish	72°C	10 min
		Hold	4°C	Indefinite

cbbM gene		Warm up		
		Denaturation	95°C	5 min
	35	Segment	95°C	5 min
		First[1]	52°C	30 s
		Last[2]	60°C	30 s
		Segment	72°C	30 s
		Finish	72°C	5 min
		Hold	4°C	Indefinite
Archaeal 16S rRNA gene		Warm up		
		Denaturation	95°C	5 min
	35	Segment	95°C	30 s
		Segment	62°C	30 s
		Segment	72°C	1 min 30 s
		Finish	72°C	10 min
		Hold	4°C	Indefinite
Eukaryote 18S rRNA gene		Warm up		
		Denaturation	86°C	3 min
	1	Segment	Pause	
	40	Segment	95°C	30 s
		Segment	55°C	30 s
		Segment	60°C	4 min
		Hold	4°C	Indefinite

[1]Temperature of second segment. [2]Temperature of last segment. Successive segments change temperature incrementally, beginning at 'First' and ending at 'Last'.

A 0.8% agarose gel was prepared by adding 0.2 g electrophoresis-grade agarose to 25 mL TBE (5.4 g tris-hydroxymethyl-methylamine, 2.75 g boric acid and 2 mL 0.5 M EDTA, pH adjusted to 8.0 with NaOH) and microwaving for 2 minutes. 0.6 µL ethidium bromide (10

mg/mL) was added once the gel had melted, then it was poured into a PCR electrophoresis gel mould and left to set. Once set 5 µL of PCR digest and 1 µL of x6 blue/orange loading dye (0.25% (w/v) bromophenol blue in 30% glycerol) was centrifuged in sterile PCR tubes for 4 seconds (to remove any droplets), and loaded in wells in the gel along with 5 µL 1kb DNA ladder (100 µg/L). The gel was run with a BioRad Power Pac 300 powerpack at 90 mV for 30 min, and visualised under UV light using a Gel-Doc EQ and Quantity One software.

2.7.3. T-RFLP (terminal restriction enzyme fragment length polymorphism) analysis

A PCR digest (using a labelled forward primer; Table 2.8) was first run on an electrophoresis gel to confirm PCR product. 3 µL PCR product was added to a T-RFLP reaction mix (6.5 µL ultra-pure water, 1 µL restriction enzyme buffer, 1 µL x10 diluted (bovine serum albumen) and 0.5 µL corresponding restriction endonuclease enzyme; Table 2.10) in a 1.5 mL Eppendorf tube, and centrifuged for 4 s. Tubes were then covered in foil to exclude light and incubated at 37°C for 1 h. 29 µL size standard was added to each well in a row of a 96-well plate. 1 µL of each digest was added to its own well and a drop of mineral oil. The corresponding row of wells in a buffer tray was filled with separation buffer. Once prepared using this protocol, digests were analysed using a CEQ machine (Beckmann and Coulter CEQ8000, USA). Peaks were compared to other known T-RFs using the T-RFLP database maintained at Bangor University (edition 3.01).

Table 2.10. List of restriction enzymes used in this study. ▼ and ▲ indicate sites where restriction enzymes cut double-stranded DNA.

Restriction Enzyme	Restriction sites
*Alu*1	5'-AG▼CT-3' 3'-TC▲GA-5'
*Cfo*1	5'-GCG▼C-3' 3'-C▲GCG-5'
*Hae*3	5'-GG▼CC-3' 3'-CC▲GG-5'

2.7.4. Analysis for confirming the presence of the gene coding for nitrogenase (nifH)

The method described by Ueda *et al.* (1995) was adapted for this purpose. DNA was extracted by cell lysis (section 2.7.1.2) 1 µL of extracted DNA and an appropriate dilution were each added to 19 µL *nifH* reagent (Tables 2.7 and 2.8). *At. ferrooxidans*^{T} was included as a positive control. This was then run through a PCR machine (*nifH* program; Table 2.9), and treated as for PCR gel electrophoresis, with visualisation on a 2% gel (1 g agarose per 50 mL water) (section 2.7.2).

2.7.5. Analysis for confirming the presence of gene coding for RuBiSCo (carbon dioxide fixation) (cbbL and cbbM)

The method described by Alfreider *et al.* (2003) was adapted for this purpose. DNA was treated as above (section 2.7.2), with *cbbL* or *cbbM* reagents (Tables 2.7 and 2.8) and corresponding PCR programs (*cbbL* or *cbbM* programs; Table 2.9) in place of those described for *nifH*.

2.7.6. Constructing phylogenetic trees

16S rRNA gene sequences (obtained this study or the NCBI) were combined in a single file. An alignment containing generated with ClustalX, and then imported to MEGA 5. Sequences were manually trimmed to the length of the shortest sequence in ClustalX, so that all sequences in the alignment were complete. The alignment was then used to generate bootstrapped (x1000) maximum likelihood (Tamura-Nei Model, with uniform rates among sites and Nearest-Neighbour-Interchange inference method), maximum parsimony (Subtree-Pruning-Regrafting inference method) and neighbour-joining (Maximum composite likelihood with uniform rates among sites among sites) trees in MEGA 5.

Chapter 3. Preparation of synthetic Spatone water

3.1. Introduction

The ferrous iron rich spring water at Trefriw is sold as a '*Natural Iron Supplement*' by site owners, Spatone Ltd. (a subsidiary of Nelsons UK). The water is collected, filtered, packaged and sold within the UK and internationally as '*Spatone', 'Ferrotone'* and '*Pur-Absorb Iron'* natural iron supplement (section 1.3.3). It is formed by meteorological water passing through a mineralogically relatively pure pyrite ore body (Ball and Bland, 1985; Botterell and Morton, 1992; Chapter 1). The spa water pH is approximately 3 and contains little or no dissolved oxygen, with iron mostly present (74 - 98%) as soluble Fe^{2+}. It also contains relatively small concentrations of dissolved solutes other than iron and sulfate, in comparison to similar acidic waters such as many acid mine drainage waters. These characteristics have allowed the Trefriw spa site to be exploited as an economically-viable entity since the 19th century.

However, the spa water iron concentration and water volume discharge is dependent on hydrological and biogeochemical conditions and so subject to variation (Fig. 3.1). The mean iron concentration in the spa water is also decreasing over time as the ore body depletes (Fig. 3.1), which is typical of acid mine drainage waters (Younger, 1997; Younger, 2000). This is undesirable in a regulated product, where each 25 mL sachet (maximum volume) is required to contain 5 mg of ferrous iron (i.e. 200 mg/L minimum concentration). One way of dealing with these issues could be varying the volume of water in each sachet, though the 25 mL maximum sachet volume places a limit on this approach. Another way would be to add solid ferrous sulfate, though this would result in the loss of the "naturally-formed" claim, which the site owners consider a marketing advantage.

The objective of the work described in this chapter was to develop a concentrated dissolved ferrous iron solution using the same microbiological processes that occur in the pyritic ore

body located above Trefriw Spa. This concentrate could then be used to supplement the spa water when the ferrous iron concentration fell to non-tenable levels for a large portion of the year. As shown in Fig. 3.1, this point is predicted to occur in the near future.

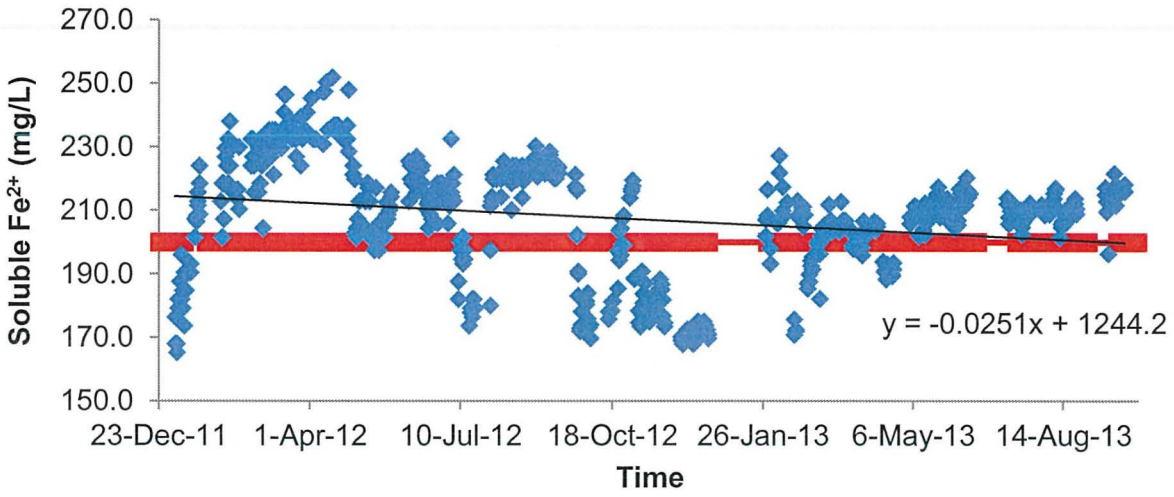


Figure 3.1. Dissolved ferrous iron concentrations (♦) at Spatone well 1, over the period January 2011 to September 2013, taken from Spatone production data. Minimum tenable concentration of iron shown in **red** and best-fit line shown in **black**.

Since the approach used to generate this potential supplement was designed to mimic the biogeochemical processes known to occur within the pyritic ore body, the modified product should also continue to maintain its classification as a natural iron supplement. An additional benefit to using this approach was that insights into the process that formes the natural water in the Cae Coch pyrite ore body might be gained.

3.2. Rationale of the experimental approach used

Iron-rich liquor similar to that of Trefriw spa water was prepared in a bioreactor, by methods pertaining to the processes by which the spa water is thought to form at the site and refined over successive experiments. A two-stage process was used. In the first stage, acidophilic sulfide mineral-oxidizing bacteria were used to solubilise iron and sulfur from pyrite via oxidative dissolution, according to equations [3.1] and [3.2]:

$$FeS_2 + 6\ Fe^{3+} + 3\ H_2O \rightarrow 7\ Fe^{2+} + S_2O_3^{2-} + 6\ H^+ \text{ (abiotic)} \quad [3.1]$$

$$4\ Fe^{2+} + 4\ H^+ + O_2 \rightarrow 4\ Fe^{3+} + 2\ H_2O \text{ (biotic)} \quad [3.2].$$

In the second stage, a combination of abiotic (equation [3.1]) and microbially-catalysed reactions (e.g. equation [3.3]) was used to regenerate ferrous iron from ferric:

$$S_2O_3^{2-} + 8\ Fe^{3+} + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 8\ Fe^{2+} + 10\ H^+ \text{ (biotic and abiotic)} \quad [3.3].$$

Since reactions [3.1] and [3.3] do not require oxygen, while reaction [3.2] does, control of aeration (by gassing with either air for aerobic conditions, or oxygen-free nitrogen (OFN) for anaerobic conditions) was used to mediate redox transformations of iron. The method was modified with a view to eventual development as a full-scale process for producing synthetic Spatone water.

3.3. General culture conditions

A 2 L (working volume) bioreactor (Electrolab, UK) fitted with pH and temperature control was used in these experiments. The reactor vessel (2.3 L total volume) was filled with 2 L of medium containing either (a) 1 mM Fe^{2+}, basal salts (#1) and trace elements for experiments 3.1 and 3.2, (b) standard synthetic iron-free Spatone water for experiments 3.3, 3.4 and 3.5, or (c) concentrated synthetic iron-free Spatone water for experiment 3.6. The rationale for the difference in liquor treatments was as follows: (a) growth media was used because the aim of experiments 3.1 and 3.2 was to discover whether the proposed system worked as hypothesised, and the maximum amount of ferrous iron produced. (b) Standard iron-free synthetic Spatone was substituted to more closely replicate the Trefriw water. (c) Concentrated iron-free synthetic Spatone was used because the general aim of this chapter was to produce concentrated synthetic Spatone.

Standard and concentrated iron-free synthetic iron-free Spatone waters were prepared by adding known volumes of sulfate salts from concentrated solutions to acidified RO-grade

water (Tables 3.1). A concentrated solution of calcium sulfate could not be prepared because it has limited solubility, so solid material added directly to the acidified water. The concentrated synthetic Spatone water contained 10-times the concentration of each analyte compared to the standard version, apart from: (i) calcium sulfate, which was only 2.5-times as concentrated (due to solubility issues), and (ii) potassium phosphate and ammonium sulfate which were included in the concentrated version but absent in the standard version.

Table 3.1. Volumes of 1 M sterile standard solutions (µL) used to prepare 1 L of iron-free standard and concentrated synthetic iron-free Spatone water.

	Standard synthetic Spatone	Concentrated synthetic Spatone
$MnSO_4$	30	300
$ZnSO_4$	3	30
$MgSO_4$	1700	17000
K_2SO_4	10	100
$CaSO_4.2H_2O$	0.73 g (solid)	1.83 (solid)
KH_2PO_4	0	1000
$(NH_4)_2SO_4$	0	1000
(pH	1.8	1.6)

Table 3.2 lists the concentrations of different metals present in the prepared liquors used in this investigation in comparison to typical concentrations in the original Spatone product. The prepared synthetic Spatone waters did not contain iron (which would be generated by dissolution of pyrite), so the concentration of sulfate was also less than that of the spa water produced on site. It was expected that with the dissolution of the pyrite, the concentration of sulfate would also increase. In addition, small concentrations of transition metals (e.g. cadmium; ~1.5 µg/L) and aluminium (~40 mg/L) present in the original Spatone were not included in the synthetic versions, as they are potentially harmful to human health.

Table 3.2. Comparison of the elemental compositions of actual Spatone water, synthetic iron-free Spatone waters, and the basal salts/trace elements used in experimental work.

Element	Concentration (mg/L)			
	Spatone*	Synthetic iron-free Spatone (standard)	Synthetic iron-free Spatone (concentrated)	Basal salts (#1) /trace elements
Iron	200	0	0	0
Calcium	170	170	426	2.4
Magnesium	41	41	410	50
Sodium	12	0	0	21
Manganese	1.6	1.6	16	0.24
Potassium	0.4	0.4	4.0	40
Zinc	0.2	0.2	2.0	2.3
Ammonium; N	0.008	0	28	95
Sulfate; S	314	192	930	190
Chloride	14	0	0	24
Nitrate; N	0.07	0	0	1.7
Phosphate; P	ND	0	31	11.4

*data supplied by Nelson's personnel

Fifty grams of research-grade finely ground pyrite (FeS_2; STREM Chemicals, Newburyport, MA, USA) was added before (experiment 3.1) or after (other experiments) sterilisation of the reactor vessel containing the liquid medium by heat sterilisation (section 2.2.1). Bioreactor temperature was maintained at 30°C (experiments 3.1-3.3) or 40°C (experiments 3.4-3.6), and the vessel volume maintained at 2 L by addition of sterile RO water as required. pH in the bioreactor was maintained at pH 1.8 (experiments 3.1- 3.3) or 1.6 (experiments 3.4 - 3.6) by automated addition of sterile 1 M NaOH or 1 M H_2SO_4. These values were different to those of Trefriw spa water itself (pH ~3, and ~10°C) to optimize rates of pyrite dissolution. Pyrite slurry was stirred at 200 rpm and the reactor liquor gassed with either sterile air or

OFN (oxygen free nitrogen) at 500 mL/min, depending on whether aerobic or anaerobic conditions were required. A redox electrode coupled to an independent pH/redox meter (Thermo Scientific Orion 3 star bench-top) was also included. Redox potentials could not be automatically controlled, though manual gassing with either air or OFN allowed some degree of control of E_H values.

A mixed *Acidithiobacillus* (*At.*) culture (containing *At. ferrooxidans*^{T} and *At. ferridurans*^{T}) was used in experiment 3.1, and a pure culture of *At. ferrooxidans*^{T} in experiments 3.2 and 3.3. *At. ferrooxidans*^{T} and *At. ferridurans*^{T} can both oxidize and reduce iron, and are metabolically active under both aerobic and anaerobic conditions. *Leptospirillum (L.) ferriphilum*^{T} was used in experiments 3.4, 3.5 and 3.6, as it is more efficient at oxidising iron at moderately thermophilic (>40°C) temperatures. *Sulfobacillus (Sb.) thermosulfidooxidans*^{T} (which can also both oxidize and reduce iron) was included in experiments 3.4-3.6 since *L. ferriphilum* oxidises iron only.

Samples were withdrawn at regular intervals to measure solution pH and redox potentials using off-line electrodes that were calibrated each day, and on-line electrode and meter values corrected where required.

3.4. Experiment 3.1

Experiment 3.1 was a preliminary experiment set up to test whether it was possible to bioleach pyrite to generate synthetic Spatone, by first producing soluble Fe^{3+} then converting this to Fe^{2+} by removing oxygen from the system.

A 2 L (working volume) bioreactor containing 50 g of pyrite and basal salts/trace elements was set up and sterilized as described above. Autoclaving the pyrite resulted in the production of large amounts of soluble iron (400 mg/L), 80% of which was present as ferrous and the remaining 20% as ferric. The reactor was then inoculated with 5 mL of mixed *At. ferrooxidans*^{T}. and *At. ferridurans*^{T}. (grown in 25 mL basal salts/trace elements liquid

medium containing 500 mg pyrite, pH 2). The bioreactor was aerated until redox potentials and total soluble iron concentrations stabilised (264 h after the start of the experiment). Concentration of total soluble iron increased by 33% during the aerated period (reaching 600 mg/L). The air supply was then turned off and changes in redox potential monitored. As predicted by equation [3.1], concentrations of total soluble iron continued to increase (to 871 mg/L). However, contrary to what would be predicted from equations [3.1] and [3.3], ferric iron became the dominant species present, reflected in increasingly positive redox potentials. This was considered due to the presence of residual dissolved oxygen in the bioreactor liquor, which would have allowed the iron-oxidising bacteria to convert ferrous iron to ferric. At 312 h after the start of the experiment, the reactor liquor was gassed with OFN, and subsequently E_H decreased as ferric iron was reduced to ferrous. The experiment was terminated at 364 h, with pyrite leachate containing 664 mg/L of total iron, 52% of which was ferrous and 48% of which was ferric.

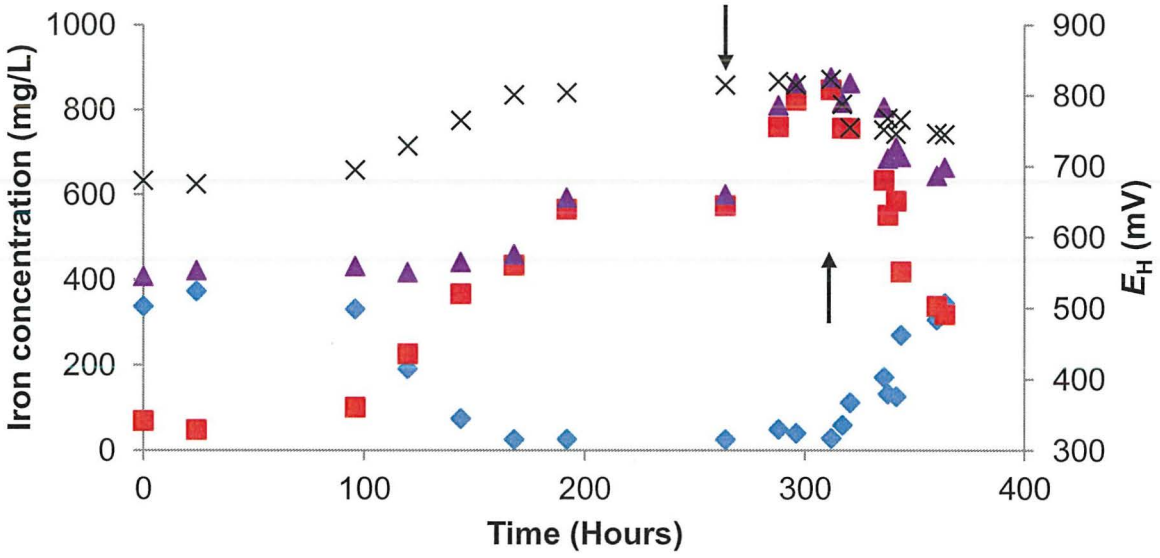


Figure 3.2. Preparation of synthetic Spatone water (data from experiment 3.1). Key: concentrations of ferrous iron (◆), ferric iron (■) and total iron (▲), and redox potential measurements (X). The downward-pointing arrow indicates the time at which aeration was turned off, and the upward-pointing arrow the time when gassing with OFN commenced.

3.5. Experiment 3.2

The aims of experiment 3.2 were: (i) to confirm and substantiate the general trends observed in experiment 3.1; (ii) to increase both the concentration of total soluble iron obtained by bioleaching the pyrite; (iii) to achieve a higher final ratio of ferrous: ferric iron in the liquor produced.

The set up was similar to Experiment 3.1, with the following changes: (i) 2 mL of a pure culture of *At. ferrooxidans*^{T} (grown in basal salts/trace elements plus 500 mg pyrite, pH 1.8) was used as inoculum; (ii) 50 g FeS_2 was sterilised separately (at 160°C for 16 h) and added to the autoclaved solution in the bioreactor vessel in order to decrease the initial amount of soluble iron present; (iii) ferrous iron (230 mg/L) was added to the reactor to initiate microbial iron oxidation and pyrite oxidation; (iv) 5 g of sterile sulfur (VWR, UK) was added to the

bioreactor at the anaerobic stage (72 h) to enhance microbially-catalysed ferric iron reduction, according to equation [3.4]:

$$S^0 + 6\ Fe^{3+} + 4H_2O \rightarrow 6\ Fe^{2+} + SO_4^{2-} + 8\ H^+ \qquad [3.4].$$

During the oxidative phase of pyrite dissolution, total soluble iron increased much more rapidly than in experiment 3.1 (by 650 mg/L over 72 hours) and, again in contrast to the first experiment, most of this was present as ferric iron (Fig. 3.3.).

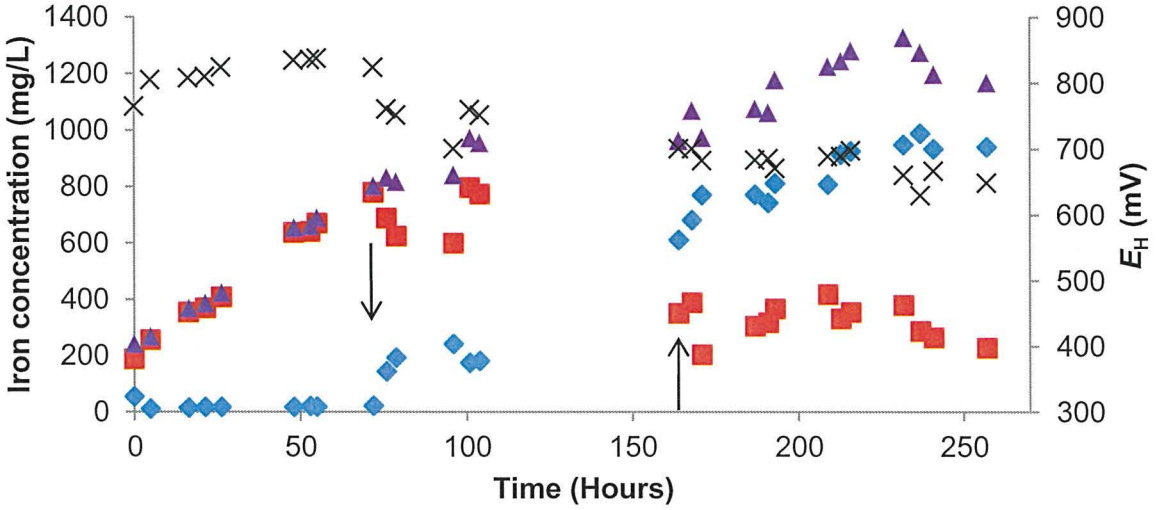


Figure 3.3. Preparation of synthetic Spatone water (experiment 3.2.). Key: concentrations of ferrous iron (♦), ferric iron (■) and total iron (▲); redox potentials (X). The downward-pointing arrow indicates the time at which aeration was turned off, and the upward-pointing arrow the time when gassing with OFN commenced.

Cutting off the air supply to the bioreactor and the addition of sterile powdered elemental sulfur resulted in redox transformation of iron (reduction of ferric to ferrous) so that by 164 h, ferrous iron was the dominant form present. Total soluble iron concentrations increased during this time, indicating that iron reduction was mediated by reaction [3.1] as well as possibly by reactions [3.3] and [3.4]. Sulfur addition was omitted from subsequent experiments as it had a relatively small effect ferric iron reduction. Ferric iron concentrations did not fall below ~200 mg/L during this period. Since minimal concentrations of this iron species were desirable in the process, the bioreactor was gassed with OFN from 164 h remove residual oxygen. However, this failed to induce a significant decline in ferric iron

concentrations, though both total soluble iron and ferrous iron concentrations increased (reflected by decreasing redox potentials). The experiment was terminated once ferric/ferrous iron speciation and redox stabilised.

The successfully prepared iron-rich, relatively low E_H leachate liquor was further processed to give a liquid with a pH and relatively low ferric iron content, similar to that of the original Spatone water. 500 mL of liquor was removed from the bioreactor and centrifuged at 3000 x g at 4°C to remove pyrite, and filtered through 25 mm diameter Whatman nitrocellulose membranes (0.2 µm pore size) fitted to Millipore filter holders. Measurements of pH, total and ferrous iron concentrations and redox potentials were recorded. The liquor pH was then increased by drop-wise addition of 1 M NaOH to 100 mL of liquor (3 replicates), until it reached approximately pH 3 (+/- 0.1). This required an average of 3.51 (+/- 0.33) mL of 1 M NaOH solution per 100 mL of liquor. On reaching pH 3, the liquor was filtered again to remove precipitated ferric iron (equation [3.5], showing schwertmannite as the solid product obtained; Hedrich and Johnson, 2012). pH, total and ferrous iron concentrations and redox potentials were again recorded.

$$8\ Fe^{3+} + SO_4^{2-} + 14\ H_2O \rightarrow Fe_8O_8(OH)_6(SO_4) + 22\ H^+ \qquad [3.5]$$

Table 3.3 lists the differences between the chemistries of the initial leach liquor and that treated with alkali.

Table 3.3. Iron speciation, pH and redox potentials of synthetic Spatone from experiment 3.2, showing values for the initial pyrite leach liquor and that of the solution after pH adjustment (3 replicates). The amounts of 1 M NaOH standard solution required to increase the extremely acidic pyrite leachate to ~ pH 3 (100 mL aliquots) are also shown.

Variable	Pyrite leachate	After pH increase		
		REPLICATE 1	REPLICATE 2	REPLICATE 3
pH	1.83	3.11	3.00	3.11
E_H (mV)	673	612	662	657
Fe^{2+} (mg/L)	937	959	952	949
Fe^{3+} (mg/L)	93	4	5	15
Total iron (mg/L)	1030	963	957	964
1 M NaOH added (mL/100 mL leachate)		3.84	3.23	3.45

Addition of NaOH caused a large portion of the ferric iron to hydrolyse and precipitate, but also resulted in some loss of ferrous iron. This was probably due to some abiotic oxidation of ferrous iron to ferric (caused by localised pH values of >3.5, generated by the addition of strong alkali solution to the leachate). Transient blue-grey ferrous hydroxide ($Fe(OH)_2$) was observed when the NaOH solution was added to the leachate liquor, indicating localised higher pH zones that would have accelerated abiotic iron oxidation. The final processed water produced in this experiment contained approximately five times the concentration of ferrous iron compared to that of the original Spatone water, and had a very similar pH value.

3.6. Experiment 3.3

The aim of experiment 3.3 was to develop the process of producing synthetic iron-containing Spatone water, though this time the standard basal salts/trace elements mixture used in

laboratory experiments was replaced with a mineral salts mixture that was similar in chemical composition to Trefriw spa water (except in its iron content). Experiment 3.3 used sterile standard iron-free synthetic Spatone water (Tables 3.1 and 3.2) as the liquor for microbially-catalysed pyrite dissolution. A major disadvantage of this was that the synthetic Spatone water solution contained far smaller concentrations of many macro- and micro-nutrients required to support the growth of iron/sulfur-oxidizing bacteria and no nitrogen and phosphorus, two important macro-nutrients. It was therefore predicted that there would be little or no growth of bacteria in this bioreactor experiment. To compensate for this, the mineral leaching acidophile used for this experiment (*At. ferrooxidans*) was pre-cultivated by growing it on hydrogen rather than on iron or pyrite.

At. ferrooxidans was cultivated in a sealed 1.3 L jar (Oxoid, UK) under an atmosphere containing hydrogen, oxygen, carbon dioxide and nitrogen, as described in section 2.2.3. 100 mL of this (containing $>10^9$ cells/mL) was added to 1.9 L of standard synthetic Spatone water, 50 g of sterile pyrite added and the bioleaching process initiated, as described for experiment 3.2 above.

Data from this experiment are shown in Fig. 3.4. Ferric iron was initially generated, but after ~100 h ferrous iron concentrations increased and those of ferrous iron declined. This was not expected as the bioreactor was aerated throughout this time, though this may have been due to problems with interruptions of air supply to the reactor. Once sufficient airflow was re-established, iron speciation reverted to ferric iron-dominated.

Once total iron in the bioreactor had reached a concentration similar to that of experiment 3.2 (~ 1000 mg/L) the air supply was turned off. Iron speciation began to change immediately. Ferric iron concentration fell from 700 mg/L (74% of total soluble iron) to 259 mg/L (28% of total) within 100 hours, where it remained stable until gassing with OFN was initiated (at 612 h). The concentration of ferric iron then fell to 147 mg/L (15% of total) at 743 h. E_H reflected this change, being close to 800 mV when ferric iron was dominant and falling

to approximately 710 mV when ferrous was the dominant ionic form of iron present. The bioleaching phase of this experiment was terminated once ferrous iron concentrations had stabilised.

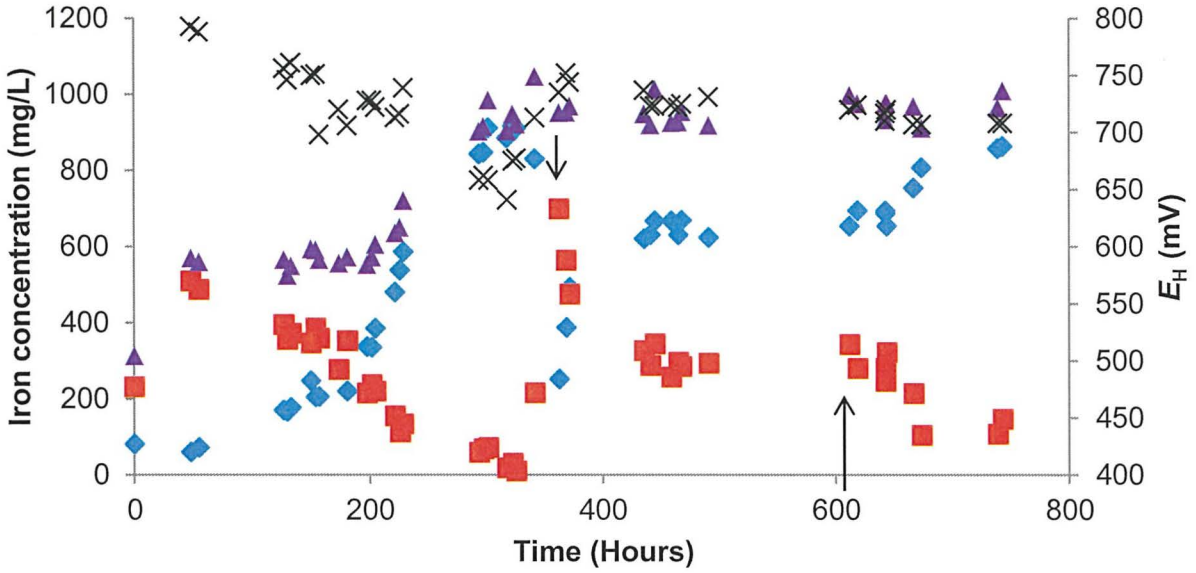


Figure 3.4. Preparation of synthetic Spatone water (data from experiment 3.3). Key: concentrations of ferrous iron (♦), ferric iron (■) and total iron (▲), and redox potential measurements (X). The downward-pointing arrow indicates the time at which aeration was turned off, and the upward-pointing arrow the time when gassing with OFN commenced.

The second phase of this experiment involved the addition of alkali to raise the leachate pH and to remove any excess ferric iron, as in experiment 3.2. As before, there was some loss in total iron (an average loss of 10%) and slightly more NaOH was required to bring the leachate to the required pH value (Table 3.4). Somewhat unexpectedly, the ferric iron concentration of the higher pH liquor was greater than that of the more acidic leachate (an average increase of 10%), confirming that some chemical oxidation of ferrous iron was occurring during this phase of the experiment.

Table 3.4. Iron speciation, pH and E_H data of filtered synthetic Spatone before (initial values) and after pH was adjusted (replicates) generated in experiment 3.3. The amounts of 1 M NaOH standard solution required to change pH of 100 mL of liquor are also shown.

Variable	Pyrite leachate	After pH increase		
		REPLICATE 1	REPLICATE 2	REPLICATE 3
pH	1.86	3.00	3.01	3.03
E_H (mV)	722	679	681	683
Fe^{2+} (mg/L)	824	649	645	640
Fe^{3+} (mg/L)	181	226	225	313
Total iron (mg/L)	1005	875	870	953
1M NaOH (mL/100 mL leachate)		4.64	4.79	4.35

3.7. Experiment 3.4

Although experiments 3.1 – 3.3 confirmed the general hypothesis that it should be possible to produce a concentrated (in terms of its ferrous iron content) version of Spatone water under laboratory conditions, it was considered that a more concentrated solution was possible. Therefore, conditions under which pyrite was oxidised and iron reduced were changed to increase efficiency of the process. A higher temperature and lower pH was used for bioleaching, and a more efficient microbial consortium used to oxidize pyrite and to catalyse ferric iron reduction. The bioreactor was maintained at 40°C and pH 1.6 throughout experiment 3.4. Different species of acidophilic bacteria than those used in experiments 3.1 – 3.3 were required to operate under these conditions. *Leptospirillum* (*L.*) *ferriphilum*^{T} was used as the pyrite-oxidizing acidophile, as it is more acidophilic and thermo-tolerant than *At. ferrooxidans* or *At. ferridurans*. The bioreactor was filled with 2 L standard-strength iron-free synthetic Spatone, 50 g sterile pyrite and 50 mL *L. ferriphilum* (grown in duplicate flasks of

25 mL basal salts medium and 50 mM Fe^{2+} at pH 1.6 for 4 days, then 50 mg sterile pyrite added and incubated for a further 5 days). 25 mL *Sulfobacillus* (*Sb.) thermosulfidooxidans* (grown in basal salts medium, 0.02% yeast extract and 0.1 mM Fe^{2+} at pH 2) was added once it became clear that the rate of iron speciation change was not sufficient under abiotic conditions (*L. ferriphilum* does not reduce iron).

These changes resulted in a three-fold increase of total iron in this experiment compared to experiment 3.3 (~1000 mg/L iron solubilised in experiment 3.3 and ~ 3000 mg/L in experiment 3.4; Figs. 3.4 and 3.5). Total iron concentrations varied more due to greater water loss by evaporation at the higher temperature used in experiment 3.4 (this being occasionally replaced with sterile RO water), but did not increase during the anaerobic phase of the experiment. The leaching phase of the experiment was terminated once nearly all of the ferric iron present had been reduced to ferrous. Experiment 3.4 was far more effective in achieving the net objectives of this part of the study than previous experiments. It produced a higher concentration of total soluble iron from the oxidative phase of pyrite leaching, and almost entirely converted this to the desired ferrous species over a time period comparable to previous experiments.

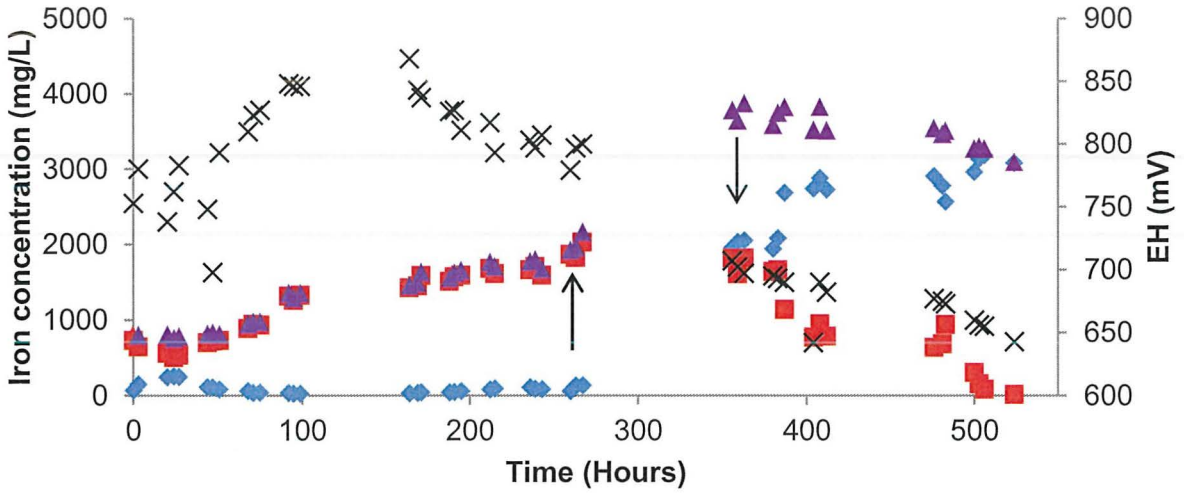


Figure 3.5. Preparation of synthetic Spatone water (data from experiment 3.4). Key: concentrations of ferrous iron (♦), ferric iron (■) and total iron (▲), and redox potential measurements (X). The upward-pointing arrow in **bold** indicates the time at which aeration was turned off, and when gassing with OFN commenced. The downward-pointing arrow indicates the time 25 mL *Sb. thermosulfidooxidans* was added.

More NaOH was required to increase the pH of the leachate generated to pH 3 for this than for previous experiments (Table 3.5), averaging 8.34 mL of 1 M NaOH per 100 ml liquor (3.75 mL per 100 mL 1 M NaOH more than experiment 3.3). This was probably due to the lower initial pH of the liquor (pH 1.6 rather than pH 1.8). Ferrous, ferric and total iron concentrations solubilised were much greater in this experiment than in previous experiments. Ferric iron was proportionally less in this experiment than in experiment 3.3 (9.3% of total iron prior to the pH increase, compared to 18% of total iron for experiment 3.3). After adjustment to pH 3 (+/- 0.007), total iron concentrations fell by an average of 415 mg/L, ferrous by 154 mg/L and ferric by 261 mg/L. The relative proportion of ferric iron dropped after the pH increase (from 9.3% to 2.3 +/- 0.6% of total iron), as opposed to the increase seen in experiment 3.3 (from 18% to 28 +/- 4,4% of total iron). This was possibly due to transient $Fe(OH)_2$ (formed in response to the addition of more NaOH), creating brief localised pH increases that allowed chemical oxidation of ferrous iron to ferric.

Table 3.5. Iron speciation, pH and redox measurements from experiment 3.4 filtered synthetic Spatone before (initial values) and after pH was adjusted (replicates). The amounts of 1 M NaOH standard solution required to increase the extremely acidic pyrite leachate to ~ pH 3 (100 mL aliquots) are also shown.

Variable	Pyrite leachate	After pH increase		
		REPLICATE 1	REPLICATE 2	REPLICATE 3
pH	1.66	3.00	3.02	3.00
E_H (mV)	656	624	622	610
Fe^{2+} (mg/L)	3226	3070	3082	3064
Fe^{3+} (mg/L)	332	87	51	75
Total iron mg/L	3558	3157	3133	3139
1 M NaOH (mL/100 mL leachate)		8.42	8.12	8.48

3.8. Experiment 3.5

The purpose of experiment 3.5 was to repeat the conditions of experiment 3.4, with the *L. ferriphilum/Sb. thermosulfidooxidans* consortium present at time zero. Experiment 3.5 was set up by draining all but ~200 mL of liquor, containing *L. ferriphilum* and *Sb. thermosulfidooxidans* and pyrite. This was unlike previous experiments, which were dismantled and re-built each time. Fresh sterile standard-strength iron-free Spatone medium (Table 3.1) was added directly on removal of the liquor from experiment 3.4, and experiment 3.5 started immediately. The purpose of this was to see what effects re-use of present pyrite and biomass would have on the system.

Total iron increased to 1761 mg/L after 900 h during aerobic phase (Fig. 3.6). This was 2108 mg/L less than the previous experiment, over approximately twice the time. This is likely partly because the most accessible FeS_2 was degraded during the previous batch, where it was also used as substrate. It may also be that the *Lp. ferriphilum* biomass was stressed from a period of anaerobic conditions in experiment 3.4, as this species is obligately aerobic. Iron speciation at experiment termination was 90% ferrous, which was less than experiment 3.4 (99%), but more than experiment 3.2 (80%) and 3.3 (85%).

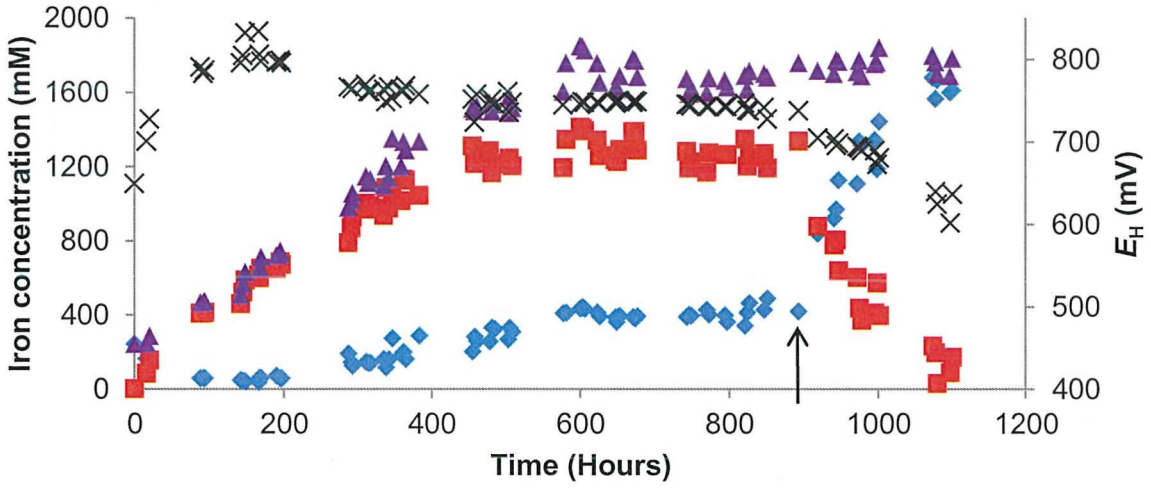


Figure 3.6 Preparation of synthetic Spatone water (data from experiment 3.5). Key: concentrations of ferrous iron (♦), ferric iron (■) and total iron (▲), and redox potential measurements (X). The upward-pointing arrow in **bold** indicates the time at which aeration was turned off, and gassing with OFN commenced.

The pH increase on termination of experiment 3.5 required more 1 M NaOH than experiment 3.4 (an average of 20.77 mL as opposed to 8.24 mL per 100 ml liquor for experiment 3.4) (Tables 3.5 and 3.6). Total iron was lower for this experiment than for experiment 3.4 (an average of 1093 mg/L after pH increase for this experiment as opposed to 3072 mg/L after pH increase for experiment 3.4). Ferrous iron concentrations were lower than those achieved in experiment 3.4 (average 1093 mg/L after pH increase, compared to 3072 mg/L for experiment 3.4). Ferric iron was present in relatively larger amounts than in experiment

3.4 (average of 103 mg/L (8.6 +/- 5.8% of total iron) after the pH increase in comparison to 71 mg/L (2.3 +/- 0.6% of total iron) in experiment 3.4).

Table 3.6. Iron speciation, pH and redox measurements from experiment 3.5 filtered synthetic Spatone before (initial values) and after pH was adjusted (replicates). The amounts of 1 M NaOH standard solution required to increase the extremely acidic pyrite leachate to ~ pH 3 (100 mL aliquots) are also shown.

Variable	Pyrite leachate	After pH increase		
		REPLICATE 1	REPLICATE 2	REPLICATE 3
pH	1.584	3.00	3.03	3.01
E_H (mV)	602	624	621	628
Fe^{2+} (mg/L)	1733	1061	1102	1116
Fe^{3+} (mg/L)	79	96	29	185
Total Fe (mg/L)	1812	1157	1131	1301
1 M NaOH (mL/100mL)		14.54	23.61	24.15

3.9. Experiment 3.6

The main objective of experiment 3.6 was to create a concentrated form of synthetic Spatone using the method developed in previous experiments. This concentrate could then be diluted before addition to Trefriw well water, or added neat to increase the iron concentration while adding the minimum liquid necessary. Experiment 3.6 was set up in a similar way to experiment 3.5 (including using ~200 mL of saved liquor from the previous batch as inoculum). The same (residual) pyrite mineral and inoculum was therefore used for experiments 3.4 - 3.6), with concentrated iron-free synthetic iron-free Spatone solution (Tables 3.1 and 3.2) replacing standard iron-free synthetic Spatone. '10x' concentration was chosen as the target concentration, because the final iron concentration of experiment 3.5

was 2000 mg/L (Fig. 3.7), which is 10x greater than that of commercial Spatone (~200 mg/L). Other components of standard synthetic Spatone were increased ten-fold to reflect this (Table 3.1) except calcium, which was increased by a factor of 2.5 (limited by the solubility of calcium sulfate in acidic solutions). A consequence of this was that the total sulfate concentration of the concentrated liquor was less than 10x than of the standard synthetic solution (Table 3.2).

Phosphate and ammonium were also included in concentrated synthetic iron-free Spatone solution. These macronutrients were not included in the standard synthetic iron-free Spatone solution, as these are present in either very low concentration (N) or below detection (P) in commercial Spatone (Table 3.2). The amounts of these added to the concentrated synthetic iron-free Spatone solution were evaluated using the following calculation:

(i) wet weight of an *At. ferrooxidans* cell: 3.75×10^{-13} g (based on its typical dimensions of 2 µm length, 0.4 µm diameter, and a density of 1.5×10^{-12} g/µm^3)

(ii) estimated number of bacteria in the pyrite leachate: 10^9/mL (10^{12}/L)

(iii) estimated wet weight of bacterial biomass: 375 mg/L

(iv) most Gram-negative bacteria are ~ 80% water, therefore the estimated dry bacterial biomass is 75 mg/L

(v) Gram-negative bacteria generally contain ~14% N and 3% P (dry weight basis)

(vi) the estimated N and P contents of the bacterial biomass in the bioreactor are therefore 10.5 and 2.25 mg, respectively

Table 3.2 shows that 28 mg/L of N and 31 mg/L of P were included in the concentrated synthetic iron-free Spatone solution. This was to provide a slight excess of both (more so in the case of P, as ferrous and ferric phosphates can form in iron-rich liquors).

The inclusion of inorganic nitrogen and phosphorus was considered a reason why total iron reached 2,409 mg/L after 306 h in aerobic conditions. This was 45% more than in

experiment 3.5 over approximately one third of the time (Figs. 3.6 and 3.7), though it was ~64% less than for experiment 3.4 over a comparable time period (Fig. 3.5). After switching the gas intake from air to OFN (at 287 h), it took a further 400 h for most of the ferric iron present to be converted to ferrous. This is approximately twice as long as for experiments 3.4 and 3.5 (both at ~200 h +/- 19 h, despite having a large difference in total iron between them).

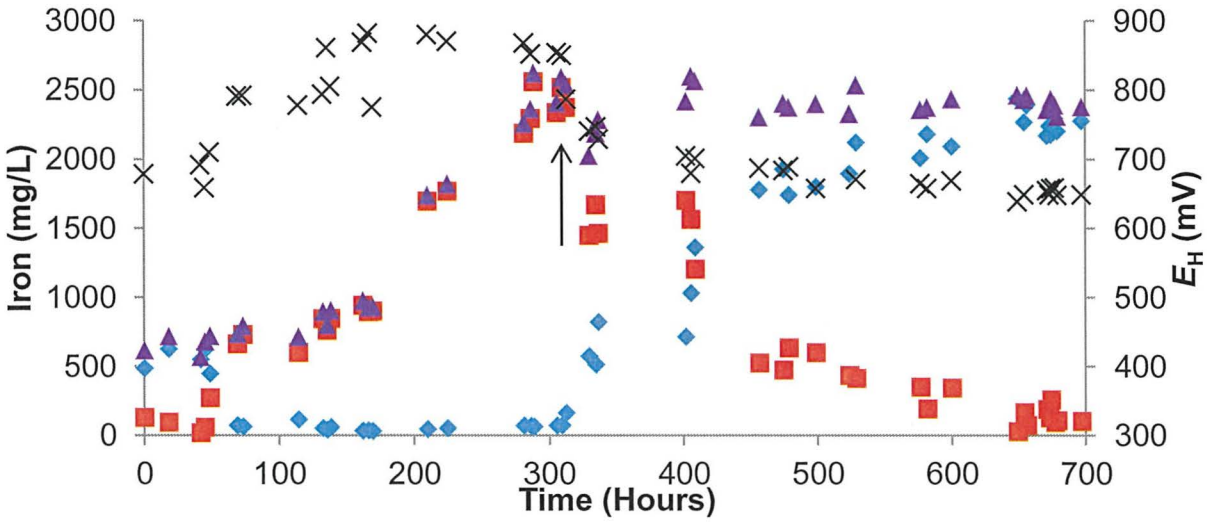


Figure 3.7. Preparation of synthetic Spatone water (data from experiment 3.6). Key: concentrations of ferrous iron (♦), ferric iron (■) and total iron (▲), and redox potential measurements (X). The black arrow indicates the time at which aeration was turned off, and gassing with OFN commenced.

Post-leaching pH adjustment in experiment 3.6 required approximately 3x more NaOH than in experiment 3.5 (Table 3.7), which may be due to greater HSO_4^-/SO_4^{2-} buffering in the concentrated synthetic Spatone solution. In this experiment, 0.7% of total soluble iron in the pyrite leachate was present as ferric, increasing to 3.7 +/- 4.0% after the pH increase. There was also a large decrease in total soluble iron in this case as a result of pH increase (817 mg/L lost), probably linked to the increased amount of 1 M NaOH required to increase the pH (as found in experiment 3.2).

Table 3.7. Iron speciation, pH and redox measurements from experiment 3.6 filtered synthetic Spatone before (initial) and after pH was adjusted (replicates). The amounts of 1 M NaOH standard solution required to increase the extremely acidic pyrite leachate to ~ pH 3 (100 mL aliquots) are also shown.

Variable	Pyrite leachate	After pH increase		
		REPLICATE 1	REPLICATE 2	REPLICATE 3
pH	1.61	3.01	3.02	3.01
E_H (mV)	643	638	640	641
Fe^{2+} (mg/L)	2173	1284	1313	1367
Fe^{3+} (mg/L)	15	4	29	118
Total Fe (mg/L)	2189	1288	1342	1485
1 M NaOH (mL/100mL leachate)		38.34	60.19	91.03

3.10. Discussion

The aim of chapter 3 was to produce a concentrated synthetic Spatone liquor that could be used to amend the original Spatone water when its ferrous iron concentration falls below a critical value, without compromising its '100% natural' claim. The final product (the original spa water amended with synthetic Spatone) would need to contain at least 200 mg/L ferrous iron, have a pH of approximately 3, and be formed by a biological processes. A target was set of generating acidic liquor containing ~2,000 mg/L of ferrous iron by bioleaching pure-grade pyrite, via a two-stage process. First, oxidative dissolution was catalysed by iron-oxidising bacteria, then the generated ferric iron was reduced by bacteria, under anaerobic conditions. This process replicated that which generated the natural spa water in a general way, though specifics such as transition between aerobic and anaerobic states was likely

more gradual at the site itself. This concentrated liquor could then be diluted with acidic water or Spatone generated on site, in order to generate a final product containing 200 mg/L ferrous iron.

A progression of six experiments was required to achieve the objective. The main differences in the operational conditions of these experiments are shown in Table 3.8, and the main analytical data summarised in Table 3.9.

Table 3.8. Comparison of microbial inocula and operational parameters used in experiments 3.1 - 3.6

Experiment	Inoculum	Temperature (°C)/pH	Leach period (h)	Basal medium	Pyrite source	S^0 addition
3.1	*At. ferrooxidans/At. ferridurans*	30/1.8	364	Basal salts (#1)/trace elements	Fresh	-
3.2	*At. ferrooxidans*	30/1.8	255	Basal salts (#1)/trace elements	Fresh	+
3.3	*At. ferrooxidans*	30/1.8	743	Standard synthetic Spatone	Fresh	-
3.4	*L. ferriphilum/(Sb. thermosulfidooxidans*)*	45/1.6	524	Standard synthetic Spatone	Fresh	-
3.5	*L. ferriphilum/Sb. thermosulfidooxidans**	45/1.6	1101	Standard synthetic Spatone	From expt. 3.4	-
3.6	*L. ferriphilum/Sb. thermosulfidooxidans**	45/1.6	697	Concentrated synthetic Spatone	From expt. 3.5	-

**Sb. thermosulfidooxidans* was added to the bioreactor immediately before commencement of anaerobic conditions (air flow stopped) in expt. 3.4, and was present from the start of mineral bioleaching in experiments 3.5 and 3.6.

Table 3.9. Concentrations of iron and pH values of pyrite leachate and synthetic Spatone liquors in each of the six experiments described.

Experiment	Pyrite leachate			Synthetic Spatone liquor*		
	pH	$[Fe]_{total}$	$[Fe^{2+}]$	pH	$[Fe]_{total}$	$[Fe^{2+}]$
3.1	1.80	664	345	-	-	-
3.2	1.83	1030	937	3.07	961	953
3.3	1.86	1005	825	3.02	899	645
3.4	1.66	3558	3226	3.01	3143	3072
3.5	1.58	1812	1733	3.01	1196	1093
3.6	1.61	2189	2173	3.01	1372	1321

*mean values of 3 replicates in each case; all Fe concentrations are mg/L

Experiment 3.1 showed that it was possible to bioleach the pure-grade pyrite mineral using the proposed system. During this experiment, it was also shown to be possible to induce reduction of ferric iron to ferrous by stopping aeration in the reactor to facilitate anaerobic, reducing conditions. The reaction shown in equation [3.1] was likely responsible for much of the ferric iron reduction (re-oxidation of the ferrous iron produced being inhibited by the absence of molecular oxygen). As experiment 3.1 was a preliminary experiment, it was terminated once it had served its purpose of testing the viability of the proposed system, despite ~50% of the soluble iron still being ferric iron.

Experiment 3.2 achieved a higher yield of soluble iron by oxidative dissolution of pyrite over a shorter period than experiment 3.1. The anaerobic phase of bioleaching was promoted in

this experiment by the addition of elemental sulfur, specifically to facilitate the reaction shown in equation [3.4]. This was effective in that the ratio of ferric:ferrous iron in the leachate generated was much lower that than that of experiment 3.1.

The yield of soluble iron achieved in experiment 3.3 was similar to that of experiment 3.2, which was not surprising as the inoculum and operating conditions were the same for both experiments. The main difference between them was that standard-strength iron-free Spatone liquor, rather than laboratory basal salts (# 1) were used in experiment 3.3. Poor bacterial growth was anticipated as the former solution contained no N or P, so a larger bacterial inoculum (in terms of numbers of *At. ferrooxidans*) was used to compensate for this. There was still reasonably good conversion of ferric iron to ferrous observed during the anaerobic leaching phase despite there being no elemental sulfur addition in this experiment, so it continued to be omitted from subsequent experiments.

Total iron concentrations in leach liquors generated in experiments 3.2 and 3.3 were approximately 1000 mg/L. It was decided at this stage to change the bioleaching conditions of the experiments, specifically to bioleach at both a higher temperature and a lower pH. This was because rates of sulfide mineral oxidation generally increase with temperature, and operating at a lower pH would allow more iron to remain in solution. This required a change in the microorganisms used, requiring species that are more suited to the new conditions (specifically, more thermo-tolerant than iron-oxidising *Acidithiobacilli)*. Therefore, a mixed culture of *L. ferriphilum* and *Sb. thermosulfidooxidans* replaced *Acidithiobacillus ferrooxidans* in the final three experiments. Specific conditions in the bioreactor were altered to reflect optimum growth conditions of this consortia: pH 1.6 and 45°C rather than pH 1.8 and 30°C. *L. ferriphilum* and *Sb. thermosulfidooxidans* bacteria are moderately thermophilic and tolerate lower pH than *At. ferrooxidans* (Coram and Rawlings, 2002; Golovacheva and Karavaiko, 1991).

These changes resulted in a significantly greater total iron concentration during the bioleaching phase of experiment 3.4 (3,500 mg/L) than was achieved in previous experiments. The ~3.5x increase in total iron was thought due to biological as well as chemical factors. *Leptospirillum* spp. have a greater affinity for ferrous iron and a higher tolerance to ferric iron than *At. ferrooxidans*, so are better adapted to conditions (including lower pH and higher temperature) under which the bioreactor was operated in the final three experiments (Rawlings *et al.*, 1999; Norris *et al.*, 1988). However, unlike *At. ferrooxidans*, *L. ferriphilum* is an obligate aerobe and does not reduce iron, so *Sb. thermosulfidooxidans* was included immediately prior to the commencement of anaerobic conditions in experiment 3.4. *Sb. thermosulfidooxidans* can catalyse the reduction of ferric iron coupled to the oxidation of elemental sulfur and sulfur oxy-anions, such as tetrathionate (Bridge and Johnson, 2002).

The amount of pyrite dissolved in experiment 3.4 was calculated to be about 14 % of that present in the reactor (approximately 7 g was leached from the 50 g FeS_2 in the 2 L solution; ~30% of the pyrite was dissolved over experiments 3.4-3.6). Therefore, it was decided to use the residual pyrite in experiment 3.5. The soluble iron concentration generated by this experiment was greater than in experiments 3.1 to 3.3, though only about half that obtained in experiment 3.4, despite experiments 3.4 and 3.5 being conducted under virtually identical conditions. It was considered that this difference was due to the more reactive grains of pyrite being preferentially dissolved in experiment 3.4 and leaving more recalcitrant mineral particles for experiment 3.5. This same explanation was thought to account for the amount of iron solubilised in experiment 3.6 being less than that of experiment 3.4, and for the general decrease in soluble ferrous iron concentration at Trefriw Wells Spa (Fig. 3.1). It is also possible that the biomass present, and *Lp. ferriphilum* in particular, were not thriving. The amount of total iron in the liquor was 21% greater for experiment 3.6 than for experiment 3.5 over a shorter time. This was attributed to the addition of N- and P-supplements to the oncentrated iron-free Spatone solution to promote bacterial growth.

The second part of each of the experiments described (apart from experiment 3.1) involved controlled addition of alkali to the leach liquors. The objectives here were: (i) to increase the pH of the liquors to a value similar to that of actual Spatone; and (ii) to remove more of the ferric iron via hydrolysis (inducing formation of schwertmannite and possibly other ferric iron minerals), which had not reduced to ferrous during anaerobic bioleaching. Objective (ii) was difficult to achieve. One possible reason for this was indicated by the transient appearance of grey-blue ferrous hydroxide as the NaOH solution was added to the leachates. This was formed by the reaction between ferrous iron and hydroxyl ions (equation [3.5]):

$$Fe^{2+} + 2\ OH^- \rightarrow Fe(OH)_2 \qquad [3.5]$$

This reaction requires a pH of 5 or above (Stumm and Lee, 1961). At pH 3, the ferrous hydroxide re-dissolved, liberating hydroxyl ions (equation [3.5] in reverse) indicated by the colour disappearing. The formation of ferrous hydroxide confirmed the transient existence of zones where the pH was sufficiently high to promote the abiotic oxidation of ferrous iron to ferric (Stumm and Morgan, 1981), which may be why ferric iron concentrations in the synthetic Spatone waters did not decrease as much as anticipated.

Two notable jumps in NaOH consumption occurred; (i) between experiments 3.3 and 3.4, where there was a large increase in pyrite dissolution and therefore iron in solution between the two experiments, and (ii) between experiments 3.5 and 3.6, when standard synthetic Spatone medium was replaced with concentrated synthetic Spatone iron-free medium. Both changes resulted in a near doubling of the amount of NaOH required to reach a final liquor pH of approximately 3. As well as proton acidity (as measured by pH), a buffering system becomes increasing import at ~pH 2 and below. This is the sulfate/bisulfate buffer, based on the reversible dissociation of the bisulfate (HSO_4^-) anion:

$$SO_4^{2-} + H^+ \leftrightarrow HSO_4^- \qquad (pK_a\ 1.92) \qquad [3.6]$$

At pH 1.6 (experiments 3.4 – 3.6) most of the sulfate would be present as HSO_4^- , releaseing protons as the pH increases to 3, thereby requiring more NaOH to neutralise. Also, the

strength of the bisulfate/sulfate buffer increases with increasing total sulfate concentrations, which is why more NaOH was required to raise the pH of leachate from experiment 3.6 (which used concentrated synthetic iron-free Spatone) than that from experiment 3.5 (Table 3.10).

Table 3.10. Total sulfate concentrations in standard and concentrated synthetic Spatone, and calculated proportions of SO_4^{2-} and HSO_4^-, assuming a dissociation constant (pK_a) of 1.92.

pH of liquor	Standard synthetic Spatone (mM)		Concentrated synthetic Spatone (mM)	
	SO_4^{2-}	HSO_4^-	SO_4^{2-}	HSO_4^-
pH 1.6	4.36	2.09	23.40	11.23
pH 3.0	0.50	5.95	2.66	31.97
Total sulfate	6.45		34.63	

Phosphoric acid would also have contributed to the buffering capacity of the mineral leachates (the pK_a of $H_3PO_4/H_2PO_4^-$, pKa being 2.15), but the effect of this would have been low as total phosphate was always present in far lower concentrations than sulfate. A further possible explanation for this is that as the pH increased, schwertmannite would be generated as ferric iron precipitated from solution. This reaction generates acidity, which would also require more NaOH to neutralise.

Finally, this chapter showed that the model for acidic ferrous iron rich water formation at the site set out in section 1.3.5 was practicable. It was shown that degradation of pyrite did result in a decrease in dissolved ferrous iron concentration, a general pattern seen at the Trefriw site (Fig. 3.1). This section also illustrated the importance of the biological component in the system. In experiments 3.3-3.6, it was shown that affecting this biological component could have a significant effect on the amount of dissolved ferrous iron in the water. However,

differences between the conditions in this chapter and that of the Trefriw and Cae Coch sites (including lower temperature, higher pH and switch from aerobic to anaerobic conditions because of continuous flow system and different bacterial community (Chapter 5)) means that the system proposed here is an illustration of the principals involved, rather than a true model of the water formation and speciation switch.

To generate a system that was closer in design to the Trefriw site, and to create a system with more scope for automation and integration into site production might involve a continuous flow system with aerobic and anaerobic sections, and bacteria fixed to a bed or growing as a streamer community (which are found at the spa site; Chapter 5). This system has the advantage of separating aerobic and anaerobic sections, thereby possibly alleviating the issue seen in section 3.5.

In conclusion, the investigation in this chapter achieved its aim of creating a concentrated synthetic Spatone water which, in principle, could be added to the original product when natural iron concentrations are or become too low without compromising its '100% natural' claim, or significantly altering its chemical properties. Using a consortium of *L. ferriphilum /Sb. thermosulfidooxidans* to bioleach pyrite at pH 1.6 and 45°C, followed by controlled alkalisation of the leach liquor, it was possible to generate a pH 3 liquor that contained up to 3,143 mg/L ferrous iron, i.e. over 15-times the concentration in the commercial product.

Chapter 4. Survival of two species of neutrophilic bacteria found as contaminants in Spatone water

4.1. Introduction

As described in Chapter 1, the acidic, iron-rich chalybeate water marketed by Nelsons Ltd. as *Spatone, Ferrotone* etc. is formed as meteoric water percolates through cracks and fissures in the pyrite ore body located within the hillside above the Trefriw Spa site, (now the site of production of the spa water in the Conwy Valley, north Wales). Prior to the 1990's water was bottled and sold without any processing. Consequently, ferrous iron in the bottled water oxidised within days or weeks due to the presence of indigenous acidophilic iron-oxidising bacteria (e.g. *Acidithiobacillus* spp. and "*Ferrovum myxofaciens*"). In the 1990's, filtration through 0.2 µm (or smaller) pore-sized membranes and the use of sterile sachets was introduced to remove this problem. The system was periodically upgraded by the then site owner Mr. Tony Rowlands and later by current site owner Nelsons Ltd. A schematic representation of the current production system installed at the Trefriw site is shown in Fig. 4.1.

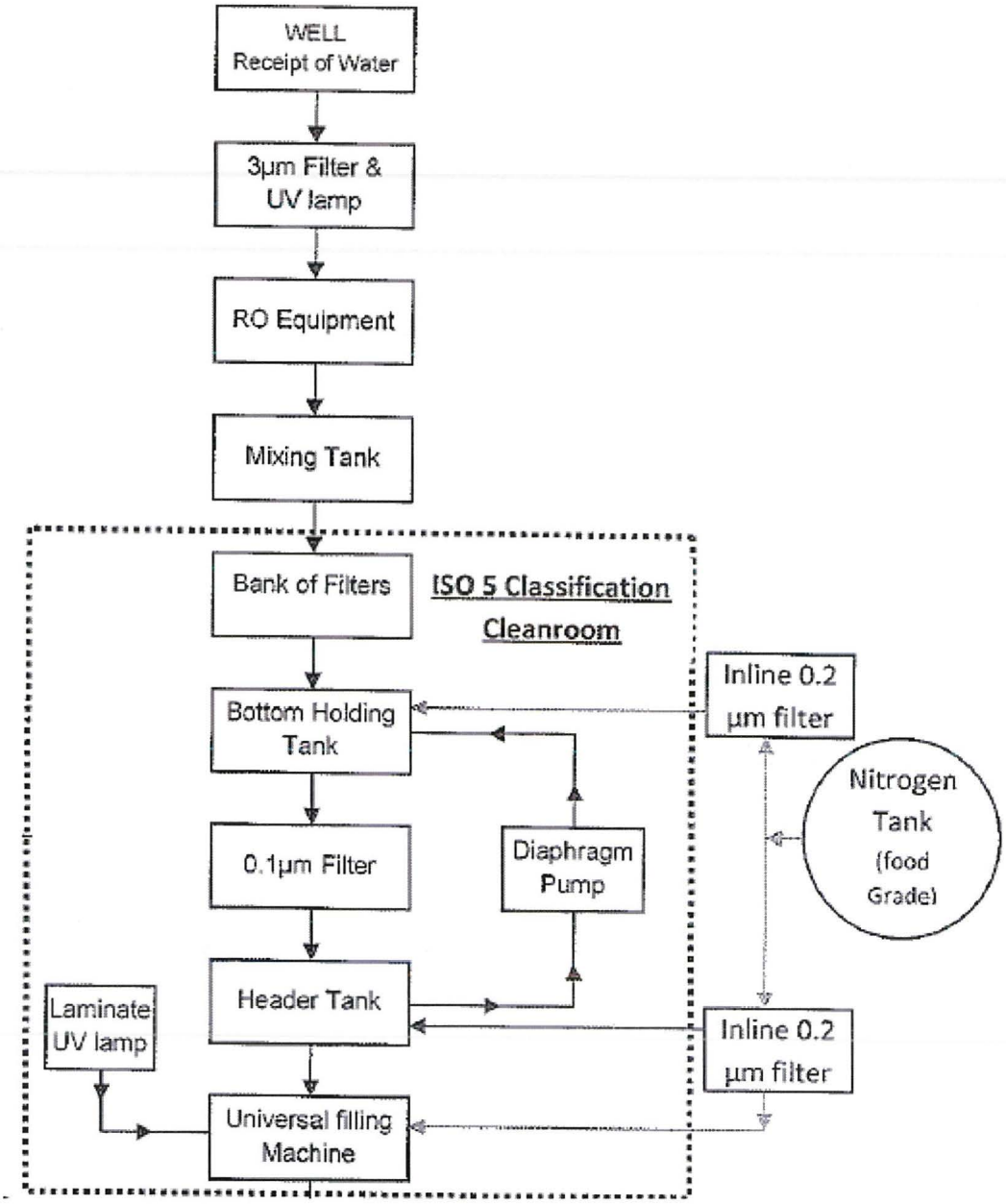


Figure 4.1. Flow diagram of water processing at Spatone Ltd (supplied by Nelsons personnel). RO is reverse osmosis water. Bank of filters includes 3 µm, 0.6 µm, 0.2 µm and 0.1 µm filters. Universal filling machine fills ~20 mL sterile sachets. Nitrogen is included in the system to maintain anaerobic conditions.

Quality control of Spatone includes regular testing of samples for the presence of contaminating microorganisms. This includes testing for acidophilic iron-oxidising and heterotrophic bacteria, yeasts, fungi, and neutrophilic bacteria (including coliforms). Although positive quality control results are rare, bacteria such as *Leptospirillum ferrooxidans,*

Acidocella spp. and acid-tolerant yeasts have occasionally been detected in (and isolated from) sachets of Spatone (D.B. Johnson, personal communication).

Spatone water is acidic (nominally pH ~3) and similar in chemical composition to some acid mine drainage waters, and low pH ferruginous waters that drain coal mines in particular, as these tend to be enriched with soluble iron but few or no other transition metals. Extreme acidophiles, moderate acidophiles and acid tolerant species live in these waters, but the low pH means that they are hostile to most neutrophilic bacteria. There have been relatively few reports on the survival of the latter in AMD impacted environments. Previous studies found that neutrophilic bacteria were transient at best in acidic environments, with Gram-negative bacteria being relatively more resistant to acidic conditions than Gram-positive bacteria (Tuttle *et al.*, 1968). Wortman *et al.* (1986) reported that the Gram-negative *Escherichia (E.) coli* exhibited damage to the cell envelope when exposed to acid mine drainage of pH 3. Richard and Foster (2003; 2004) found four acid-resistance mechanisms in *E. coli* cells for surviving at pH 2.5 - 3. These varied in effectiveness, and their use was often dependant on the availability of specific amino acids. Other responses by both Gram-positive and Gram-negative bacteria on exposure to acidity include proton removal, attempts at alkalinisation of the external environment, changes in cell envelope composition and production of acid shock proteins (Cotter and Hill, 2003).

During the present study, samples of a batch of Spatone was identified as being contaminated with unknown bacteria and sent to the laboratory at Bangor University for analysis. These were inoculated onto solid R2A medium (an agar-gelled neutral pH medium formulated to promote the growth of bacteria present in non-saline waters; Reasoner and Geldrich, 1985) and incubated at 30ºC. Three colony variants were identified: (i) isolate Y1, which formed yellow, round, domed, entire colonies; (ii) isolate W2, which formed white, round, irregular, domed colonies; (iii) isolate B3, which formed cream, round, irregular, domed colonies.

Samples of each representative colony were removed, a PCR performed (section 2.7.1.2 and 2.7.2). Amplified 16S rRNA genes were sent to MacroGen (Korea) for sequencing, and BLAST analysis of the data sent to Bangor University confirmed the identities of the contaminant bacteria as (i) isolate Y1, a strain of *Kocuria marina;* (ii) isolates W2 and B3, both strains of *Dermacoccus nishinomiyaensis* (B.M. Grail, personal communication).

Kokuria marina is a neutrophilic and halotolerant Gram-positive actinobacterium (Kim *et al.*, 2004). *Kokuria* spp. are found on mammalian skin, soil, fresh water and salt water (Kim *et al.*, 2004, Kocur, 1986). Some, including *K. marina,* can be pathogenic to immunocompromised patients, (Lee *et al.*, 2009; Purity *et al.*, 2013). *Dermacoccus nishinomiyaensis* is Gram-negative, mesophilic, neutrophilic and found primarily on mammalian skin and fresh water (Stackebrandt, 2012). There are no reported cases of pathogenicity caused by this bacterium.

To date, there is no reported evidence of either species being indigenous to extremely acidic environments, and their likely origin in the Spatone sachets was considered as from human skin, introduced during the production process. However, since they were isolated from Spatone water of approximately pH 3, it was apparent that they could survive for a time in low pH water, at least under some conditions. Information on the behaviour of these bacteria under acidic conditions is sparse. *D. nishinomiyaensis* has been reported to grow as low as pH 5.4 (Stackebrandt, 2012), though this is not quoted as its pH minimum. No data for tolerance to acidity have been reported for *K. marina*.

Survival rates of these contaminants in Spatone water products would be useful information for Nelsons Ltd., given that *K. marina* has been reported as pathogenic, albeit in rare cases . Knowledge of contaminants in Spatone water would allow more informed decisions on proceeding with avoiding contamination in the future. This information would also clarify the role (if any) of neutrophilic bacteria in the waters resulting from the Cae Coch ore body.

4.2. Methods and Results

Two experiments were set up in order to determine the viability of the isolated strains of *D. nishinomiyaensis* (isolates W2 and B3) and *K. marina* (isolate Y1) in Spatone water.

The first experiment looked at whether the bacteria could survive for between 1 and 3 days in processed Spatone. The processed Spatone used as medium was from Spatone samples sent in commercial sachets to Bangor University by Nelsons' personnel from the Trefriw site and were confirmed free of any microbial contamination.

Samples of each isolate were taken from colonies grown for three days on R2 medium and placed in 5 mL of sterile basal salts (# 1) medium (pH 7). Cell suspensions were vortexed to separate cells, and their numbers counted using a Hawksley Thoma counting chamber. Different volumes of these suspensions were then diluted with pH 7 basal salts and different volumes assed to a known volume of pH 7 basal salts to give an inoculum suspension. The aim of this step was to add similar numbers of total bacterial cells (between 2.4 and 3.6 x 10^6) to each Spatone sample.

25 mL aliquots of Spatone water were placed into sterile 100 mL conical flasks, and then each flask inoculated with 50 µL of bacterial suspension. The flasks were incubated with shaking at 30°C. In parallel, 50 µL of bacterial cell suspensions were inoculated into 15 mL sterile Falcon tubes filled to capacity with Spatone waters (16 mL). These were wrapped in aluminium foil (to exclude light) and incubated at room temperature (~22ºC). The Falcon tube experimental set-up was thought to more closely resemble conditions in a Spatone sachet.

Immediately following inoculation and once each day thereafter, 20 µL aliquots were taken from each flask and Falcon tube (T_0). These were spread over R2 agar plates using a sterile plastic spreader, and incubated at 30°C. Plates were examined regularly for the appearance of colonies (typically after 2 days), at which point colony forming units (CFUs) were counted.

An assumption was made that each colony represented one viable bacterial cell in the Spatone sample.

Colonies from both flask and Falcon tube cultures grew only for inocula taken at T_0. No colony growth was observed on plates inoculated with samples beyond 24 h of incubation. It was concluded that neither contaminant could not survive for longer than 24 h in Trefriw Spa water, under either aerobic at 30°C or "anaerobic" conditions at room temperature.

A second experiment was set up to determine how rapidly the two neutrophilic contaminants lost viability when put into Spatone. The experimental set-up was as described previously, with samples plated approximately every two hours after T_0 for up to 10 hours, and again at 23.5 hours after T_0.

Colony numbers from both flask cultures and Falcon tubes decreased rapidly over time and no colonies grew on plates inoculated with contaminated Spatone held for 24 hours in either flasks or Falcon tubes (Fig. 4.2).

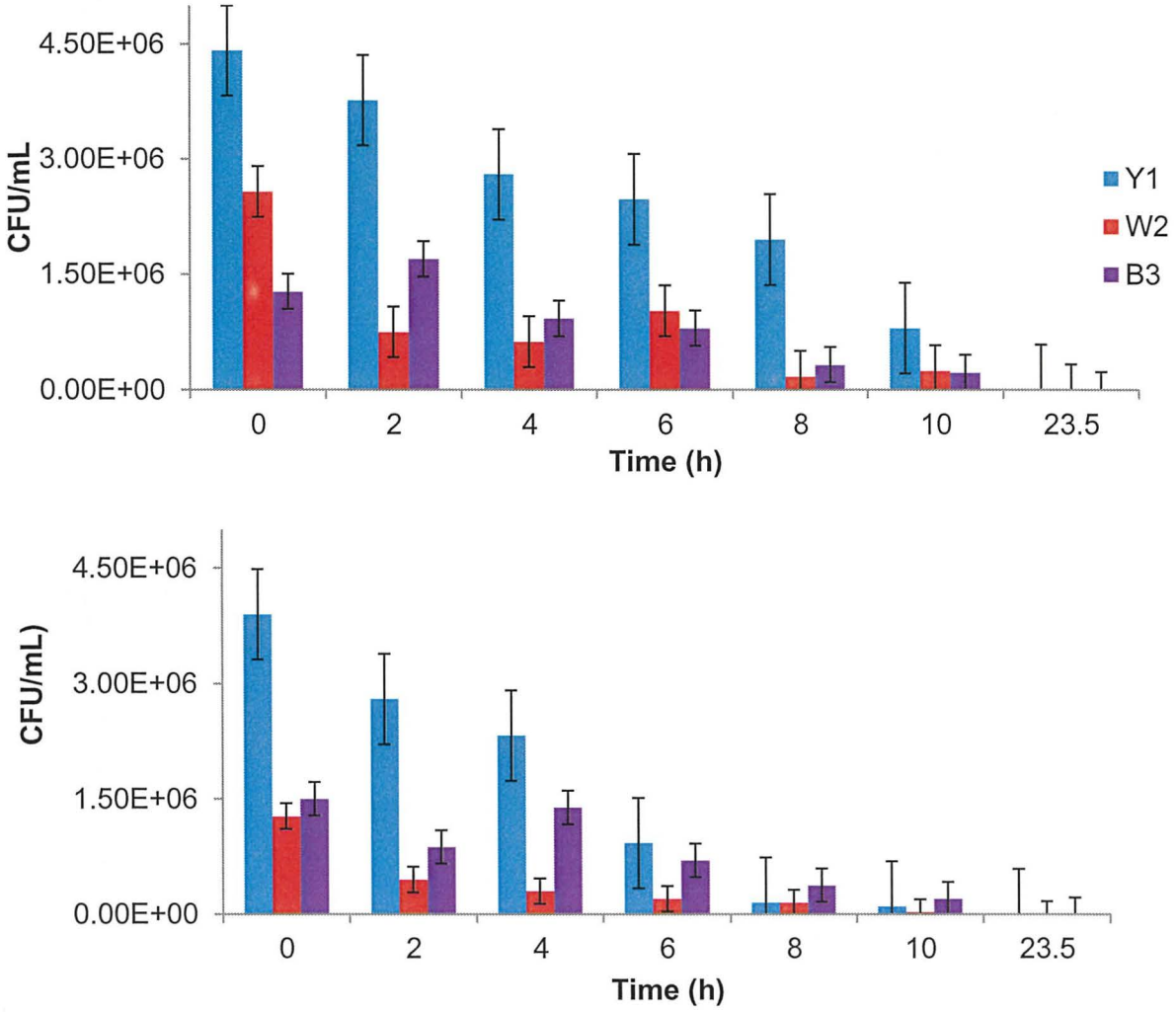


Figure 4.2. Mean CFUs of *Kokuria marina* (Y1) and *Dermacoccus nishinomiyaensis* (W2 and B3) on R2 plates, from samples of inoculated Spatone: (a) flask cultures; (b) Falcon tube cultures. Error bars represent standard error.

Fig. 4.3 showes a steady decrease in CFUs of all three isolates during the first 10 hours of the experiment. *K. marina* Y1 displayed a clear, almost linear decrease in both flask (r^2 = 0.98) and Falcon tube (r^2 = 0.95) cultures (Fig. 4.2 (a)). Mean numbers of CFUs (i.e. viable bacteria) were 8.0 x10^5 cells/mL in the Falcon tube cultures after 10 hours, and 1.0 x10^6 cells/mL active in the flask cultures, indicating the numbers of viable bacteria decreased more quickly under aerobic conditions at 30°C than under "anaerobic" conditions at room temperature.

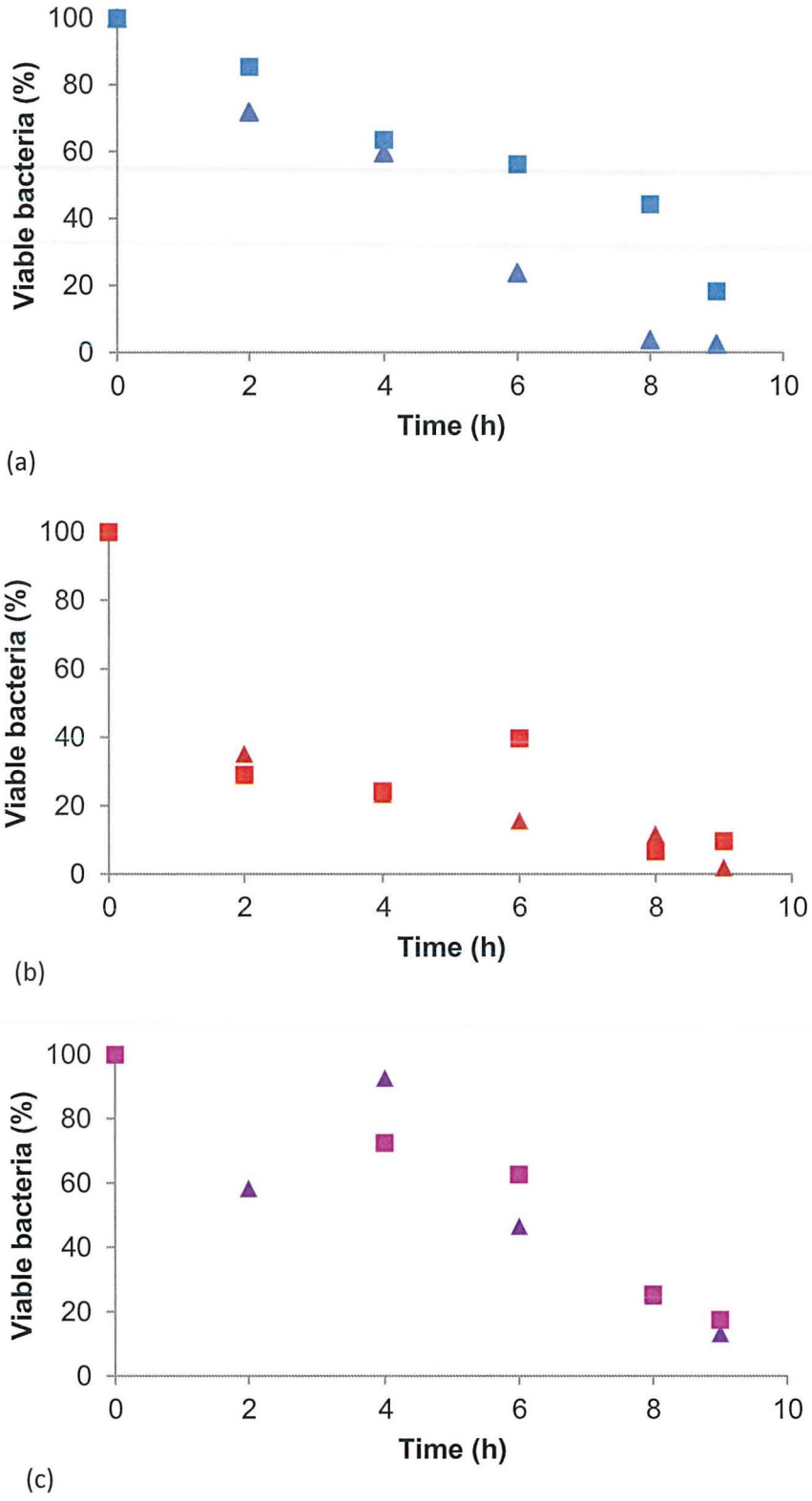


Figure 4.3. Changes in relative numbers of mean viable bacteria/CFUs (as % of those at T_0) of: (a) *Kokuria marina* (Y1) in flask (▲) and Falcon tube cultures (■); (b) *Dermacoccus nishinomiyaensis* isolate W2 in flask (▲) and Falcon tube cultures (■) and (c) *Dermacoccus nishinomiyaensis* isolate B3 in flask (▲) and Falcon tube cultures (■).

D. nishinomiyaensis isolates (W2 and B3) CFU numbers also decreased over time in both flask and Falcon tube cultures, with a faster decrease rate again observed in the flask cultures. However, CFU numbers of both isolates W2 and B3 fluctuated more (isolate W2 flasks $r^2 = 0.76$ and Falcon tubes $r^2 = 0.64$; and isolate B3 flasks $r^2 = 0.77$ and Falcon tubes $r^2 = 0.81$) than those of *K. marina* Y1, possibly due to uneven cell distribution (e.g. resulting from aggregation of cells) in the samples. It may also be due to violation of the assumption that an individual viable bacterium was always able to form a single colony.

4.3. Discussion

Two species of bacteria discovered as contaminants of commercial Spatone sachets were identified as strains of known neutrophilic prokaryotes that had not previously been reported in extremely acidic environments, including Trefriw Wells Spa. The probable source of these contaminants was via sloughing off or through direct contact with mammalian skin. The two *D. nishinomiyaensis* isolates are not considered pathogenic and therefore not of concern. However, *K. marina* is pathogenic in patients with very weak immune systems, and so is of more concern when present in an ingestible product.

The results of the experiments conducted in this study showed that neither bacterial species were culturable after 24 hours in Spatone waters, including in experimental conditions similar to those inside a Spatone sachet. CFUs of both isolates were obtained only up to 10 h of being present in the Trefriw Spa water, and numbers of viable bacteria decreased significantly within that period. Viable counts of *K. marina* Y1 were higher than those of the *D. nishinomiyaensis isolates*, particularly in the Falcon tubes. This observation correlates with reports by Tuttle *et al*. (1968) and Cotter and Hill (2003), who reported that Gram-negative neutrophilic bacteria were relatively more resistant to acidic conditions than Gram-positive neutrophilic bacteria. The more persistent viability of the contaminants in the Falcon tubes is considered a probable consequence of the lower temperature at which the tubes

were incubated, relative to the shake flasks. As both contaminating bacterial species are aerobes, exposure to oxygen would not inhibit viability. Importantly, it was not possible to isolate either bacterium after placement in Trefriw Wells Spa water for 24 hours, therefore the problem of contamination from these three bacterial strains appears very short term.

However, a caveat of this approach is the assumption that viability equals cultivability. This is not always so (Oliver, 2005; Li *et al.*, 2014). Cells enter the VBNC (viable but not culturable) state as a stress response, though this ability has not yet been reported for *Dermacoccus* or *Kocuria* spp.. It is possible that the extremely low pH of the Spatone samples could induce this stress response. Li *et al.* (2014) review some methods of detecting the proportion VBNC cells, which includes fluorescent staining for viable and non-viable cells. Any further studies on neutrophilic contaminant viability in Spatone water should consider this aspect.

The presence of viable populations of the contaminants could be related to the temperatures at which the sachets of Spatone were held, these being <10ºC (winter samples) at the Trefriw storage depot and 4ºC in the Bangor laboratory. The limited evidence from the experiments described in this chapter suggests that survival of both contaminant bacteria in Spatone may be temperature-related.

The general conclusion drawn from the work described in this chapter and data published on similar themes is that neutrophilic bacteria are expected to survive for only short periods in acidic Trefriw spa water. This is particularly important when considering potential contamination with coliforms and other pathogenic bacteria. They are also unlikely to contribute significantly to the bacterial community at the site. However, if the contaminant is a spore-producing or an acid-tolerant microorganism (neither of which applies to *K. marina* or *D. nishinomiyaensis*) then greater longevity in Spatone would be anticipated.

Chapter 5. Identification and isolation of acidophilic bacteria from the Trefriw Wells spa

5.1. Introduction

Acidophilic bacteria have critical roles in the formation and modification of the chemistry of Trefriw spa water (section 1.3.5; chapter 2). The site is interesting as a subterranean ecosystem that relies almost exclusively on chemolithotrophy. The pyrite deposit responsible for the chalybeate waters at Trefriw Wells Spa was actively mined until the end of the 1st World War (the abandoned Cae Coch mine; section 1.3.1). Some new bacteria were detected in (and isolated from) the Cae Coch mine including isolate "CCH7", an iron-oxidising heterotroph that was inhibited by light (Johnson *et al.*, 1992). A clone related to the neutrophile *Gallionella ferruginea* and a number of other novel strains and species were also identified at the Cae Coch site, some of which are not normally associated with acidic mine environments (Kimura *et al.*, 2011).

Most microbiological research carried out on the Cae Coch ore deposit has focused on the abandoned mine itself. However, Hallberg *et al.* (2006) conducted a study on acid streamers found in the Trefriw Wells site. They noted that the microbial community was relatively simple and dominated by *At. ferridurans* (referred to at the time as "*At. ferrooxidans* NO-17"). Other bacteria isolated from these streamers included an *Actinobacterium*, characterised and named in the current study as *Acidithrix ferrooxidans* but described at the time as an "iron-oxidising heterotroph" related to *Ferrimicrobium acidiphilum* (Table 5.1). Kimura (2005) noted that microbial community analysis using T-RFLP showed that there were a number of T-RFs present that could not be identified at the time of analysis.

Table 5.1. Trefriw Wells acid streamer community (Hallberg *et al.*, 2006)

Isolate	Description	Closest relative (% relatedness[1])
CCW10	Iron-oxidizing autotroph	*Acidithiobacillus ferrivorans*^{T} (100%)
CCW68	Iron-oxidizing autotroph	*Acidithiobacillus ferrooxidans*^{T} (99.5%)
CS11	Iron-oxidizing heterotroph	*Ferrimicrobium acidiphilum*^{T} (93%)
CCP3	Heterotroph	*Acidiphilium* sp. strain NO-17 (99.5)
CCW30	Heterotroph	*Acidocella* sp. strain NO-12 (99.4)[2]

[1]based on 16S rRNA gene sequence similarity; [2]97% 16S rRNA gene sequence similarity to *Ac. facilis* ATCC 35904^T

The study described in this chapter aimed to characterise the bacterial community at the Trefriw Wells Spa site, some 10 years after the study of Kimura (2005; data incorporated into Hallberg *et al.*, 2006), combining molecular and cultivation-based approaches (Section 1.2).

5.2. Sample collection

Samples from the small grotto in the Trefriw Wells spa were collected on 2nd January 2014. Sampling time was chosen to coincide with a production shut-down period (five days) at the Spatone site, as biomass was reported to build up in the grotto during such periods.

Section 1.3 gives a general description of the site. Water samples were taken at 30 cm depth from well 1 (W1) and well 2 (W2) in the grotto by dipping 20 mL sterile plastic bottles into the water and filling them so as to exclude oxygen. Two acid streamers samples that were attached to grotto substrate were also taken: one from a shallow drain in the floor in the vicinity of the passage to well 1 (Str1) and another from the end of a plastic overflow pipe connected to well 1 (Str2). A brown, gelatinous "snottite" (small microbial stalactite growth) was collected from the grotto roof above well 2 in a plastic petri dish. Each sample was

sealed with Parafilm-M (Sigma, UK), and placed immediately on ice. The samples were transported to the Bangor laboratory within one hour, and stored at 4°C until processed. Table 5.1 summarises the various samples that were taken on this occasion, Fig. 5.1 shows a schematic of the sampling locations and Fig. 5.2 shows images of the sampling locations.

Table 5.2. Summary of the water and streamer samples taken from the Trefriw Wells spa grotto (on 02/01/2104)

Sample code	Type	Location
W1	Water sample	Well #1
W2	Water sample	Well #2
Str1	Streamer growth	Drainage stream in the floor of the passage to Well #1
Str2	Streamer growth	Water surrounding the end of a drainage pipe
Snt	"Snottite"	Roof of Well #2

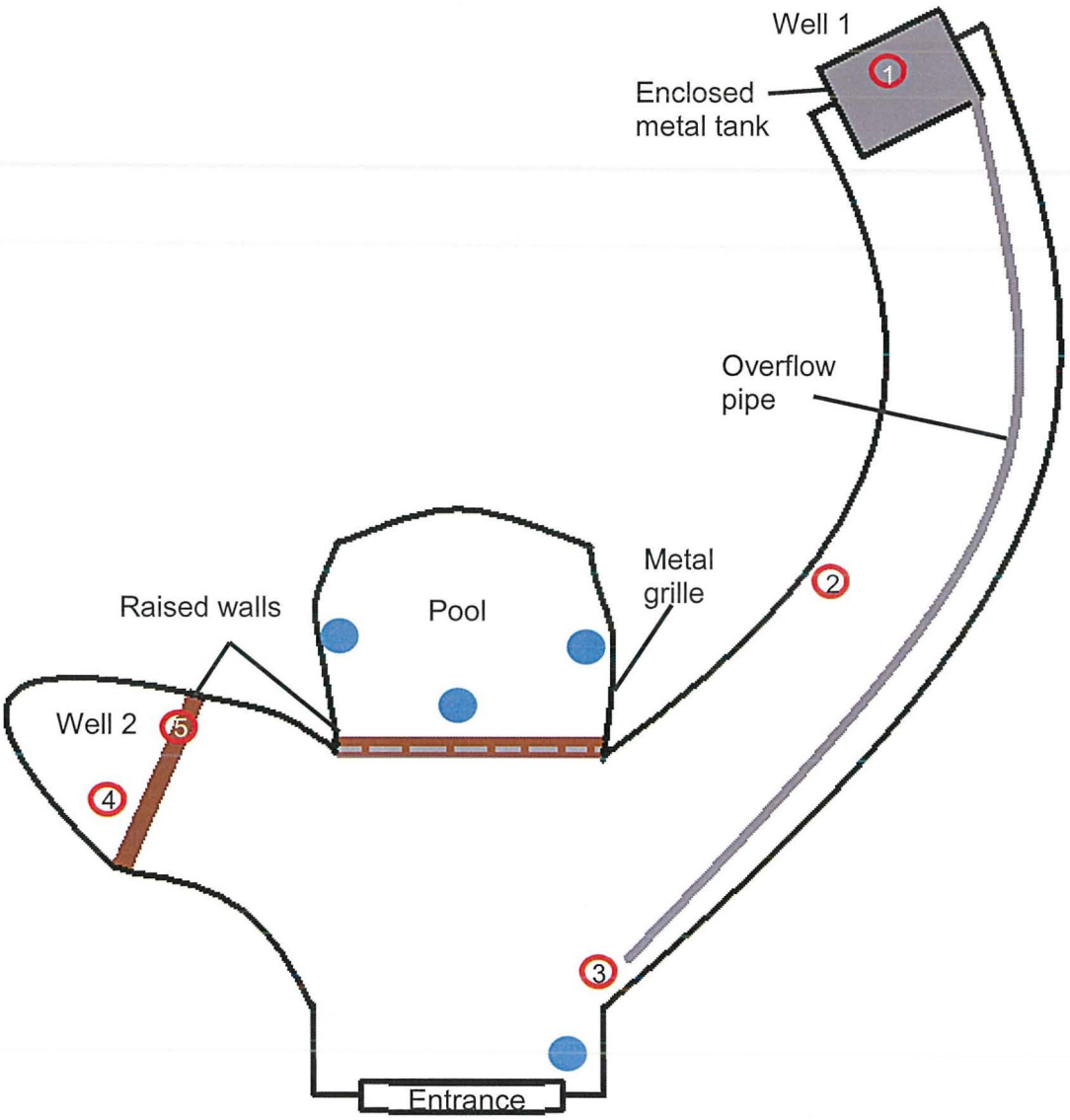


Figure 5.1. Schematic of the Trefriw Wells spa grotto as of 02/01/2014. The locations of the sampling points on that day are indicated by the symbol ○. 1 indicates the location of well water sample W1; 2 indicates the location of acid streamer sample Str1; sampling point, 3 indicates the location of acid streamer sample Str2 sampling point, 4 indicates the location of well water sample W2; 5 indicates the location of the snottite sample Snt. Locations of samples taken by Kimura (2005) are indicated by the symbol ●.

Figure 5.2. (a) image of well #2; (b) the floor close to well #1, showing streamer growths (sample Str1); (c) the roof of well #2, showing ferric iron-encrusted stalactite and gelatinous "snottites" (sample Snt); (d) floor of the grotto close to the entrance, showing the end of the plastic pipe (source of sample Str2).

5.3. Chemical analysis

5.3.1. Methods

pH, redox potential, concentrations of ferrous iron, total iron and sulfate contents of the water samples were measured for in the laboratory immediately on return from the site (sections 2.6.1, 2.6.2 and 2.6.5). However, since there was clear evidence of ferrous iron oxidation in the W1 sample (though less in the W2 sample) between the time when the sample was taken and arrival back in the laboratory, some of the laboratory data (ferrous and ferric iron concentrations, and redox potentials) were considered to be inaccurate. The data shown in

Table 5.3 include the ferrous iron concentration of well 1 water taken from the *Spatone Quality Control* records for 02/01/2014 (determined by collecting water in a sterile 50 mL Falcon tube and titrating the sample against 1 mM potassium permanganate).

5.3.2. Results

Iron concentrations in both waters are known to change with time (section 3.1). Fluctuations appear linked to extended wet and dry periods of weather, though this has not been statistically proven (Trefriw Spa Chief Engineer, personal communication). Sampling time in this study coincided with a period of particularly high rainfall.

Table 5.3. Laboratory analysis of water from Trefriw spa wells 1 and 2 (sampled on 02/01/2014).

	W1	W2
pH	2.92	2.71
Fe^{2+} (mg/L)	128*	47
Fe^{3+} (mg/L)	7*	103
Total iron (mg/L)	135	150
Sulfate-S (mg/L)	147	112

*from *Spatone Quality Control* records for that day

5.4. Preparation of streamer and snottite samples

Streamer samples (Str1 and Str2) were harvested by centrifugation in 50 mL Falcon tubes (3000 x g; 10 min) to form a pellet, then washed and lightly homogenised in sterile basal salts solution (8 mL basal salts #1/L, adjusted to pH 3 with H_2SO_4). Sections of the snottite sample (Snt) was sliced from the main body with a sterile scalpel, placed in a 1.5 mL Eppendorf tube and treated as above. All samples were stored at 4°C.

5.5. Culture-independent study of bacterial populations in Trefriw Spa water and streamer/snottite growths

5.5.1. Bacterial community phylogenetic profile

5.5.1.1. Methods

Sample preparation is described in section 5.4. DNA was extracted from portions of Str1, Str2 and Snt using the lysis method (section 2.7.1.2). Biomass from water samples were collected by filtering 50 mL of W1 and W2 water samples through 25 mm diameter Whatman 0.2 µL (pore size) nitro-cellulose membranes and extracting DNA from the membranes using an UltraClean soil DNA kit (MIOBIO, UK). Amplification of 16S rRNA genes was carried out using these DNA templates and labelled bacterial and archaeal 16S rRNA gene primers (sections 2.7.2. and 2.7.3). Amplified genes were digested with the restriction enzymes AluI, CfoI and HaeIII for T-RFLP analysis.

5.5.1.2. Results

No archaeal T-RFs were identified in any sample. The bacterial T-RFLP profile showed few matches (12-19%) with T-RFs in the T-RFLP database maintained at Bangor University (Table 5.4). The bacterial community profiles for the Trefriw water, streamer and snottite samples obtained using the restriction enzyme AluI (Fig. 5.3) showed that "*Fv. myxofaciens*" was dominant (~33% relative abundance) in W1, and also present (~5%) in the snottite sample. T-RFs corresponding to 232 nt and 233 nt (either of which could correspond *At. ferrivorans*) were present at 9% and 5% relative abundance in W1 and Str2, and 6% relative abundance in Str2. A T-RF at 70 nt, identified as corresponding to *Atx. ferrooxidans* (68 +/- 2 nt) occurred in all samples except Str1, at between 20-24% relative abundance. However, about a fifth of T-RFs in T-RFLP profiles found in the Trefriw spa samples using restriction enzyme AluI were not identified, including the dominant T-RFs; 99 nt; ~60% relative abundance in Str1, 129 nt T-RF in Str2 (~29% relative abundance), 94 nt T-RF in Snt (~ 20% relative abundance) and 56 and 94 nt T-RFs in W2 (both ~21% relative abundance).

Table 5.4. Table of T-RFs from the BART database that matched T-RFs from Trefriw spa samples (+/- 2 nt)

Bacterial species	T-RF (nt)	Enzyme
At. ferrivorans	233	AluI
	206	CfoI
	70	HaeIII
"Fv. myxofaciens"	170	AluI
	206	CfoI
	200	HaeIII
Atx. ferrivorans	68	AluI
	139	CfoI
	225	HaeIII
Ac. aromatica	208	AluI
	174	CfoI
	228	HaeIII

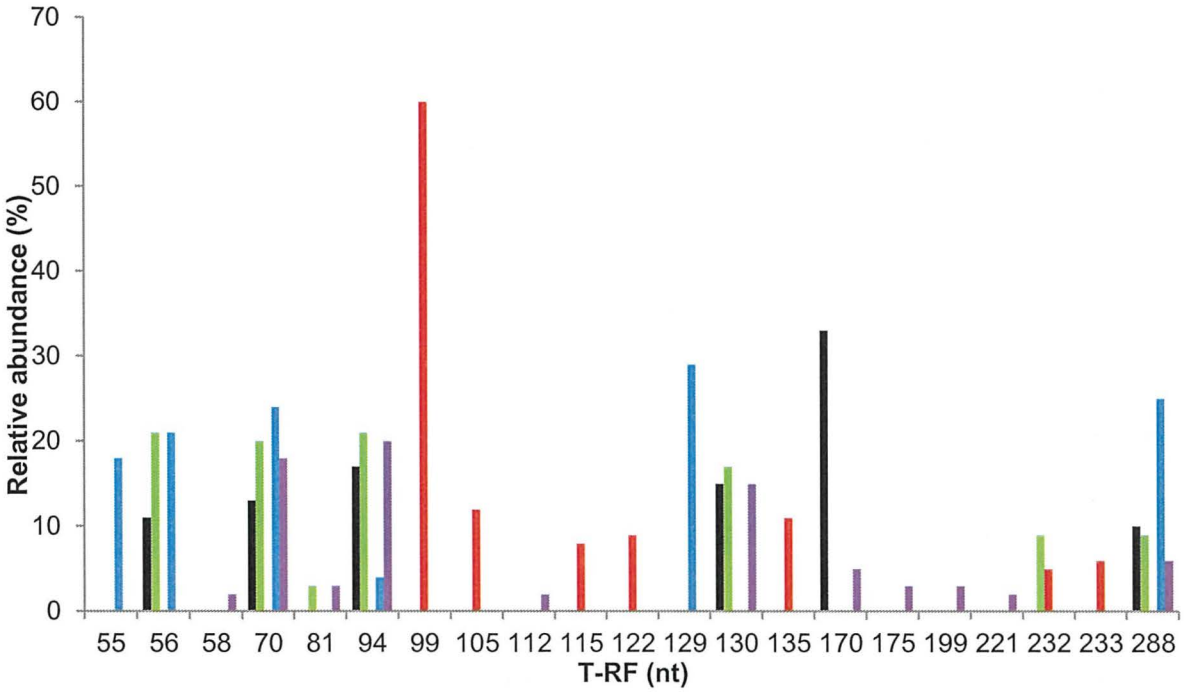


Figure 5.3. T-RFLP profile of amplified bacterial 16S rRNA genes from Trefriw spa samples, digested with AluI. Black = W1, Green = W2, Red = Str1, Blue = Str2, purple = Snt.

T-RFLP profiles generated with digestion by restriction enzyme CfoI showed a minor T-RF at 206 nt, (1 - 2% relative abundance; Fig. 5.4). This enzyme does not differentiate between "*Fv. myxofaciens*" and *At. ferrivorans*, both of which generate T-RFs at 206. The T-RF at 175 was identified as *Ac. aromatica*, and which appeared in all samples except Str2 with a relative abundance of ~12-23%. However, T-RFs for *Ac. aromatica* did not appear in bacterial community profiles of amplified 16S rRNA genes digested with either AluI or HaeIII, indicating that this T-RF may not correspond to this bacterial species in these samples. As with the bacterial community profile generated by digestion of amplified genes with the restriction enzyme AluI, only a small percentage (~12%) of the total was identified by the BART database. Those not identified included T-RFs generated at 112 nt and 65 nt, which occurred in all streamer samples.

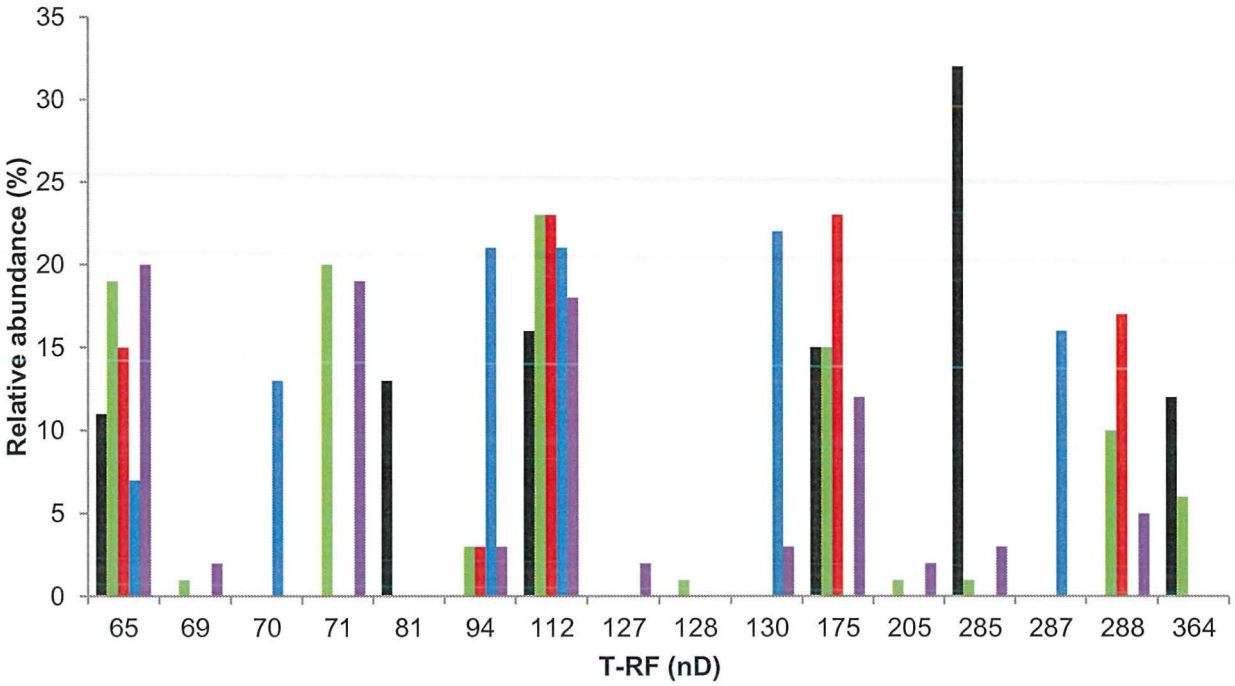


Figure 5.4 T-RFLP profile of amplified bacterial 16S rRNA genes from Trefriw spa samples, digested with CfoI. Black = W1, Green = W2, Red = Str1, Blue = Str2, purple = Snt.

T-RFLP of amplified 16S rRNA bacterial genes digested using the restriction enzyme HaeIII generated T-RFs at 69, 70 and 71 nt, all of which could be *At. ferrivorans* (Fig. 5.5). There was a "*Fv. myxofaciens*" T-RF at 199 nt and a 225 nt which was identified as *Atx. ferrooxidans*, both from W1. All other T-RFs (71%) were unidentified. T-RFs at 69, 94 and 130 appeared in all samples, and constituted between 11-29% relative abundance.

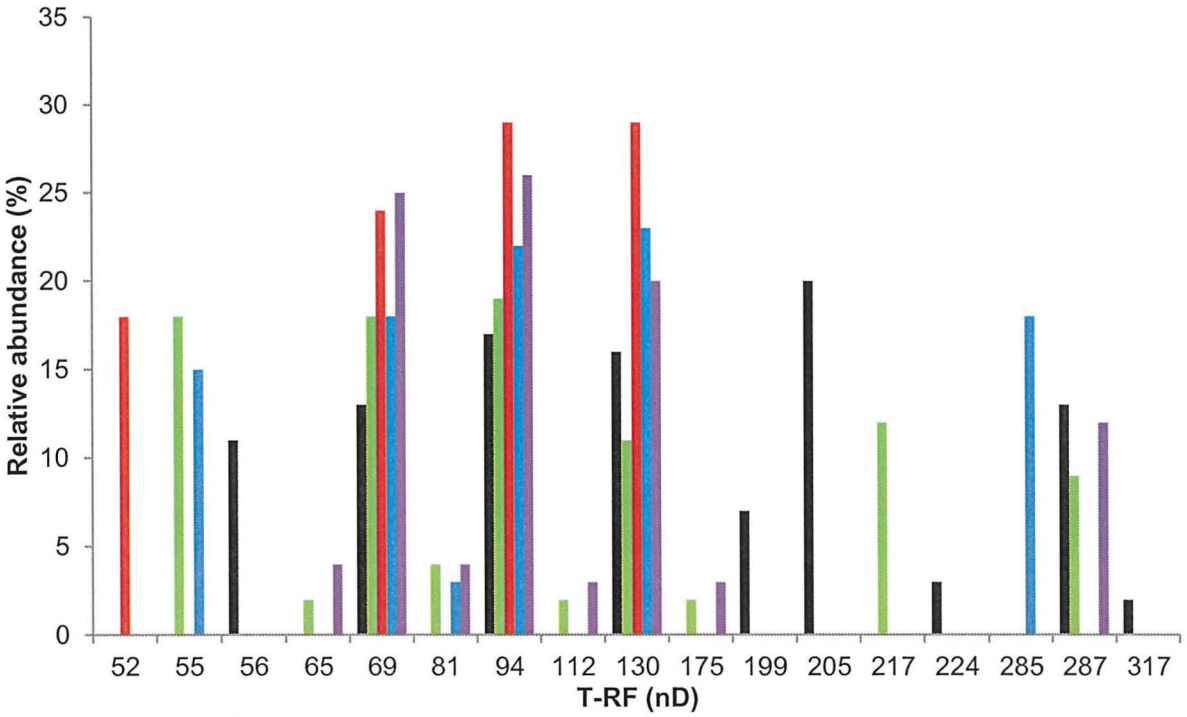


Figure 5.5. T-RFLP profile of amplified bacterial 16S rRNA genes from Trefriw spa samples, digested with HaeIII. Black = W1, Green = W2, Red = Str1, Blue = Str2, purple = Snt.

5.5.2. Analysis of phylogenetic community and geochemical data

5.5.2.1. Methods

A Brae-Curtis distance matrix was generated from T-RFLP data (section 5.5.1) in Microsoft Excel using macro tools as described in Fredriksson *et al.* (2013). Briefly, with these macros, raw data was subjected to noise removal, normalization, peak alignment and binning, relative abundance calculated and a Brae-Curtis dissimilatory matrix generated. Data was calculated based on peak area. This matrix was then used in a non-metric multi-dimensional scaling plot (MDS) in PAST 3.08 (< http://folk.uio.no/ohammer/past/>). A non-metric MDS plot that included geochemical data also generated for W1 and W2.

5.5.2.2. Results

The non-metric MDS for all sites showed that there were some T-RF profile clustering, but it was within the 95% confidence interval (Fig. 5.6). This shows that differences between these groups were small.

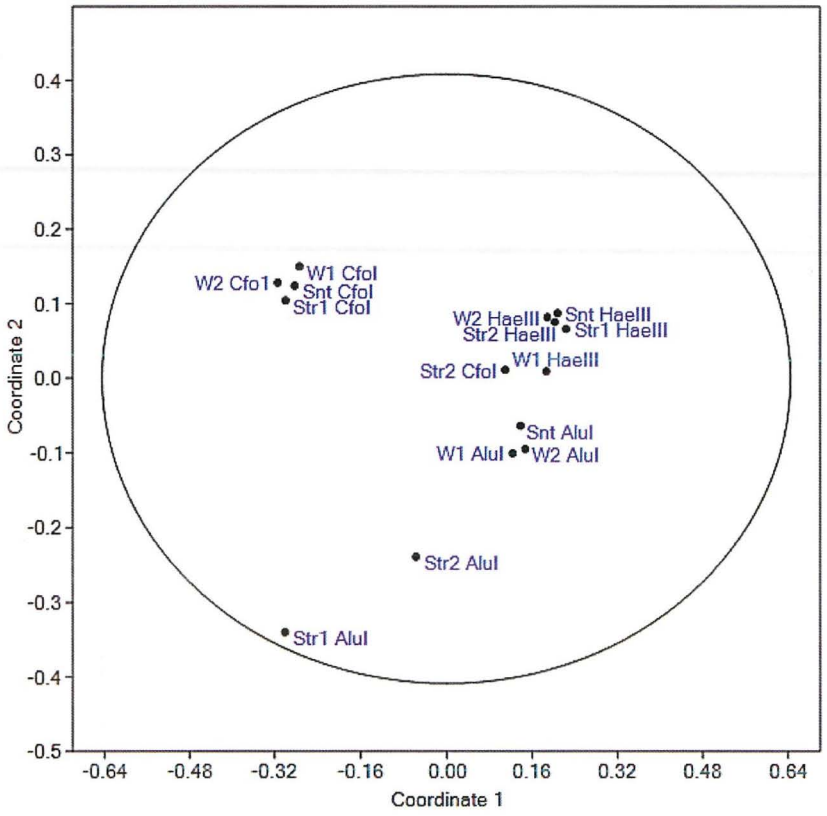


Figure 5.6. Non-metric MDS plot of all Trefriw Wells Spa samples. Ellipse shows 95% confidence interval of groupings.

The clusters were (1) CfoI restriction enzyme T-RF profiles (except Str 2), (2) HaeIII restriction enzyme T-RF profiles and (3) W1, W2 and Snt AluI restriction enzyme T-RF profiles. This clustering shows that Str 2, and to a lesser extent Str1 are less similar to each other and W1, W2 and Snt profiles, as these cluster together in each restriction enzyme T-RF profile. The separation of the AluI profiles also suggests that this enzyme provides better separation between peaks (that is, that each peak has more chance of representing a single species) in the Trefriw Wells Spa T-RF profiles than other enzymes used.

The non-metric MDS with geochemical data also showed that T-RF profiles of all three restriction enzymes for W1 and W2 samples were not significantly different from each other (Fig. 5.7). That is, that no distinct groups were identified, and no environmental factor gradient of significance was identified. T-RF profiles cluster together by enzyme, suggesting that the profiles for both sites are similar.

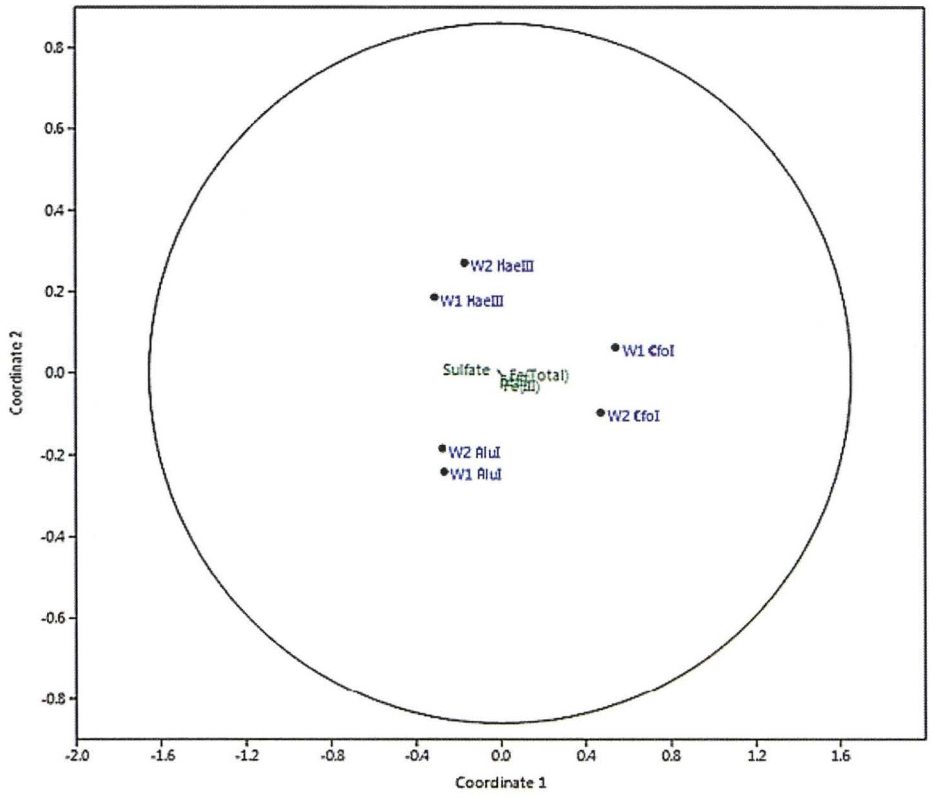


Figure 5.7. Non-metric MDS plot of W1 and W2 samples and environmental factors. Ellipse shows 95% confidence interval of groupings.

Therefore, the T-RF profiles of W1, W2 and Snt were similar to each other, whereas Str1 and, to a lesser extent, Str2 were less similar. However, these differences were not statistically significant. The small difference between the W1 and W2 T-RF profiles likely meant that they were not sufficiently different for the effect of environmental factors to be distinguishable. These observations may partly be due to the similarity of conditions between sites, particularly pH and temperature. Geochemical data from the other sampling sites, particularly the floor samples may have expanded this analysis. This would then allow the creation of non-metric MDS plots with single enzymes, which might show more clearly the relationship between environmental factors and T-RF plots. However, this data was not measured in this study and should therefore be investigated in further studies. It is also possible that microclimates within the streamers mean that the conditions surrounding the bacteria present are different from those of the surrounding liquor and that the geochemical factors presented are not representative of those surrounding the bacteria.

5.6. Cultivation-based study of bacteria from Trefriw Spa samples

5.6.1. Isolation of bacteria from Trefriw spa samples

5.6.1.1. Methods

A portion of each prepared sample (section 5.4) was used to streak inoculated onto a range of solid media that promote the growth of acidophilic microorganisms (iFeo; So; YE3o; FeTo; Feo/PFBC; Fru/YE; section 2.2.2.3). Enrichment cultures were also set up, containing 10 mM fructose as sole carbon/energy source (in medium containing basal salts #1, trace elements, 0.1 mM Fe^{2+}; pH 3) and inoculated from the W1 sample. Solid media and enrichment cultures were incubated aerobically at ~23°C (room temperature). When grown, these were inoculated onto Fru/YEo plates (section 2.2.2.3 (ii)).

Colonies that grew on solid media were differentiated by their colour and morphology, and representative single colonies were transferred to fresh solid medium of the same type. This process was repeated until a single colony- and cell-type was observed on each solid media. This method assumed that visible colony and cell morphology differences potentially indicated different isolates, and therefore those of similar morphology were more likely to be the same species (Table 5.2). Culture purity was then assessed by T-RFLP analysis (section 2.7.3). Attempts were made to transfer isolates to liquid culture corresponding to the solid media (e.g. ferrous iron medium for colonies that grew on iFeo plates). This met with variable success. Those isolates that did grow in liquid media were sub-cultured every 7-20 days. Those that did not grow were maintained on solid media.

T-RFs for Trefriw Wells spa isolates were estimated by using the software program GeneDoc to search for the cutting sites of restriction enzymes. The theoretical T-RFs were confirmed by T-RFLP where possible. Partial 16S rRNA gene sequences were aligned with the most similar (98% or more) full 16S rRNA gene sequence (as determined by BLAST analysis and aligned by ClustalX).

5.6.1.2. Results

Table 5.5 lists all colonies found on inoculated solid media after 4 weeks. The solid media that included organic substrates (Feo/PFBC and Fru/Yeo) were mostly contaminated with fungi and yeasts (8/10 plates). Isolation of heterotrophic bacteria from fructose enrichments was more successful (4 isolates from 3 enrichments compared to 2 isolates from 10 directly-inoculated solid heterotrophic media). No growth was observed on plates inoculated with W2 water, and only a small number of cells were observed in this water sample under phase contrast microscope, in comparison to the W1 sample where more bacteria were evident. In total, 11 acidophilic strains were successfully identified from Trefriw Wells spa samples.

Table 5.5. Table of colonies that appeared on inoculated solid media. (-) indicates isolates that were lost before identification could be performed.

Inoculum	Solid medium	Colony description	Isolate code
Str1	iFeo	Round, Fe^{3+}-encrusted, 1-2 mm ("*Ferrovum*"-like)	RF1 (-)
	So	Fe^{3+} encrusted (*Acidithiobacillus*-like)	RF7
		Round, entire, white	RF8 (-)*
	FeTo	Entire, translucent white, convex	RF2 (-)
	Fru/YEo	Entire, convex, cream (*Acidocella*-like)	RF3
Str2	iFeo	Fe^{3+}-encrusted, 1-2 mm ("*Ferrovum*"-like)	RF4 (-)
	So	Irregular, Fe^{3+}-encrusted, 1-2 mm	RF5 (-)
	FeTo	Fe^{3+} encrusted (*Acidithiobacillus*-like)	RF6
		Fe^{3+} encrusted, irregular, lobed	RF13a
Snt	iFeo	Fe^{3+} encrusted, 1-2 mm ("*Ferrovum*"-like)	RR1 (-)
		Entire, white, 1-2 mm	RR5 (-)*
	So	Fe^{3+} encrusted (*Acidithiobacillus*-like)	RF12
W1	iFeo	Fe^{3+} encrusted, 1-2 mm ("*Ferrovum*"-like)	RW1
	So	Irregular, Fe^{3+} encrusted	RW3 (-)
		Round, entire, smooth, white	RW2
Fructose enrichment 1	Fru/YEo	Entire, convex, smooth, white (*Acidocella*-like), 1-2 mM	RW11
		Entire, convex, smooth, white (*Acidocella*-like), 3 mM	FE1
Fructose enrichment 2	Fru/YEo	Entire, convex, smooth, cream, (*Acidocella*-like), 1-2 mM	RW22 (-)

Fructose enrichment 2	Fru/YEo	Entire, convex, smooth, white (*Acidocella*-like), 1-2 mM Entire, convex, smooth, white (*Acidocella*-like), 3 mM	RF13 FE3

*transferred to a Fru/YEo plate

Table 5.6. Trefriw Spa isolates, their media and nearest relative (shown in percentage 16S rRNA gene sequence similarity to closest named relative). NS (not sub-cultured) indicates where attempts to transfer to liquid media were unsuccessful.

Isolate code	Solid media	Liquid medium	Closest described relative
RF7	iFeo	Basal salts #1, trace elements, pH 2.5, 10mM Fe^{2+}	*Acidithiobacillus ferrivorans*^{T} (100%)
RF12			
RW2	So	Basal salts #3, trace elements, pH 3, 0.05 mM Fe^{2+}, 5% w/v S^0	*Acidithiobacillus thiooxidans*^{T}/*albertensis*^{T} (99%)
RW1	iFeo	NS	*"Ferrovum myxofaciens"* P3G^T (99%)
RF6	FeTo	NS	*Thiomonas intermedia*^{T}/*perometabolis*^{T} (99%)
RF13a	FeTo	NS	*Thiomonas cuprinus*^{T} (97%)
RF3	Fru/YEo	Fructose/basal salts	*Acidocella facilis*^{T} (99%)
RF13			
FE1			
FE3	Fru/YEo	Fructose/basal salts	*Acidocella facilis*^{T} (97%)
RW11	Fru/YEo	Fructose/basal salts	*Acidocella aluminiidurans*^{T} (97%)

Isolates FE1, RF3 and RF13 had 100% 16S rRNA gene sequence similarity to each other, as did isolates RF7 and RF12. Therefore, only RF7 and RF13, as representatives of each of these two groups, were examined further. Isolates RF6 and RF13a (both were identified as

Thiomonas spp.) could not be transferred successfully into liquid media. Isolate RW1 (a "*Ferrovum*"-like iron-oxidiser) grew in liquid culture, but was in co-culture with an unidentified heterotrophic acidophile and attempts as separation were unsuccessful. Isolation and purification of "*Ferrovum*"-like bacteria has been reported elsewhere to be particularly difficult (Johnson *et al.*, 2014). Characterisation of isolate RW11 is described elsewhere in this study (chapter 7).

5.7.1. Phylogenetic analysis of Trefriw spa isolates

5.7.1.1. Methods

DNA was extracted from pure cultures of isolates (pelleted cells extracted from liquid cultures or single colonies grown on plates) using the lysis method (section 2.7.1.2), except for isolate RW11, where a MIOBIO soil DNA kit was used. T-RFLP was performed routinely (with three restriction enzymes) to ensure that only single T-RFs were obtained, indicating purity of cultures (section 2.7.2 and 2.7.3). The 16S rRNA genes were then amplified by PCR with unlabelled primers, and sent to MACROGEN, South Korea for 16S rRNA gene sequencing. The sequences were then manually edited and BLAST analysis performed. Phylogenetic trees and a BLAST matrix was constructed for each isolate (section 2.7.6).

5.7.1.2. Results

(i) Trefriw isolates identified by 16S rRNA gene sequence similarity as *Acidithiobacillus* spp.

Isolates RF12 and RF7 were 100% related to each other and to *At. ferrivorans*, a bacterium also identified in T-RFLP profiles of Trefriw Wells spa samples (Figs. 5.2, 5.3 and 5.4 above). Isolate RW2 was also an *Acidithiobacillus* spp., 99% related to both *At. thiooxidans* and *Acidithiobacillus albertensis*, and 98% related to isolates RF7 and RF12 (Table 5.7). A neighbour-joining bootstrapped phylogenetic tree of all characterised *Acidithiobacillus* spp.

concurred with the BLAST matrix (Fig. 5.8), which is unsurprising given its 16S rRNA gene sequence similarity to both mesophilic sulfur-oxidising acidophiles.

Table 5.7. BLAST matrix (in percent 16S rRNA gene sequence similarity) showing the relatedness of Trefriw Wells spa isolates to type strains of currently described *Acidithiobacillus* species.

	RF7	RF12	RW2	*At. ferrivorans*^{T}	*At. ferridurans*^{T}	*At. ferrooxidans*^{T}	*At. thiooxidans*^{T}	*At. albertensis*^{T}	*At. caldus*^{T}
RF7	100	100	98	100	99	97	98	98	96
RF12	100	100	98	100	99	97	98	98	96
RW2	98	98	100	98	98	97	99	99	95

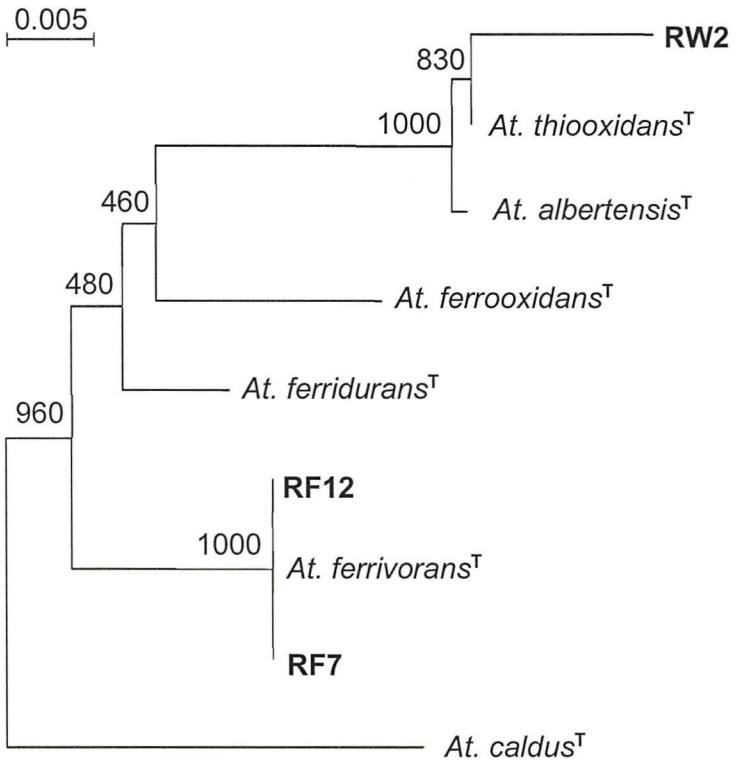


Figure 5.8. Bootstrapped (x1000) neighbour-joining phylogenetic tree of *Acidithiobacillus* species based on 16S rRNA gene sequences, with *Sb. thermosulfidooxidans* as outgroup (not shown) and Trefriw Wells spa isolates highlighted in **bold**. Sequence length 1075 nt.

(ii) Trefriw isolate identified as related *"Ferrovum myxofaciens"*

Isolate RW1 was 99% related to "*Fv. myxofaciens*" P3G^T, the only acidophilic *Betaproteobacterium* yet described. RW1 displayed similar characteristics to "*Fv. myxofaciens*"; it displayed similar morphology and it was isolated from iFeo plates, which were originally devised to isolate that particular organism and showed the same colony morphology (Johnson *et al.*, 2014). Isolate RW1 was also challenging to transfer successfully into pure liquid cultures, which was also reported for "*Fv. myxofaciens*".

(iii) Trefriw isolates identified by 16S rRNA gene sequence similarity as *Acidocella* spp.

Five bacteria isolated from Trefriw Wells spa were identified as being related to known *Acidocella* spp. (Table 5.8). Isolates RF3, RW13 and FE1 were 100% similar to each other, and 99% similar to *Ac. facilis*. Isolate FE3 has a 97% 16S rRNA gene sequence similarity to *Ac. facilis* (its closest validly described relative), but was more closely related to three of the other Trefriw Wells spa *Acidocella* isolates.

Fig. 5.7 shows that all Trefriw isolates except RW11 clustered together in a neighbour-joining phylogenetic tree, reflecting the BLAST results. Isolate RW11 is most closely related to *Ac. aluminiidurans* (97% 16S rRNA gene similarity). In the phylogenetic tree (Fig. 5.9), isolate RW11 was placed within the *Acidocella* cluster but clearly separate from the other species (bootstrap value of 944). This, along with the 16S rRNA gene sequence similarity between isolate RW11 and the other *Acidocella* spp. and Trefriw Wells isolates indicates that it is a novel species, and is described further in chapter 6.

Table 5.8. BLAST matrix (as percent 16S rRNA gene sequence similarity) showing the relatedness of *Acidocella*-like isolates from Trefriw Wells spa to type strains of currently described *Acidocella* species.

	FE3	FE1	RF3	RF13	RW11	*Ac. facilis*^{T}	*Ac. aminolytica*^{T}	*Ac. aluminiidurans*^{T}	*Ac. aromatica*^{T}
FE3	100	99	99	99	96	97	95	96	95
FE1	99	100	100	100	96	99	98	98	97
RF3	99	100	100	100	96	100	98	99	97
RW13	99	100	100	100	96	99	98	99	97
RW11	96	96	96	96	100	96	94	97	96

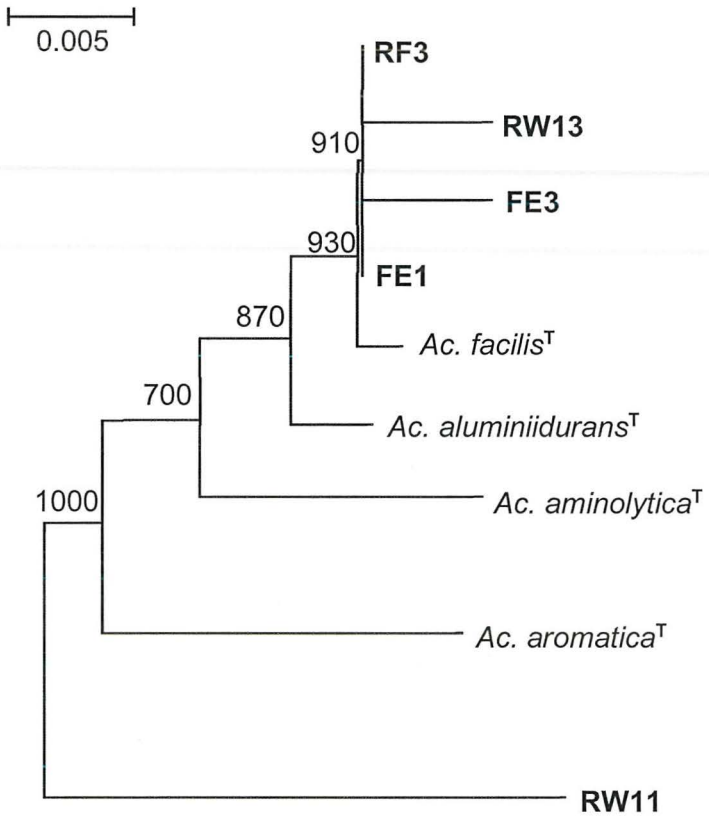


Figure 5.9. Bootstrapped (x1000) neighbour-joining phylogenetic tree of *Acidocella* spp. based on 16S rRNA gene sequences, with *Sb. thermosulfidooxidans* as outgroup (not shown) and Trefriw Wells spa isolates highlighted in **bold**. Sequence length 1134 nt.

(iv) Trefriw isolates identified by 16S rRNA gene sequence similarity as *Thiomonas* (*Tm.*) spp.

Isolates RF6 and RF13a were most closely related to *Thiomonas intermedia* (99%) and *Thiomonas delicata* (97%) respectively, according to a BLAST analysis. Table 5.9 shows the results of multiple BLAST analysis comparing Trefriw Wells spa isolates to described *Thiomonas* spp.. The Trefriw strains were isolated from plates with a pH of ~4.5, a typical pH for growth of moderately acidophilic *Thiomonas* species.

Table 5.9. BLAST matrix (percent 16S rRNA gene sequence similarity) showing the relatedness of Trefriw Wells spa isolates to type strains of currently described *Thiomonas* species.

	RF6	RF13a	*Tm. intermedia*^{T}	*Tm. arsenitoxydans*^{T}	*Tm. perometabolis*^{T}	*Tm. delicata*^{T}	*Tm. bhubaneswarensis*^{T}	*Tm. islandica*^{T}	*Tm. thermosulfata*^{T}
RF6	100	91	99	99	99	93	98	97	94
RF13a	91	100	92	92	91	97	92	93	92

The phylogenetic tree shown in Fig. 5.10 places isolate RF13a into one group of *Thiomonas* spp. (along with *Tm. delicata, Tm. arsenivorans* and *Tm. cuprina,* all of which are moderate acidophiles) while isolate RF6 was in a separate branch of *Thiomonas* spp. that also included *Tm. arsenitoxydans*, *Tm. bhubaneswarensis*, *Tm. intermedia*, *Tm. permetabolis*, *Tm. thermosulfata* and *Tm. islandica*. These spp. are all moderately acidophilic, with the exception of *Tm. bhubaneswarensis*, which is acid-tolerant (optimum pH of above 5), and all are mesophilic, except *Tm. thermosulfata* and *Tm. islandica* which are a moderate thermophiles.

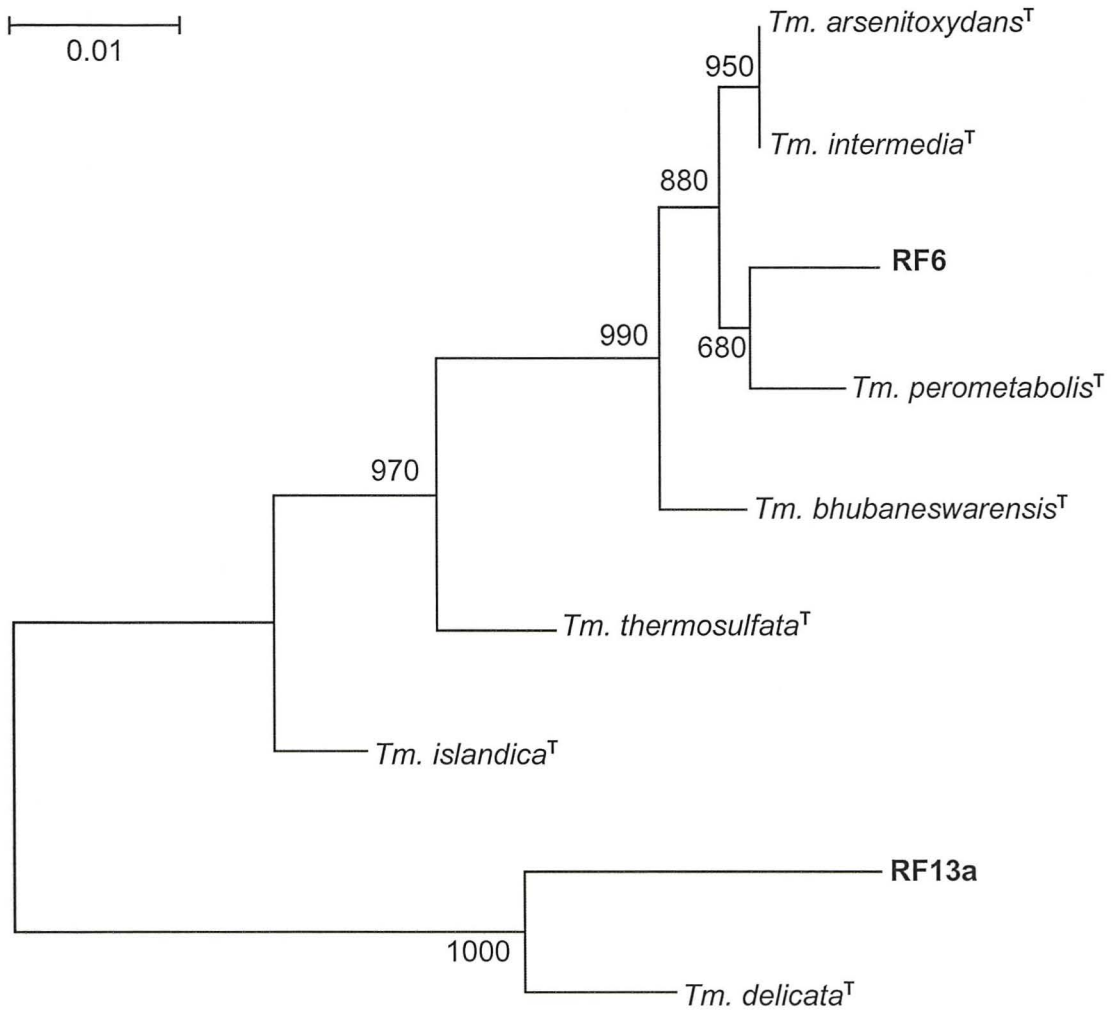


Fig. 5.10. Bootstrapped (x1000) neighbour-joining phylogenetic tree of *Thiomonas* spp. based on 16S rRNA gene sequences, with *Sb. thermosulfidooxidans* as outgroup (not shown) and Trefriw isolates highlighted in **bold**. Sequence length 1075 bp.

5.7.2. Determination of some physiological characteristics of Trefriw Wells spa isolates

Four of the isolates obtained from Trefriw Wells spa samples were partly characterised. These were: isolate RF7 (a strain of *At. ferrivorans*), RW2 (*At. thiooxidans/albertensis*), RF13 and FE3 (both strains of *Acidocella*).

5.7.2.1. Oxidation of iron and sulfur, and growth on organic substrates

(i) Methods

All four isolates were tested for oxidation of ferrous iron (inoculated cultures of ferrous iron medium (section 2.2.2.2) incubated at 30°C then the decrease in ferrous iron concentration measured by Ferrozine assay) and elemental sulfur (section 8.5.1). Isolates that tested positive to either of these were then examined for whether their growth on sulfur was enhanced by adding yeast extract (at 0.02%, w/v) to the otherwise organic C-free liquid medium.

Growth on different organic substrates was tested in the case of the heterotrophic isolates RF13 and FE3. This involved inoculating active cultures into a basal salts (# 1)/trace elements liquid medium (pH 3) containing the following compounds (at different concentrations, designed to give approximately the same "carbon equivalents", except in the case of acetic acid).

(i) Carbohydrates: glucose, fructose, ribose, cellobiose (all at 5 mM),

(ii) Aromatic compounds: phenol (1 mM), benzyl alcohol (5 mM)

(iii) Alcohols: ethanol (15 mM), methanol (20 mM), glycerol (10 mM)

(iv) Organic acids: acetic acid (1 mM); pyruvic acid, lactic acid, glycine (5 mM)

(v) (yeast extract, 0.02% w/v)

Cultures were incubated at 30ºC, with shaking, and optical densities at 600 nm (OD_{600}) determined after 7 and 14 days.

(ii) Results

Both isolates RF7 and RW2 grew autotrophically on elemental sulfur (and on ferrous iron, in the case of isolate RF7) but growth of neither isolate was enhanced by the addition of yeast extract, confirming that they (like their nearest relatives) were obligate autotrophs.

Table 5.10. Table of substrates used by Trefriw isolates. Key: + growth; - no growth. Blank cells in the table indicates that substrates were not tested (isolates RF7 and RW2 were confirmed to be obligate autotrophs)

	Isolate			
Oxidation of:	RF13	FE3	RF7	RW2
S^0	-	-	+	+
Fe^{2+}	-	-	+	-
Growth on:				
Yeast extract	+	+	-	-
Cellobiose	-	-		
Glucose	+	+		
Fructose	+	+		
Glycerol	+	-		
Ribose	+	+		
Ethanol	+	-		
Methanol	-	-		
Glycine	-	-		
Citric acid	+	-		
Acetic acid	-	-		
Pyruvic acid	-	-		
Lactic acid	+	+		
Benzyl alcohol	+	-		
Phenol	-	-		

The *Acidocella*-like isolates RF13 and FE3 showed some differences in the substrates that they could metabolise (Table 5.10). Both isolates metabolised monomeric sugars such as glucose, but not the glucose dimer cellobiose. They could also grow in yeast extract medium, similar to most other *Acidocella* species (except *Ac. aromatica*; chapter 6). These isolates also grew on some unusual substrates (for acidophiles) such as organic acids and aromatic compounds. Isolate RF13 (but not isolate FE3) could use the aromatic compound benzyl alcohol, in common with all other known *Acidocella* spp. (chapter 6 and 7). Interestingly, both of these isolated heterotrophic isolates and isolate RW11 (chapter 6)

could also grow in liquid medium containing 10 mM lactic acid. The only other extremely acidophilic bacteria previously reported to do so are some strains of *Ac. aromatica* (Jones *et al.*, 2013; chapter 7).

5.7.2.2. Temperature range, pH minima and growth under oxygen limitation of isolates RF13 and FE3

To identify pH minima, isolates RF13 and FE3 were grown in fructose liquid medium (section 2.2.2.2) at between pH 2.0 and 3.0. Growth in pH 3 fructose liquid medium was also tested between 7°C and 45°C. Growth of these two isolates was also compared in cultures grown under aerobic, micro-aerobic and anaerobic conditions (section 2.2.3).

Isolates RF13 and FE3 had pH minima for growth of 2.5. The maximum growth temperature for both isolates was 40°C, and they grew at 10°C but not at 7°C. This temperature minimum is lower than previously quoted for *Acidocella* spp., though most growth temperatures quoted in the literature appear to be the lowest temperature that were tested, rather than actual minima temperature for growth.

Both isolates RF13 and FE3 grew under aerobic and micro-aerobic conditions but not in the absence of oxygen (as other *Acidocella* species), confirming that they were obligate aerobes.

5.8. Discussion

This investigation into the Trefriw Wells Spa bacterial community identified bacterial species that included iron- and sulfur-oxidising autotrophic species and some heterotrophic bacteria, using an approach that combined culture-dependant and molecular methods. No archaea were isolated by culturing methods, nor were any archaeal 16S rRNA gene sequences amplified by PCR using archaea-specific primers. This result was also reported by Kimura

(2005), Hallberg *et al.*, (2006) and Kimura *et al.* (2011) using samples from the Trefriw Wells Spa.

The bacterial community (as identified by T-RFLP analysis using three restriction enzymes; AluI, CfoI and HaeIII), showed a relatively low bacterial diversity in all sites sampled (6-11 T-RFs). Table 5.11 lists the numbers of T-RFs in each of the five samples.

Table 5.11. Number of T-RFs detected in each sample, as digested using the restriction enzymes AluI, CfoI and HaeIII.

	Sample				
Restriction enzyme	W1	W2	Str1	Str2	Snt
AluI	6	6	7	6	10
CfoI	6	6	5	6	11
HaeIII	9	10	4	6	8

This variation in T-RF numbers between enzymes in each sample site is due to the limitations of T-RFLP analysis. Limitations are present because the base assumption of the analysis is that one peak represents one species, and that relative abundance of the fragment is representative of the relative abundance of the species in the sample. This assumption relies on a number of conditions being true. These are: 1) A single peak represents a single species. 2) the position of restriction enzyme cutting sites for each 16S rRNA gene sequence is unique to each species. 3) the T-RF fragments present are all within the analysis length (600 bp long, in this study). 4) the proportion of T-RF fragments is correlated to the amount of biomass in the sample (that is, one fragment per cell). These conditions are not always true. T-RFs of the same length are possible for different species, multiple fragments may be generated from one sequence and reproducible pseudo-T-RF peaks can be generated. T-RFs can also be absent through long length or because the peak

is below detection limit because its relative abundance is low. Finally, some prior information is required on the identity of the species corresponding to the peaks produced by each enzyme. Multiple enzyme digests and a database of T-RFs are required to compensate for some of the deviations from these assumptions. For example, if a species produced T-RFs that do not confirm to the assumptions outlined above when digested with one enzyme, it may do when digested by another. However, this approach still contains caveats, such as the loss of low abundance peaks.

In this study, three T-RFs of Trefriw Spa streamer samples in at least two out of three T-RF profiles were the same as those of known acidophilic bacteria in the BART database. One species (*Acidocella aromatica*) was matched to a T-RF in only one of the three profiles, making it an ambiguous result. Previous studies had identified *Acidocella* spp. at the site, though not *Ac. aromatica*. This suggested that possibly one or more other *Acidocella* spp. were present (Hallberg *et al.*, 2006; Kimura, 2005). This study also identified *Acidocella* spp at the site.

In this study, 12-25% of T-RFs were identified using the BART database. When T-RFs of bacteria isolated in the present were included, this increased slightly (19-25%). The small increase in the percentage of T-RFs identified was thought due to T-RFs of the new Trefriw isolates being the same as those already in the BART database in some instances. Table 5.12 shows the T-RFs corresponding to these new isolates, and Fig. 5.11 a phylogenetic tree of all of the Trefriw isolates obtained in the present study.

Table 5.12. Table of T-RFs of Trefriw isolates, using the restriction enzymes AluI, CfoI and HaeIII. Values in parentheses were estimated as described in section 5.6.1.1, but did not appear in T-RFLP profiles.

Isolate designation	T-RF	Restriction enzyme
RF7	233	AluI
RF12	206	CfoI
	70	HaeIII
RW2	206	AluI
	(1047)	CfoI
	(1146)	HaeIII
RW1	170	AluI
	206	CfoI
	200	HaeIII
RW13	(585)	AluI
RF3	340	CfoI
FE1	226	HaeIII
RW11	206	AluI
	174	CfoI
	228	HaeIII
FE3	(587)	AluI
	340	CfoI
	227	HaeIII
RF6	230	AluI
	68	CfoI
	215	HaeIII
RF13a	232	AluI
	144	CfoI
	197	HaeIII

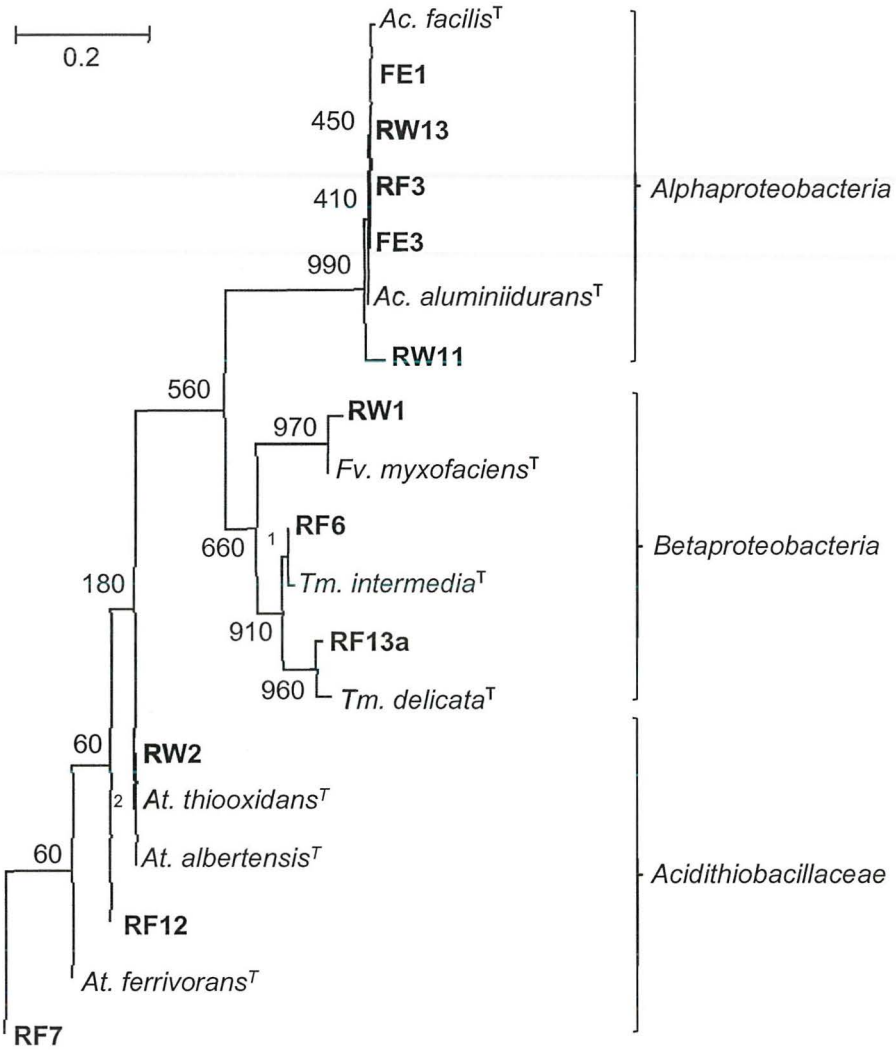


Figure 5.11. Neighbour joining phylogenetic tree showing all Trefriw Wells Spa isolates and their nearest relatives *Ferroplasma acidiphilum* (GenBank accession number AJ224936) is the tree root (not shown). Sequence length 1002 nt. Bootstrap values appear on their respective nodes. [1] is 790, [2] is 870.

In a previous study of the Trefriw spa grotto Kimura (2005), identified 0-20% of T-RFs using the restriction enzymes HaeI and MspI. The single identified species was a *Thiomonas* spp.. Kimura *et al.* (2011) found that plating efficiency in comparison to live cells (counted by fluorescent *in situ* hybridisation; FISH) was approximately 20% for Cae Coch mine. The same pyrite ore body is responsible for both sites, though the physiochemistry of the water bodies within Cae Coch differ from those in the Trefriw Wells grotto. That is, the Cae Coch conditions are more acidic, more oxidised and generally more enriched with soluble metals

(Kimura *et al.*, 2011; Hallberg *et al.*, 2006). These differences are likely to be why the bacterial community of the sites are somewhat different, despite originating from the same ore body. Isolates of *Fv. myxofaciens, At. ferrivorans* and *Acidocella* spp. were identified at both sites, though other species such as *Leptospirillum* spp., and Archaea were identified in Cae Coch mine only (Kimura *et al.*, 2011; Hallberg *et al.*, 2006).

Hallberg *et al.* (2006) isolated a strain of *At. ferrivorans*, and also an *Acidocella* sp. that shared 97% 16S rRNA gene sequence with *Ac. facilis* from the Trefriw Wells Spa site; both of these are similar to strains isolated in this study. They also isolated a strain of *Atx. ferrooxidans* (Table 5.1); this *Actinobacterium* was also detected by T-RFLP analysis in the current study, though it was not isolated. Elsewhere (chapter 8), it is shown that *Atx. ferrooxidans* can grow on the solid medium that was used in this study to attempt to isolate this acidophilic *Actinobacterium* from Trefriw Wells spa samples. Hallberg *et al.* (2006) also isolated an *Acidiphilum* sp., and a strain of *At. ferrooxidans*, neither of which was identified by T-RFLP or isolated in this study.

Variations between the bacterial communities in the five sampling sites were also observed. T-RFLP profiles from single restriction enzymes showed that some T-RFs occurred in all samples, and some occurred in only one sample site. This included T-RFs that were assigned to isolates from this study. Both *Thiomonas* isolates were relatively rare (less than 5%), but occurred in all samples, while the *"Ferrovum"* isolate RW1 was relatively abundant in some samples but absent in others. This variation in bacterial communities between sites that are geographically close is likely due to small variations in the physicochemical conditions of the habitats. However, differences between site profiles were shown to be non-significant by non-metric MDS. Differences between the chemistries of waters in well 1 and well 2 are shown in Table 5.2, and include pH and iron concentration. Given that the samples were not filtered on site, chemical parameters such as ferrous iron and redox changed in transit and so is of limited value. However, data from elsewhere in this study

(Table 1.2; Hallberg *et al.*, 2006, and Table 3.2; Nelsons personnel) also found differences between water bodies at the site.

According to Hallberg *et al.* (2006), the dissolved oxygen concentration in water in well 2 was higher than that in well 1 at that time, which may possibly account for the higher concentration of ferric iron in well 1. This difference in ferric/ferrous iron proportions between well 1 and well 2 is relatively constant (data not shown). Conditions can also vary at micro scale within streamers and snottites (Kimura *et al.*, 2011). Different bacteria can also compliment and compete with each other (section 1.1.5), so that the constituent members of a community can be greatly impacted by the presence of others.

One other interesting point is that all isolated heterotrophic bacteria from Trefriw spa could metabolise some small molecular weight aliphatic acids and (with the exception of RF3) the aromatic compound benzyl alcohol. All described *Acidocella* species can also metabolise these compounds to a greater or lesser extent, as shown elsewhere in this study (Chapters 6 and 7). Also interesting is that all heterotrophic bacteria isolated from Trefriw Wells Spa, including FE3, were capable of using lactic acid as a substrate at a concentration ~x5 higher than the previously reported highest value for an extreme acidophile (2 mM; *Acidobacterium capsulatum;* Kishimoto *et al.*, 1991). This topic of the ability of *Acidocella* spp. to metabolise small weight organic acids and aromatic compounds are discussed further in chapters 6, 7 and 10.

In summary, this study identified 19-25% of the bacterial streamer community diversity in samples from the Trefriw Spa, as determined by T-RFLP analysis and culture-dependant methods. Overall, bacterial diversity was found to be broadly similar to those reported in studies carried out at the site, but shows some new observations. These include the isolation of novel strains and species of *Acidocella*, and the apparent universal ability of all *Acidocella* spp. isolated from the site to tolerate and metabolise lactic acid at concentrations that are lethal to most acidophilic bacteria.

Chapter 6 "*Acidocella acidivorans*": a proposed novel species of the genus *Acidocella*

6.1. Introduction

Prior to this study, the genus *Acidocella* comprised four validated species (*Ac. facilis*, *Ac. aminolytica*, *Ac. aluminiidurans* and *Ac. aromatica*). The novel species *Ac. aromatica* was ascribed to the genus as part of the current research programme (chapter 7). Several *Acidocella*-related strains were isolated from the Trefriw Wells Spa site (chapter 5), including one (strain RW11) that was very atypical of this genus in terms of its cellular morphology. Isolate RW11 also displayed the unusual property of tolerating and using organic acids that are generally toxic to acidophilic bacteria such as pyruvic acid, in concentrations far higher than previously reported (5 mM as opposed to 0.5 µM; Rawlings and Johnson, 2004). A similar characteristic was identified as present in *Ac. aromatica* (~10 mM acetic acid as opposed to 1 mM repoted previously; chapter 7), and other *Acidocella* spp. isolated from Trefriw Wells Spa (chapter 5).

The isolate was therefore further characterised, and the data obtained provided firm evidence that this was a strain of a novel species of *Acidocella*, proposed as "*Acidocella acidivorans*" sp. nov..

6.2. Isolation and cultivation

Strain RW11 was isolated from a fructose enrichment culture inoculated with a water sample (pH ~2.5) from the Trefriw Wells spa grotto (section 5). A pure culture was obtained by repeated single colony isolation on fructose overlay solid medium (Fruo; section 2.2.2.3) and confirmed by T-RFLP analysis (section 2.7.3).

Strain RW11 formed smooth, domed, round/convex, white colonies on solid medium, similar to those of known *Acidocella* spp.. Strain RW11 grew as single cells or short chains of motile non spore-forming rods (2-3 µm wide and 0.4-0.8 µm long) that were often refractile when viewed under the phase contrast microscope, and occasionally irregular in shape. Individual cells were notably larger (in particular, in width) than *Acidocella* species previously described (Fig. 6.1).

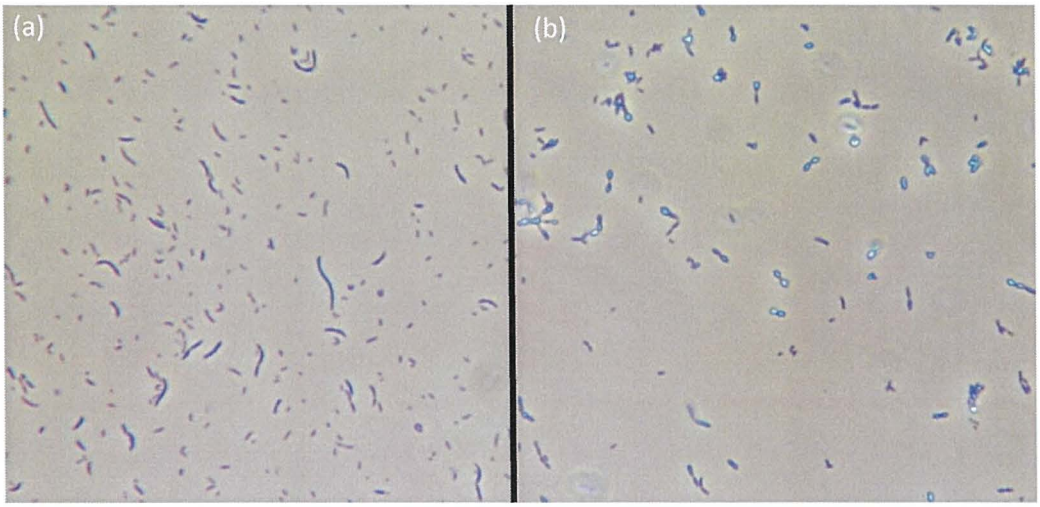


Figure 6.1. Phase contrast images (x1000 magnification) of (a) *Acidocella facilis*^{T} and (b) isolate RW11.

6.3. Physiological characteristics of isolate RW11 in comparison with those of previously described *Acidocella* spp.

6.3.1. Determination of the effect of pH and temperature on the growth of RW11

6.3.1.1. Methods

Isolate RW11 was grown in a 2 L bioreactor (as described in section 6.3.1.1) to determine its temperature and pH optima and mean generation times. The reactor vessel contained 1.5 L of fructose/basal salts medium (section 2.2.2.2) and was maintained at either pH 3.0 (+/- 0.05) when varying temperature (22.5 - 37°C), or at 30°C (+/- 0.5°C) and varying pH (pH 2.4

– 4.0). The bioreactor was aerated at ~1 L air/min and stirred at 100 rpm. Samples were removed at regular intervals, and OD_{600} values recorded. Semi-logarithmic plots of changes in OD_{600} values against time were used to calculate mean generation times.

Shake flasks were used to determine growth of isolate RW11 outside of the above pH and temperature ranges. To determine its pH minimum for growth, duplicate 100 mL flasks containing 25 mL of inoculated fructose/basal salts medium (pH adjusted to 1.9-2.4) were incubated with shaking at 30°C for 14 days. To establish its growth temperature limits, isolate RW11 was grown in duplicate 100 mL flasks containing 25 mL of fructose/basal salts medium (pH 3) at temperatures between 4.0 and 42.5°C.

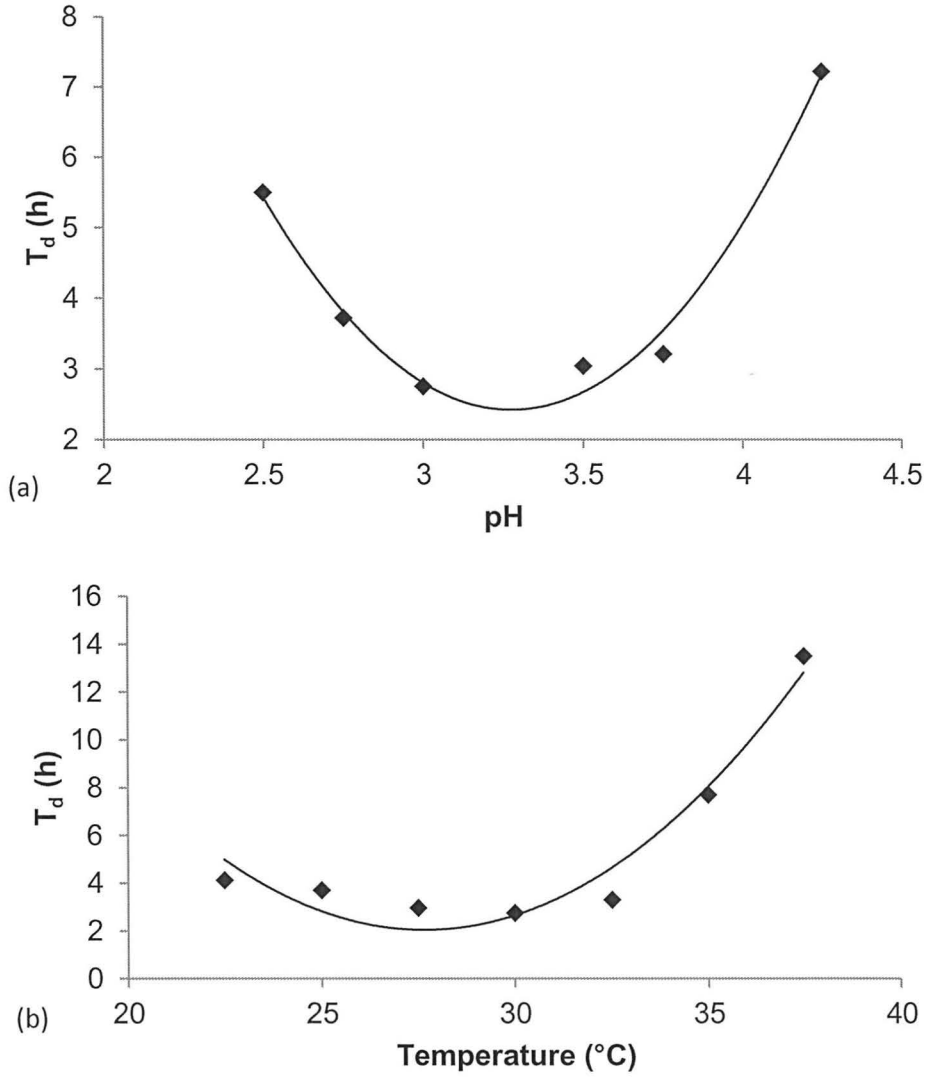


Figure 6.1. Culture doubling times for isolate RW11 in a bioreactor for (a) a pH range of 2.5-4.25 at 30°C and (b) a temperature range of 22-37°C at pH 3.

6.3.1.2. Results

Isolate RW11 had an optimum pH of 3.2 (Fig. 6.1. (a)), and a temperature optimum of 30°C (Fig. 6.1. (b)). In flask cultures, it grew at pH 2.4 but not at pH 2.3. It also grew in flask cultures at 15°C (but not 10°C), and did not grow at 40°C (it grew at 37.5°C in the bioreactor). Under optimum conditions, its culture doubling time was 2.75 h, corresponding to a mean growth rate of 0.36 h^{-1}.

6.3.2. Determination of the substrates metabolised by isolate RW11

6.3.2.1. Methods

To examine which substrates isolate RW11 could metabolise, it was grown in duplicate 5 mL liquid cultures in 25 mL universal bottles containing basal salts/trace elements, 0.1 mM Fe^{2+} adjusted to pH 3.0 (minimal salts medium; section 2.2.2.2) and amended with one of the following substrates, amounts of which were adjusted (in most cases) to be similar in terms of carbon-equivalents;

(i) Carbohydrates: cellobiose, sucrose, galactose, glucose, fructose, mannose, arabinose and rhamnose (all 5 mM).

(ii) Alcohols: glycerol (10 mM), ethanol (15 mM) and methanol (20 mM).

(iii) Aromatic compounds: benzyl alcohol, sodium benzoate (both at 5 mM), and phenol (1 mM).

(iv) Organic/amino acids: acetic acid, butyric acid, pyruvic acid, citric acid, glucuronic acid, glutamic acid, proline, alanine, sodium formate (all at 5 mM); lactic acid, glycolic acid and 10 mM propionic acid (all at 10 mM).

(v) Complex media; tryptone and yeast extract (both at 0.02%, w/v).

Cultures were incubated, with shaking, at 30°C for 7-14 days, and OD_{600} values recorded. These were compared with both positive (minimal salts medium amended with 5 mM fructose) and negative (minimal salts medium with no added substrates) controls.

6.3.2.2. Results

Isolate RW11 metabolised a range of organic substrates including some monosaccharides (glucose, fructose and mannose) and disaccharides (cellobiose and sucrose) but, like *Ac. aromatica*, did not use complex media (yeast extract and tryptone; Table 6.1). Isolate RW11 also used the aromatic compounds phenol (at 1 mM but not 5 mM) and benzyl alcohol.

Isolate RW11 notably metabolised many aliphatic acids including some (such as pyruvic and lactic acid) that are toxic to most acidophiles when present at micromolar concentrations. Most of these acids were not used by other *Acidocella* spp. when provided at similar concentrations. This observation is explored further in section 6.3.3.

Table 6.1. Growth of isolate RW11 on different organic substrates. The type strains of *Ac. aromatica, Ac. facilis, Ac. aminolytica* and *Ac. aluminiidurans* included for comparison. Key: +, growth; –, no growth.

Substrate	Bacterium				
	RW11	*Ac. aromatica*^{T}[1]	*Ac. facilis*^{T}	*Ac. aminolytica*^{T}	*Ac. aluminiidurans*^{T}
Cellobiose	+	-	-	-	-
Galactose	-	-	+	+	+
Glucose	+	-	+	+	+
Fructose	+	+	+[3]	+[4]	-[5]
Mannose	+	-	+[3]	-[5]	-[5]
Sucrose	+	+	+	+	+
Arabinose	-	-	-[5]	-	-[5]
Rhamnose	-	-	-[3]	-[4]	-[5]
Acetic acid	+	+	-[3]	-[4]	-[5]
Butyric acid	+	-	-	-	-
Pyruvic acid	+	-	-	-	-
Citric acid	+	-	-[3]	-[4]	-[5]
Glucuronic acid	-	-	-[3]	-[4]	-[5]
Glutamic acid	+	-	-[3]	-[4]	+[5]
Glycolic acid	+	-	-	-	-

Proline	-	-	-	-	-
Alanine	-	-	-	-	-
Lactic acid	+	-	-[3]	-[4]	-[5]
Propionic acid	+	-	-[3]	-[4]	-[5]
Glycerol	+	-	+[3]	-[4]	-[5]
Ethanol	+	-	+	+	+
Methanol	-	-	-[4]	-[4]	
Phenol	+	+	-	-	-
Benzyl alcohol	+	+	+	+	+
Sodium benzoate	+	+	-	+	-
Tryptone	-	-	+	+	-
Yeast extract	-	-	+	+	+

[1]Section 7, Jones *et al.*, 2013; Gemmell and Knowles (2000); [2]Hallberg *et al.* (1999); [3]Wichlacz *et al.* (1987); [4]Kishimoto *et al.* (1993); [5]Kimoto *et al.* (2010).

6.3.3. Growth of RW11 on small molecular weight aliphatic acids

6.3.3.1. Methods

The relative toxicities of pyruvic, lactic, acetic and oxalic acids to isolate RW11 were assessed initially by adding sodium pyruvate (1 – 60 mM, final concentrations), lactic acid (1 – 20 mM), sodium acetate (1 – 10 mM) or oxalic acid (0.5 - 5 mM) to 5 mL of minimal salts medium pH 3.0 (section 2.2.2.2) in 25 mL universal bottles. These were inoculated with an active culture of strain RW11 and incubating at 30°C. After 7-14 days, OD_{600} and pH values were recorded.

Following on from this, a bioreactor was set up (section 6.3.1) to examine growth of isolate RW11 on pyruvate in more detail. The bioreactor vessel contained 1.5 L basal salts #1, trace

elements, 0.1 mM Fe^{2+} and 1 - 15 mM sodium pyruvate, and was maintained at 30ºC and pH 3.0. Growth of strain RW11 was determined by recording OD_{600} values.

6.3.3.2. Results

In the small volume, non pH-controlled universal bottle cultures, strain RW11 grew in the presence of 50 mM pyruvate, 20 mM lactate and 1 mM acetate. No growth was observed in the presence of 60 mM pyruvate, 20 mM lactate or 5 mM acetate, or in the presence of oxalic acid at any concentration tested. However, where positive growth occurred, it was accompanied by a large increase in the pH of the culture.

In the pH-controlled bioreactor set at pH 3.0, isolate RW11 grew on 10 mM but not on 15 mM sodium pyruvate. Under optimum conditions (pH 3.0, 30°C and 5 mM pyruvate) its culture doubling time was 3.4 h (corresponding to a growth rate of 0.25 h^{-1}).

6.3.4. Screening of isolate RW11 for oxidation of elemental sulfur, ferrous iron and hydrogen, and growth under anaerobic conditions

6.3.4.1. Methods

Aerobic oxidation of S^0 was examined by amending duplicate inoculated 5 mL cultures of minimal salts medium containing 1 mM fructose with ~5 mg sterile elemental sulfur in 25 mL universal bottles. Controls were not amended with sulfur. After 7 days of incubation at 30°C, turbidity (by optical density at 600 nm) and pH of each sample was measured. Ferrous iron oxidation was examined by amending 5 mL cultures of minimal salts medium (pH 2.5) with 1 mM fructose and 10 mM sterile $FeSO_4$ in 25 mL universal bottles. Ferrous iron concentrations in the cultures were measured using the Ferrozine assay (section 2.6.2) at days 0 and 7.

Screening for growth on hydrogen was carried out as described in section 2.2.2.4. In brief, duplicate inoculated cultures containing 5 mL minimal salts medium were amended with 0.5 µM fructose and sealed in a 2.5 L jar. This jar contained an aerobic atmosphere, and a H_2-generating mixture of chemicals which activated on addition of water. Duplicate inoculated controls were set up in tandem and incubated aerobically in the absence of hydrogen. Bacterial growth was estimated from OD_{600} measurements after 14 days incubation at 30°C.

Screening for fermentative growth was carried out by placing duplicate 5 mL cultures of inoculated fructose medium in 25 mL universal bottles (section 2.2.2.2) in an anaerobic jar (section 2.2.3) for 14 days. Growth was determined by recording OD_{600} values, and compared with those of fructose-free control cultures.

6.3.4.2. Results

Growth of isolate RW11 in the presence of elemental sulfur, ferrous iron or hydrogen was the same as that observed in control cultures. There was also no evidence of ferrous iron oxidation (accumulation of ferric iron) or sulfur oxidation (decrease in culture pH) in inoculated cultures. It was therefore concluded that strain RW11 neither grew on nor oxidised any of these three inorganic electron donors. As with other *Acidocella* spp., strain RW11 did not grow in the absence of oxygen via fermentation.

6.3.5. Isolate RW11 and the ability to perform dissimilatory reduction of Fe^{3+} under micro- and anaerobic conditions

6.3.5.1. Methods

Duplicate 50 mL flasks (anaerobic) or 100 mL flasks (micro-aerobic) containing 50 mL fructose liquid medium (section 2.2.2.2) were amended with 50 mg amorphous ferric hydroxide. The latter was prepared by adding 25 mM ferric sulfate to 2 L acidified RO water, autoclaving the liquor to cause precipitation of amorphous ferric iron hydroxide and filtering

the liquor through Whatman Grade 1 filter papers (Bridge, 1995). The solid material was washed with RO water, and ground to a fine powder. Duplicate flasks were inoculated with 0.5 mL RW11 culture ($4.06x10^8$ cells/mL) grown in fructose medium. Non-inoculated flasks containing the same medium were used as controls. Both sets of flasks were placed in 2.5 L atmosphere jars where either microaerobic (6% oxygen) or anaerobic atmospheres were generated using CampyGen® or AnaeroGen sachets® (Oxoid, UK), respectively. Samples were withdrawn on days 0, 4 and 8, and cell numbers (Thoma cell counting chamber) and ferrous iron concentrations (Ferrozine assay) recorded.

6.3.5.2. Results

Isolate RW11 catalysed the dissimilatory reduction of ferric iron. This was accompanied by growth under micro-aerobic conditions but not anaerobic conditions (Fig. 6.2). Ferrous iron concentration and cell numbers increased with time in these cultures, after a four day lag period. Under anaerobic conditions, ferric iron was reduced at a much slower rate than under micro-aerobic conditions with a shorter lag time. Cell numbers did not increase over time under anaerobic conditions. This observation and those of section 6.3.4 indicate that isolate RW11 is an obligate aerobe that can grow under micro-aerobic but not strictly anoxic conditions. However, strain RW11 can catalyse the dissimilatory reduction of ferric iron under microaerobic and anaerobic conditions.

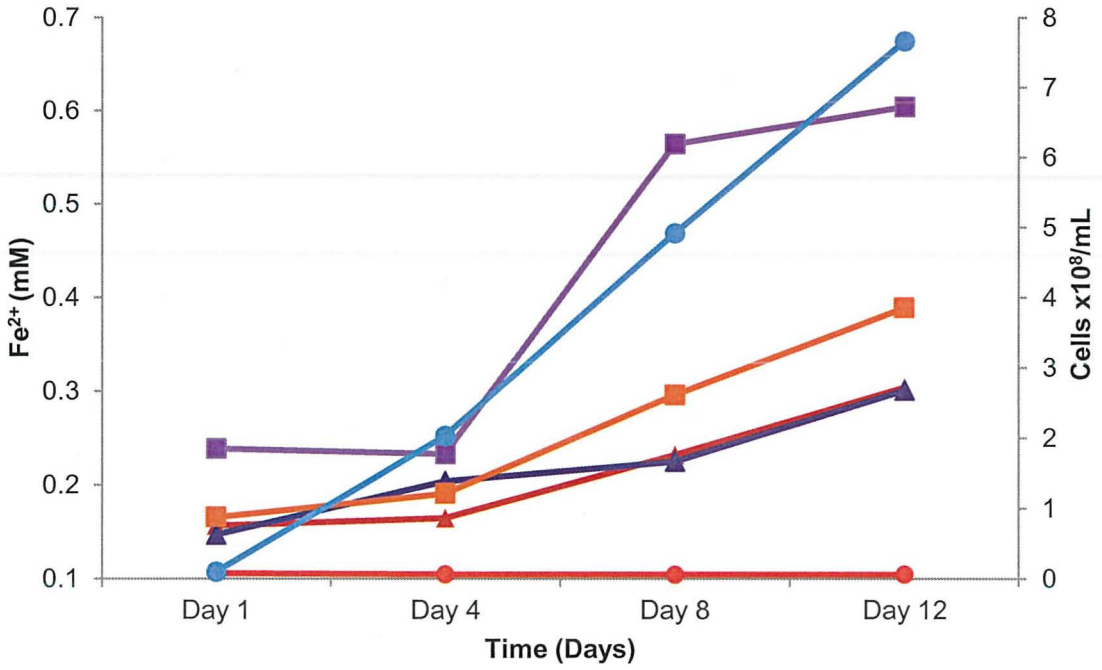


Figure 6.2. Dissimilatory ferric iron reduction (as increase in Fe^{2+}) and growth of RW11 (as cell numbers) under microaerobic and anaerobic conditions. Data points are mean values from duplicate cultures. Symbols are as follows; (▲) control Fe^{2+} concentrations under anaerobic conditions (no inoculum); (■) Fe^{2+} concentrations under anaerobic conditions; (●) cell numbers under anaerobic conditions; (▲) control Fe^{2+} concentrations under microaerobic conditions (no inoculum); (■) Fe^{2+} concentrations under microaerobic conditions; (●) cell numbers under microaerobic conditions.

6.3.6. Tolerance of isolate RW11 to some transition metals and aluminium

6.3.6.1. Methods

Fructose liquid medium (section 2.2.2.2) at pH 3.0 was amended with sterile metal sulfate solutions to give final concentrations of: Al^{3+} (100 - 600 mM), Cu^{2+} (1 - 40 mM), Mn^{2+} (100 - 500mM), Ni^{2+} (1 - 400 mM), Zn^{2+} (50 - 300 mM) and Fe^{2+} (50 - 200 mM; medium adjusted was at pH 2.5 to minimise precipitation of ferrous phosphate and chemical oxidation of ferrous iron). Universal bottles were inoculated with an active culture of strain RW11, incubated at 30°C for 7 days then growth assessed by measuring turbidity (OD_{600}). To

account for any loss of ferrous iron loss by precipitation and/or oxidation, ferrous iron concentrations were measured using the Ferrozine assay. Data were compared with those of the other *Acidocella* spp. (section 6).

6.6.3.2. Results

In general, tolerance of RW11 to certain transition metals and aluminium was similar to those of other *Acidocella* spp. (Table 6.2), with some exceptions.

Table 6.2. Comparison of metal tolerance of strain RW11 with those of the type strains of other *Acidocella* spp. Metal concentrations (in mM) are minimum inhibitory concentrations (MIC) and those in parenthesis are the highest concentration where growth was observed.

Metal	RW11	*Ac. aromatica*	*Ac. facilis*	*Ac. aminolytica*	*Ac. aluminiidurans*
[1]Fe^{2+}	49 (26)	195 (122)	151 (12)	57 (46)	164 (58)
Ni^{2+}	300 (200)	200 (100)	3 (1)	3 (1)	3 (1)
Al^{3+}	500 (400)	300 (200)	(300)	(300)	(500)
Cu^{2+}	10 (5)	5 (3)	(1)	20 (15)	(1)
Zn^{2+}	300 (200)	300 (200)	200 (100)	200 (100)	100 (50)
Mn^{2+}	300 (200)	200 (100)	(300)	200 (100)	(300)

[1] measured concentrations (Ferrozine assay) at day 7

Isolate RW11 was particularly tolerant of aluminium (minimum inhibitory concentration of 500 mM, which is similar to that of *Acidocella aluminiidurans*) and nickel (even more so than strains of *Ac. aromatica*). Like other *Acidocella* spp., it displayed much greater sensitivity to copper.

6.4. Determination of chemotaxonomic and molecular biological characteristics of isolate RW11 in comparison to previously described *Acidocella* spp.

6.4.1. Chemotaxonomic analysis of isolate RW11

6.4.1.1. Methods

RW11 biomass was grown in fructose liquid medium at 30°C, and sent to the DSMZ (*Deutsche Sammlung von Mikrooganismen und Zellkulturen*, Braunschweig, Germany), where its polar lipids, fatty acids, respiratory quinones and G+C content were determined.

6.4.1.2. Results

The chromosomal DNA content of isolate RW11 was G+C 61.58 mol%, which is similar to other described *Acidocella* spp. (1.17-2.88 mol% difference between isolate RW11 and the described *Acidocella*; section 6.4.1.2). The major quinone reported for isolate RW11 was Q-10, the major quinone reported for all other currently described *Acidocella* spp. (Jones *et al.*, 2013; Kishimoto *et al.*, 1993; Kishimoto *et al.*, 1995; Kimoto *et al.* 2010). The main polar lipids of isolate RW11 are: four different glycolipids; two aminolipids; diphosphatidylglycerol; phosphoglycerol; phosphatidylethanolamine and phosphatidylmonomethylethanolamine. Wichlacz *at al.*, (1986) and Jones *et al.*, 2013 (section 6.4.1.2) also reported four phospholipids and two aminolipids for *Ac. facilis* and *Ac. aromatica*. Kishimoto *et al.* (1993) reported that *Ac. aminolytica* had two ornithine amide lipids. The major fatty acids found in strain RW11 grown on fructose at 30°C were cyclo- $C_{18:1}$ ω7c and $C_{16:0}$.

6.4.2. Phylogenetic analysis of isolate RW11

6.4.2.1. Methods

Attempts at extracting DNA from harvested RW1 cells using the lysis (section 2.7.1.2), CTAB (section 2.7.1.1.) and lysozyme methods (section 2.7.1.3) were all unsuccessful. Template

DNA was successfully extracted from biomass grown in 100 mL fructose medium using an UltraClean Soil DNA kit (section 5.5.1).

The 16S rRNA gene of strain RW11 was amplified by PCR using unlabelled primers (section 2.7.2) and sent to Macrogen Inc. (Korea) for sequencing. The returned sequence was manually edited using Chromas lite and ClustalX, and then subjected to a BLAST analysis (http://ncbi.nlm.nih.gov/BLAST) to compare the 16S rRNA gene sequence similarity of isolate RW11 against others in the NCBI sequence database. An alignment containing this sequence and those of other acidophilic heterotrophic bacteria (trimmed to 1236 bp) was then constructed and bootstrapped (x1000) maximum likelihood, maximum parsimony and neighbour-joining trees were generated from this alignment as detailed in section 2.7.6. As tree topologies were nearly identical, only the neighbour-joining tree is shown.

6.4.2.2. Results

The accession number of the 16S rRNA gene sequence of isolate RW11, designated type strain (and currently the sole representative) of "*Acidocella acidivorans*" is KP216527. Its closest relative, based on 16S rRNA gene sequence similarity, is *Ac aluminiidurans* (97% 16S rRNA gene sequence similarity). Gene similarity values to other *Acidocella* spp. are 94-6%. Since a value of >97% 16S rRNA gene sequence similarity is considered the threshold to distinguish separate species (Tindall *et al.*, 2010) the case for a novel species designation is strong, particularly as "*Ac. acidivorans*" and *Ac. aluminiidurans* are very different in many physiological traits.

BLAST analysis revealed that isolate RW11 shared 99% gene sequence similarity with some undescribed isolates such as isolate CCW30, an *Acidocella* strain also previously isolated from Trefriw Spa (Hallberg *et al.*, 2006). Isolates of 99% gene sequence similarity were also isolated from Mynydd Parys, North Wales (isolate Py-H1; Kay *et al.*, 2013), a copper mine, Norway (Isolate NO-12; Johnson *et al.*, 2001), an AMD lake, Germany (Isolate YE4-N1-5-

CH; Lu *et al.*, 2010) and a related clone was obtained from a pit lake in Spain (CN7m-bac_f1; Santofimia *et al.*, 2013).

Fig. 6.3 shows the constructed phylogenetic tree, which places isolate RW11 in the *Acidocella* group. The tree also clearly places isolate RW11 on its own lineage within the genus, indicating that is sufficiently distinct from the other *Acidocella* spp. to be considered a separate species (bootstrap value 1000).

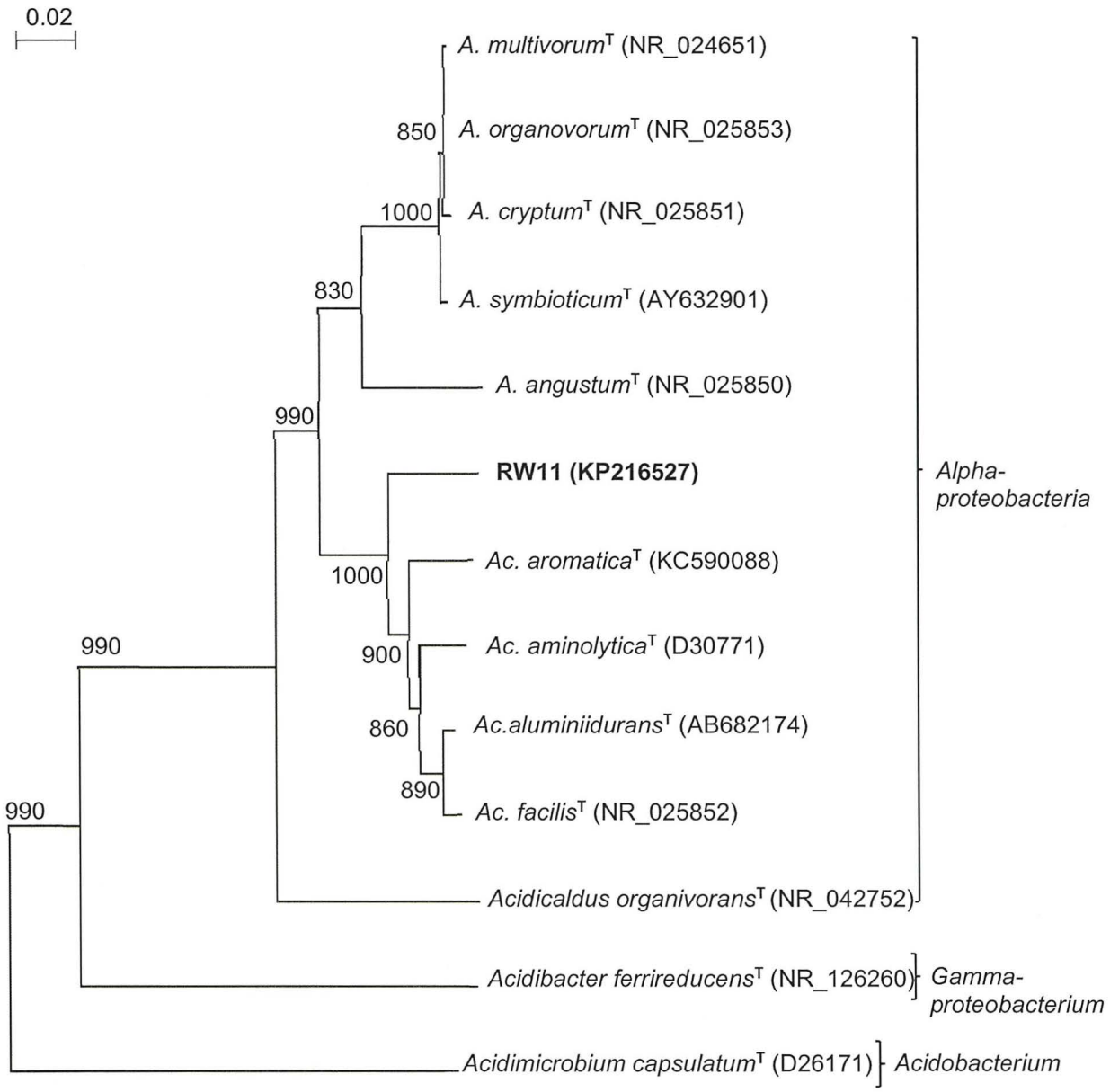


Figure 6.3. Bootstrapped (x1000) neighbour-joining phylogenetic tree showing isolate RW11, the designated type species of the proposed novel species "*Ac. acidivorans*" in relation to all currently described *Acidocella* species, and other extremely acidophilic heterotrophs of the *Proteobacteria* and *Acidobacteria*. The tree was rooted with *Ferroplasma acidiphilum* (GenBank accession number AJ224936; not shown). Bootstrap values appear on their respective nodes. Values in parenthesis are GenBank accession numbers.

6.5. Discussion

Results from this part of the study showed that strain RW11, isolated from the Trefriw Wells spa, has physiological and phylogenetic characteristics that distinguish it from other heterotrophic acidophiles, including other currently described species of the genus *Acidocella*. The proposed name of the new species is "*Ac. acidivorans*", reflecting its propensity to grow on a variety of small molecular weight aliphatic acids.

Growth on small molecular weight aliphatic acids was the most notable physiological trait identified in strain RW11. As shown in Table 6.1, it grew in cultures with no pH regulation on acetic, glycolic, propionic, pyruvic, butyric, lactic and citric acids. None of the other *Acidocella* spp. were able to grow on any of these organic acids except *Ac. aromatica*, which was able to grow on acetic acid. Small molecular weight aliphatic acids are generally highly toxic to acidophiles, due to the pH of environments in which acidophiles grow being close to or below the pK_a values of monovalent organic acids, where the latter exist predominantly in their undissociated, lipophilic forms. This is discussed further in chapter 7.

The ability of strain RW11 to tolerate and grow on pyruvic acid at concentrations that are either inhibitory or lethal to other acidophiles (including many iron-oxidising chemolithotrophs of commercial importance) is particularly remarkable. The maximum growth rate of isolate RW11 growing on pyruvate in a bioreactor was 0.25 h^{-1}. Under the same (optimum) conditions and experimental set-up but with 5 mM fructose, its growth rate was 0.36 h^{-1}. In contrast, while *Ac. aromatica*^{T} had a faster growth rate than isolate RW11 on fructose (maximum of 0.41 h^{-1}), it grew much more slowly on acetic acid (growth rate of 0.007 h^{-1} at pH 3.5 and 5 mM acetic acid/acetate) (chapter 7). In non-pH-regulated culture, strain RW11 grew in the presence of 50 mM pyruvate. However, the addition of sodium pyruvate will have increased the initial pH of the media in the following ways; (i) the substrate was added (to pH 3 basal salts) from a circum-neutral pH solution of sodium pyruvate, increasing the start pH

value, and (ii) the pH of the culture increased dramatically during growth, as predicted by equation [6.1]:

$$CH_3COCOO^- + Na^+ + 2.5\ O_2 \rightarrow 3\ CO_2 + H_2O + Na^+ + OH^- \quad [6.1].$$

The pH increase would have induced dissociation of the relatively toxic acid form to the less toxic (and presumably metabolisable) pyruvate anion. Strain RW11 also grew on pyruvate in bioreactor culture, where pH was controlled and its sensitivity to pyruvic acid more accurately assessed (Table 6.3). This was still an approximate order of magnitude greater than the tolerance of most acidophiles (Rawlings and Johnson, 2004).

Table 6.3. Summary of the undissociated acid tolerance of isolate RW11 for some small-weight aliphatic organic acids. Calculations based on pK_a values of the acids and the pH at which the tests were carried out (pH 3).

Organic acid	pK_a (s)	MIC (mM)	Maximum concentration for positive growth (mM)
Pyruvic	2.50	14.42	12.02
Pyruvic**	2.50	3.61	2.40
Lactic	3.08	13.64	9.09
Acetic	4.75	2.48	0.50
Oxalic	1.25, 4.14	0.45	ND

** bioreactor culture (all other data refer to batch cultures in universal bottles with no pH regulation)

Strain RW11 grew on acetate but was more sensitive to this aliphatic acid than *Ac. aromatica*, which had the highest tolerance yet described to acetic acid of any extremely

acidophilic bacterium (chapter 7). Strain RW11 could also grow on some aromatic compounds such as phenol, benzoate and benzyl alcohol, an ability also identified in *Ac. aromatica,*. Metabolism of aromatic compounds appears to be more widespread among *Acidocella* spp. than other genera of heterotrophic acidophiles, as all currently described *Acidocella* spp. also metabolise benzyl alcohol (chapter 7). Strain RW11 did not grow on yeast extract or tryptone, which is also similar to *Ac. aromatica*. It did not grow in oxalic acid, as with all currently known acidophiles. This organic acid was included in the analysis as pyruvic acid was also previously thought to be toxic above micro-molar concentrations to extremely acidophilic bacteria.

Glycolic acid (hydroxyacetic acid) a C_2 acid, was another notable aliphatic acid that supported the growth of strain RW11. Ňancucheo and Johnson (2010) identified this aliphatic acid in cultures of the chemolithotrophic acidophiles *L. ferriphilum, At. ferrooxidans* and *At. caldus*, possibly as a waste product of RuBisCO activity (oxidation of ribulose bisphosphate to phosphoglyceric acid and phosphoglycolate, then enzymatic hydrolysis of the phosphoglycolate to glycolate, which is exported of out the cells). They identified some *Firmicutes* (chiefly *Sulfobacillus* spp., which are not present at Trefriw Wells Spa) as the only extremely acidophilic organic carbon-metabolising bacteria and archaea that could metabolise this organic acid. The observation that strain RW11 could also grow on this aliphatic acid is therefore significant.

The ability to metabolise organic acids that are toxic to many extremely acidophilic bacteria in relatively small concentrations can be useful (This is also discussed in chapter 7). This trait is used in the design of "overlay" plates, as described in chapter 2. *Acidiphilium* SJH was used mostly in overlay media designed for acidophiles because of its tolerance of extremely low pH (pH less than 2) conditions. However, *Acidiphilium* spp. (and most *Acidocella* spp.) metabolise yeast extract, a complex substrate often included in solid media that are used to isolate heterotrophic acidophiles, including sulfate reducers. Solid media with *Ac. aromatica* as the under-layer component were designed (Johnson and Hallberg,

2007) because this *Acidocella* sp. left the yeast extract available for the microorganisms while degrading micromolar concentrations of pyruvic acid (identified as probably the most important inhibitory compounds in low pH agarose gelled media; Rawlings and Johnson, 2004). The characteristics of isolate RW11 make potentially useful in this regard.

Another characteristic that identified isolate RW11 as different from the other *Acidocella* spp. was that cells appeared refractile (white) when viewed under the phase contrast microscope. All other *Acidocella* spp., were observed as typical (for small bacteria) dark blue "coloured" cells. Strain RW11 was also recalcitrant to DNA extraction of from harvested biomass, except by MIOBIO soil DNA kit. Whether these observations are linked in some way is unclear. It could be that the cell wall structure of strain RW11 differs from that of other *Acidocella* spp., though this was not investigated in the present study.

Metal tolerance in strain RW11 was also interesting. Its tolerance of aluminium was exceptionally high, close to that of *Ac. aluminiidurans* (named for this particular trait). However, its tolerance to nickel exceeded even that of *Ac. aromatica*, the most Ni-tolerant heterotrophic acidophile reported to date. Isolate RW11 also had a relatively low tolerance to copper, in common with all other described *Acidocella* spp. (MIC 5 mM compared to 3-20 mM for the other currently described *Acidocella* spp.).

In conclusion, isolate RW11 is proposed as the type strain of novel species "*Acidocella acidivorans*", which refers to its ability to metabolise organic acids. It is considered novel because of its 16S rRNA gene sequence similarity of 97% to *Ac. aluminiidurans*, which is within the recommended species threshold of 97% (Tindall, 2010). It also possesses novel phylogenetic characteristics that differentiate it from the other *Acidocella* species, including the ability to metabolise relatively high concentrations of some organic acids. These characteristics show that this proposed novel species contributes to the Trefriw Wells Spa community by reducing iron and metabolising waste organic acids that are toxic to other community members. Isolates of this bacterium in particular were not identified at the Cae

Coch site in previous studies though *Acidocella* spp are present (Hallberg *et al.*, 2006; Kimura *et al.*, 2011), so it is not clear whether RW11 is present across the site or confined to the Trefriw Wells Spa section of the system.

6.6. Description of the proposed novel species, "*Acidocella acidivorans*"

Acidocella acidivorans [a.ci.di.vo'rans]. N.L. n. ***acidus*** an acid; L. part. adj. ***vorans*** eating, devouring; N.L. part. adj. ***acidivorans*** acid-devouring.

Cells are irregular rods, 2-3 µm long, 0.4-0.8 µm wide and motile. They are Gram-negative, and do not form endospores. Colonies are white, smooth, entire, domed and round/convex on acidic (pH 3) solid media. Mesophilic (optimum 30°C, maximum 37°C) and extremely acidophilic (optimum pH 3.0, minimum 2.4). Obligately heterotrophic, growing on glucose, fructose, mannose, glycerol, ethanol, benzyl alcohol and sodium benzoate, but not complex organic substrates such as yeast extract. Strains also use many aliphatic acids, including butyric acid, pyruvic acid, glycolic acid, glutamic acid, lactic acid and propionic acid. Aerobic, capable of ferric iron reduction under microaerobic conditions. No growth by fermentation or other anaerobic means. Quinones are Q-9 (8%) and Q-10 (92%). Major fatty acids of type strain RW11 are cyclo- $C_{18:1}$ ω7c and $C_{16:0}$. Its main polar lipids are: four different glycolipids; two aminolipids; diphosphatidylglycerol; phosphoglycerol; phosphatidylethanolamine and phosphatidylmonomethylethanolamine. Chromosomal DNA base composition of type strain RW11 is 61.58 mol% G+C. 16S rRNA gene sequencing (GenBank accession number KP216527) places "*Acidocella acidivorans*" in phylum *Proteobacteria*, subclass *alpha-proteobacteria*. Type strain RW11 (=DSM 100119, = NCCB 100557) was isolated from a cave at Trefriw Spa, North Wales.

Chapter 7. *Acidocella aromatica* sp. nov.: an acidophilic heterotrophic *alphaproteobacterium* with unusual phenotypic traits

7.1. Introduction

Three isolates (coded WJB-3, LGS-3 and PFBC) shared 99% sequence similarities of their 16S rRNA genes and were affiliated to the *Acidocella* genus. They were identified and partially characterised prior to the present study (Kimura *et al.*, 2006; Hallberg *et al.*, 1999; Gemmell and Knowles, 2000). Strain WJB-3 was isolated from an acid mine drainage in Cornwall (at the former Wheal Jane tin mine), strain LGS-3 from an acidic geothermal site on Montserrat (West Indies). Strain PFBC was isolated from a mixed (anaerobic) enrichment culture inoculated with sediment from the abandoned Mynydd Parys copper mine in north Wales, which also included an uncharacterised *Desulfosporosinus* sp. (strain PFB). The three strains were reported as having a relatively high tolerance to some small molecular weight organic acids, and could grow on some aromatic compounds. These are unusual traits among extremely acidophilic bacteria (chapter 6). These three isolates were proposed as strains of a novel species of *Acidocella* ("*Ac. aromatica*") and were fully characterised and named in the present study.

Acidocella spp. are indigenous to the Trefriw Wells Spa and Cae Coch waters (Hallberg *et al.*, 2006; chapter 5; Kimura *et al.*, 2011), though these strains are a different species to *Ac. aromatica*. However, it was considered pertinent to study the previously non-validated *Ac. aromatica* within the context of the present study as isolates of the *Acidocella* genus from Trefriw Wells Spa used organic acids and some aromatic compounds (chapters 5 and 6), which is one of the defining traits of *Ac. aromatica*. This acidophile also has an important function in sulfidogenic bioreactors that have been developed to selectively remove transition metals from acid mine drainage (Ňancucheo and Johnson, 2011).

7.2. Bacteria and culture conditions

Strain PFBC was originally isolated on a low pH solid medium incubated anaerobically, where colonies were observed growing adjacent to those of *Desulfosporosinus* PFB (Kimura and Johnson, 2004). It was isolated and grown under aerobic conditions in fructose liquid medium (section 2.2.2.2), and maintained as an active and cold-stored culture at the *Bangor Acidophile Culture Collection*. Strains WJB-3 (NCIB 13670) and LGS-3 (NCIB 13671), *Ac. facilis*^{T} (NCCB 86038; isolated from acidic coal mine drainage, Pennsylvania USA.), *Ac. aminolytica*^{T} (DSM 11237; isolated from acid mine drainage, Okayama, Japan) and *Ac. aluminiidurans*^{T} (NBRC 10430; isolated from waterweed growing in an acidic swamp, Can Tho, Vietnam) were obtained from national culture collections (the National Collections of Industrial and Marine Bacteria (Aberdeen, Scotland), the Netherlands Culture Collection of Bacteria (The Netherlands), the National Collection of Technology and Evaluation (Japan) and the DSMZ, Germany), and maintained in fructose or glucose media (section 2.2.2.2).

Frozen stocks of *Acidocella* strain WJB3, *Acidocella* strain LGS3, *Ac. facilis*, *Ac. aminolytica* and *Ac. aluminiidurans* were created, using glycerol and dimethylsulfoxide (DMSO) as cryoprotectants (section 2.3).

7.3. Determination of physical characteristics of isolate PFBC in comparison to previously-described *Acidocella* spp.

7.3.1. Effect of pH and temperature on the growth of strain PFBC

7.3.1.1. Methods

Strain PFBC was grown in a 2 L bioreactor fitted with the following: pH control (internal pH electrode, Electrolab control box, adjusted by addition of sterile 0.1 M NaOH or 0.1 M H_2SO_4), temperature control (Electrolab control box, temperature probe and heating unit), aeration (~1 L air/min), and internal stirring (100 rpm). The reactor vessel contained 1.5 L

fructose medium at between pH 2.5 and 5.0 (with temperature fixed at 30°C), or between 22.5°C and 37.5°C (pH fixed at 3.5).

Samples were taken at regular intervals, and turbidity measured from optical densities at 600 nm (OD_{600}) against distilled water blanks. pH was also measured with an external pH electrode (section 2.6.1) and the bioreactor values adjusted when necessary. Data were graphed as semi-logarithmic plots of optical density against time to calculate mean generation times and growth rates from the linear exponential phases of growth that were identified.

To assess the ability of strain PFBC to grow outside of the above pH and temperature ranges, duplicate 250 mL conical flasks containing 100 mL of fructose medium were incubated (unshaken) at 42°C and 47°C for 7 days, or at 15°C and 7°C with shaking for 14 days. Samples were removed at regular intervals and treated as above, to determine mean generation times and growth rates. Minimum growth pH was determined in a similar way, with pH of media adjusted to pH of 2.0 to 2.6, and incubated cultures shaken at 30°C.

7.3.1.2. Results

Optimum growth of strain PFBC occurred at pH 3.8 (Fig. 7.1 (a)). Temperature optimum was 30°C (Fig. 7.1 (b)). Under optimum conditions (pH 3.8 and 30°C), the culture doubling time of strain PFBC was 1.71 h, with a growth rate of 0.41 h^{-1}.

In conical flasks, strain PFBC grew at 15°C but not 7°C, and did not grow above 42°C. The lowest pH at which this isolate would grow was 2.4.

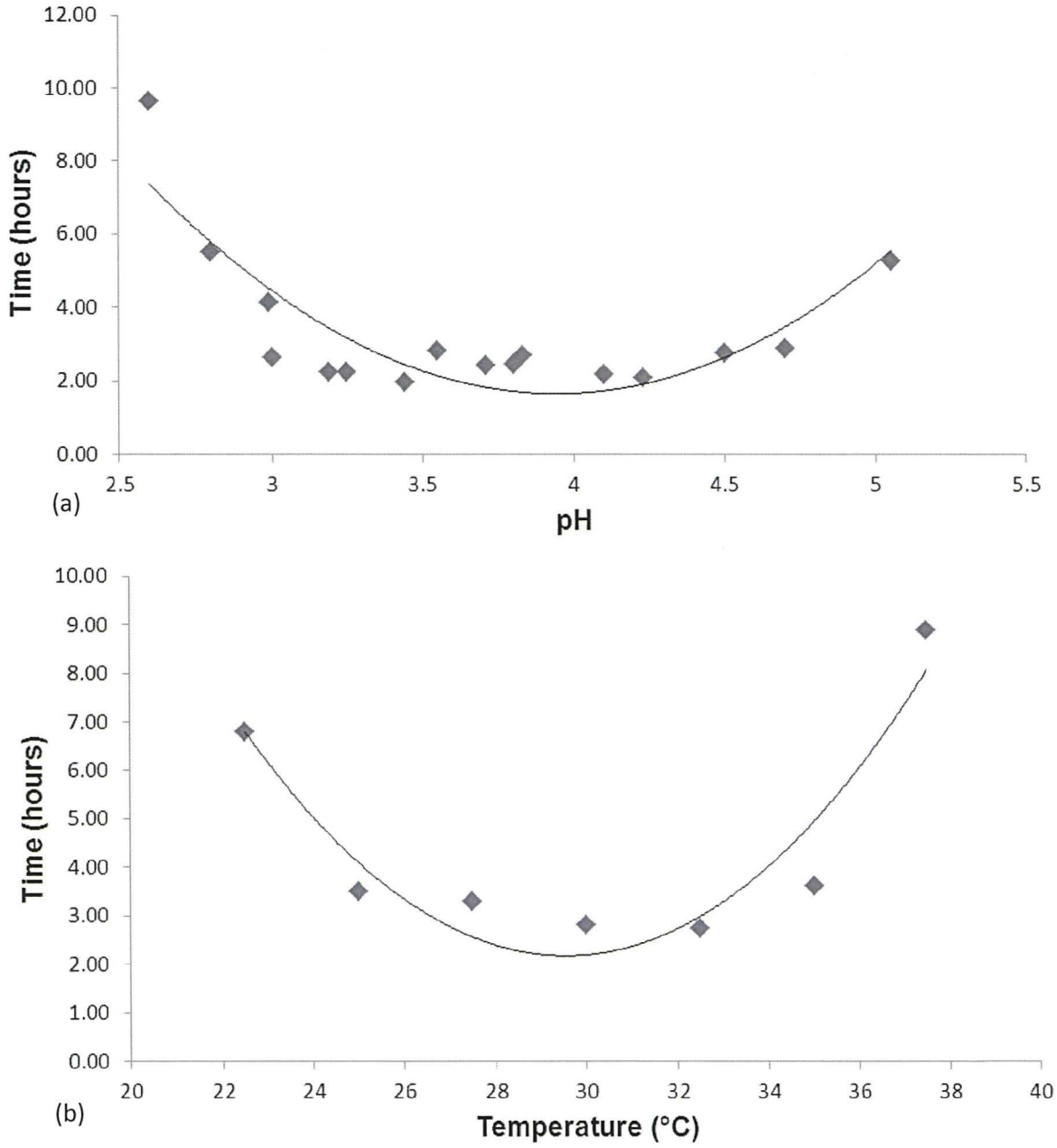


Figure 7.1. Culture doubling times of strain PFBC in a bioreactor at (a) pH 2.5 to 5.0 (at 30°C) and (b) 22 to 38ºC (at pH 3.5).

7.3.2. Determination of the organic substrates metabolised by Acidocella spp.

7.3.2.1. Methods

To test growth of strains PFBC, WJB3 and LGS-3 on various substrates, duplicate cultures of minimal salts medium at pH 3.0 (section 2.2.2.2) were amended with one of the organic

compounds listed below (at different concentrations to give similar carbon equivalents) and incubated, shaken, at 30˚C:

i) *Carbohydrates*: cellobiose and sucrose (disaccharides; 5 mM); galactose, glucose, fructose, mannose, sorbose (hexoses; 5 mM); arabinose, rhamnose ribose and xylose (pentoses; 5 mM); glucosamine (sugar amine; 5 mM), erythrulose (C_4 carbohydrate; 8 mM); glyceraldehyde (both at 8 mM), dihydroxyacetate (10 mM);

ii) *Organic/amino acids*: butyric acid, citric acid, pyruvic acid, glucuronic acid, glutamic acid (all at 5 mM), lactic acid, propanoic acid, glycine (all at 10 mM);

iii) *Alcohols:* mannitol (10 mM), sorbitol (5 mM), glycerol (10 mM), ethanol (15 mM), methanol (20 mM);

iv) *Aromatic compounds*: benzyl alcohol, phenol (both at 5 mM), sodium benzoate (tested at both pH 3.0 and 4.0, and at both1 and 5 mM);

v) *Complex organic substrates*: tryptone, yeast extract (both at 0.02%, w/v),

After 14 days, the optical density of samples of the cultures was recorded (OD_{600} values) and compared with those of negative controls (no organic additions) and positive controls containing 5 mM fructose.

Where the published literature disagreed with each other on substrates used by validated species of *Acidocella,* minimal salts medium was amended with the organic compounds in question. Tests with validated *Acidocella* spp. were also carried out using the aromatic compounds listed above. These cultures were then treated as described previously, except that positive controls contained 5 mM glucose rather than fructose.

7.3.2.2. Results

Strain PFBC grew using a restricted range of substrates at the concentrations supplied (fructose, sucrose and acetic acid), including some aromatic compounds (phenol, benzoate and benzyl alcohol). Strains WJB-3 and LGS-3 used a wider substrate range, which included ethanol and lactic acid (Table 7.1). OD_{600} values for cultures grown on sucrose was

equivalent to those of the fructose control (both substrates added at 5 mM), indicating that this strain used the fructose portion of the glucose-fructose disaccharide only. Since strain PFBC used fructose but not glucose, it was hypothesised that it might metabolise ketoses but not aldose sugars. This was not found, as strain PFBC did not use any other ketose tested.

Table 7.1. Growth of *Acidocella* spp. on different organic substrates. + indicates growth, (+) indicates limited growth, - indicates no growth and blank cells indicate no available data.

Substrate	*Ac. aromatica*			*Ac.*	*Ac.*	*Ac.*
	PFBC	WJB-3	LGS-3	*facilis*	*aminolytica*	*aluminiidurans*
Cellobiose	-	-	-	-	-	-
Galactose	-			+	+	+
Glucose	-	-	-	+	+	+
Fructose	+	+	+	+[3]	+[4]	-[5]
Mannose	-			+[3]	-[5]	-[5]
Sorbose	-					
Sucrose	+	+	+	+	+	+
Arabinose	-			-[5]	-	-[5]
Rhamnose	-			-[3]	-[4]	-[5]
Ribose	-			+[3]		
Xylose	-			+[3]	+[4]	
Erythrulose	-					
Glyceraldehyde	-					
Dihydroxyacetate	-					
Acetic acid	+	+[1]	+[1]	-[3]	-[4]	-[5]
Butyric acid	-					
Pyruvic acid	-	-	-			

Citric acid	-			-[3]	-[4]	-[5]
Glucuronic acid	-			-[3]	-[4]	-[5]
Glutamic acid	-			-[3]	-[4]	+[5]
Glucosamine	-					
Lactic acid	-	+	+	-[3]	-[4]	-[5]
Propanoic acid	-	+[1]	+[1]	-[3]	-[4]	-[5]
Glycine	-			-[3]		
Mannitol	-	-[2]	-	+	+[4]	+
Sorbitol	-			-[3]	+[4]	-[5]
Glycerol	-			+[3]	-[4]	-[5]
Ethanol	-	+	+	+	+	+
Methanol	-			-[4]	-[4]	
Phenol	+	+	-	-	-	-
Benzyl alcohol	+	+	+	+	+	+
Sodium benzoate	+	+	+	-	+	-
Tryptone	-	-	-	+	+	-
Yeast extract	-	-	-	+	+	+

[1]Gemmell and Knowles (2000); [2]Hallberg *et al.* (1999); [3]Wichlacz *et al.* (1987); [4]Kishimoto *et al.* (1993); [5]Kimoto *et al.* (2010).

Similar OD_{600} values were obtained with cultures of PFBC grown on 1 mM and 5 mM sodium benzoate at pH 3.0. At pH 4.0, much greater (about 10-fold) OD_{600} values were recorded, and these correlated with the concentration of benzoate supplied (Table 7.2).

Table 7.2. Growth of strain PFBC in shake flasks containing 1 mM or 5 mM sodium benzoate at pH 3.0 and 4.0.

Benzoate (mM)	OD_{600}	
	pH 3.0	pH 4.0
1.0	0.016	0.158
5.0	0.023	0.518

Growth on sodium benzoate was accompanied by an increase in culture pH, as predicted by equation [7.2], since CO_2 (rather than bicarbonate) is generated in acidic liquors:

$$Na^+ + C_7H_5O_2^- + 7.5\ O_2 \rightarrow 7\ CO_2 + 2\ H_2O + Na^+ + OH^- \qquad [7.2]$$

Further investigation of this phenomenon and the effect of bacterial growth using acetic acid and sodium benzoate are described below.

7.3.3. Growth of strain PFBC on benzoate and acetate

7.3.3.1. Methods

An initial investigation into growth of strain PFBC on sodium benzoate used duplicate 250 mL flasks containing 100 mL substrate free medium amended with 1 mM sodium benzoate at pH 3.0 and 4.0, inoculated with fructose-grown PFBC and incubated with shaking at 30˚C. Samples were removed at regular intervals for cell enumeration, OD_{600} measurement (both to determine biomass), and pH measurement. A bioreactor was also set up as described in section 7.3.1, with sodium benzoate (at 1.0 – 5.0 mM) as sole carbon and energy source. pH was at either pH 4.0 or 5.0 and temperature maintained at 30°C. The reactor was inoculated with a shake flask culture of strain PFBC grown on 5 mM benzoate at pH 4.0. Samples were removed at regular intervals and analysed for turbidity and pH.

The toxicity of acetic acid to strain PFBC was initially examined in shake flask cultures containing either fructose medium (pH 3.0) and amended with either 10 or 20 mM acetic acid. To examine growth on acetate, a bioreactor culture was set up at either pH 3.5 or 5.0 (at 30°C) containing acetic acid (10 or 20 mM) as sole carbon and energy source. This was inoculated with a culture pre-grown in 10 mM acetate liquid medium. Samples were withdrawn and turbidity (OD_{600}) and pH determined.

7.3.3.2. Results

In flask cultures, strain PFBC grew in medium containing 1 mM sodium benzoate initially at pH 4.0, but not at pH 3.0. Strain PFBC was noted to grow in liquid medium containing up to 2.5 mM sodium benzoate at pH 5 in the fixed pH bioreactor but not at pH 4.0, though again it grew on 1 mM sodium benzoate at pH 4 (Table 7.3).

Table 7.3. Growth of strain PFBC on benzoate and acetate in fixed pH bioreactor cultures (ND, not determined).

Benzoate/benzoic acid				
	1 mM	2.5 mM	4 mM	5 mM
pH 4.0	+	-	-	ND
pH 5.0	+	+	-	-
Acetate/acetic acid				
	10 mM		20 mM	
pH 3.5	+		-	
pH 5.0	+		+	

In shake flask cultures, strain PFBC grew in fructose medium (initially at pH 3) containing 10 mM acetic acid/acetate, but not in the same medium containing 20 mM acetic acid/acetate.

However, as there was no pH control in the flasks the acetic acid/acetate concentration in the (initially) 10 mM acetic acid flask would change as the pH did.

In the pH-controlled bioreactor (which allowed undissociated:dissociated acetic acid ratio to remain constant) strain PFBC grew in medium containing 20 mM combined acetate/acetic acid (as sole carbon and energy source) at pH 5. It also grew in medium containing 10 mM acetic acid/acetate, at pH 3.5, but it did not grow at pH 3.5 with 20 mM acetic acid/acetate (Table 7.3). It was also observed that culture-doubling times on acetate (~70 h) were far longer than for growth on fructose.

7.3.4. Dissimilatory reduction of ferric iron by strain PFBC under anaerobic and micro-aerobic conditions, and screening for growth under anaerobic conditions

7.3.4.1. Methods

Growth of strain PFBC coupled to the dissimilatory reduction of ferric iron was examined using the mineral schwertmannite ($Fe_8O_8(OH)_6(SO_4)$) as ferric iron source. Results from experiments using soluble ferric citrate (duplicate 25 mL universal bottles containing inoculated 15 mL fructose medium amended with ferric citrate solution to give 0, 5, 20 and 50 mM concentrations, incubated in microaerobic or anaerobic conditions (section 2.2.3)) were found to be non-reproducible.

Duplicate 50 mL conical flasks containing 50 mL fructose medium (section 2.2.2.2) and 50 mg dry weight schwertmannite (($Fe_8O_8(OH)_6(SO_4)$; kindly supplied by Dr. Catherine Kay, Bangor University) were inoculated with 0.5 mL fructose-grown culture (approximately 8.0 x 10^9 cells/mL). Two identical but non-inoculated flasks were also set up as controls. All of the flasks were placed into 2.5 L jars containing micro-aerobic or anaerobic atmospheres (section 2.2.3). Samples were removed at days 0, 5 and 11 so that numbers of planktonic cells (Thoma counting chamber; section 2.4.3) and concentrations of ferrous iron (Ferrozine

assay; section 2.6.2) could be measured. A second anaerobic experiment was set up, again using schwertmannite as the source of ferric iron, using a large inoculum of strain PFBC (6.3 x 10^{10} cells). Biomass was harvested from a 100 mL fructose-grown culture (section 2.5.1) and resuspended in 0.5 mL sterile basal salts medium (8 mL/L basal salts #1 in RO water).

To screen strain isolate PFBC for anaerobic growth by fermentation, duplicate 25 mL universal bottles containing 5 mL fructose medium but no ferric iron were inoculated, placed in a 2.5 L anaerobic jar (AnaeroGen™ AN25 system; section 2.2.3) and incubated at 30°C. To screen for anaerobic growth of strain PFBC by dissimilatory reduction of sulfur, duplicate cultures of 5 mL substrate free medium amended with 0.5% (w/v) elemental sulfur and 2.5 mM zinc sulfate (to act as a sink of any H_2S generated) were incubated under anaerobic conditions at 30°C.

7.3.4.2. Results

Strain PFBC did not grow anaerobically by fermentation of fructose or by oxidation of fructose coupled to the dissimilatory reduction of elemental sulfur. Strain PFBC grew by dissimilatory reduction of ferric iron under micro-aerobic conditions (both cell numbers and ferrous iron concentration increased in parallel). This acidophile also catalysed the reduction of ferric iron under anaerobic conditions (Fig. 7.2), but did not grow (cell numbers decreased, possibly due to settling on the schwertmannite mineral). Approximately six-fold more reductive dissolution of schwertmannite was observed with the larger biomass inoculum, which also showed the same trends observed with the smaller inoculum.

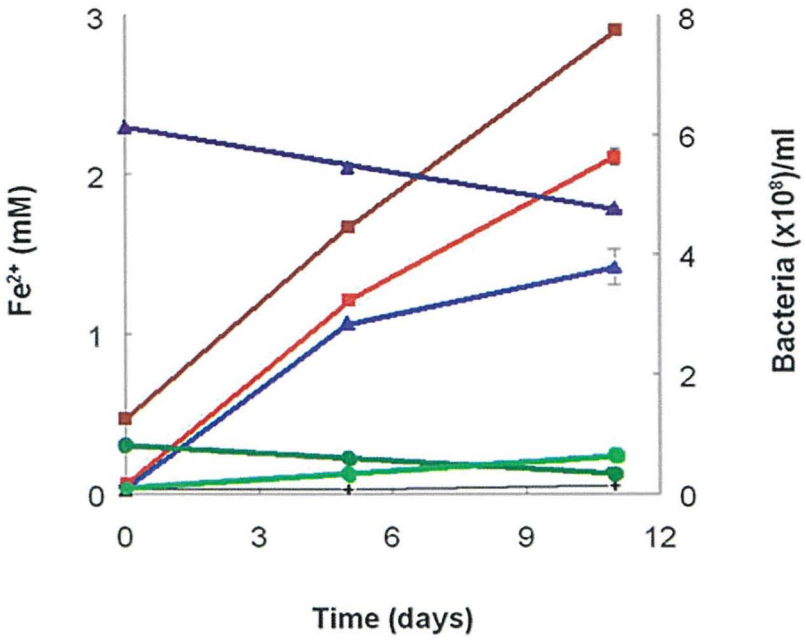


Figure 7.2. Graph of strain PFBC growth (as cell numbers) and reductive dissolution of the ferric iron mineral schwertmannite under anaerobic and microaerobic conditions, with schwertmannite amended medium. Symbols: (+), control Fe^{2+} concentrations; (■), Fe^{2+} concentrations under microaerobic conditions (small cell inoculum); (■) cell numbers under microaerobic conditions (small cell inoculum); (▲), Fe^{2+} concentrations under anaerobic conditions (large cell inoculum); (▲) cell numbers under anaerobic conditions (large cell inoculum); (●) Fe^{2+} concentrations anaerobic conditions (small cell inoculum) and; (●) cell numbers under anaerobic conditions (small cell inoculum). Data points are mean values from duplicate cultures. Error bars, where seen, depict standard deviations.

7.3.5. Tolerance of Acidocella spp. to some transition metals and aluminium

7.3.5.1. Methods

Liquid medium (pH 3.0) containing 5 mM fructose (strains PFBC, LGS-3 and WJB-3) or 5 mM glucose (*Ac. facilis, Ac. aminolytica* and *Ac. aluminiidurans*) was amended with metal sulfate standard solutions to give final concentrations of Al^{3+} (100-600 mM), Cu^{2+} (1-20 mM), Mn^{2+} (100-500 mM), Ni^{2+} (1-400 mM), Zn^{2+} (50-300 mM) and Fe^{2+} (100-200 mM) (at pH 2.7 to minimize chemical oxidation to ferric and precipitation of ferrous/ferric phosphates). Cultures were incubated at 30°C for 7 days, OD_{600} recorded, and ferrous iron measured

using the Ferrozine assay to determine the amount remaining in solution as some ferric phosphate precipitation had occurred over the 7 days of incubation.

7.3.5.2. Results

Table 7.4 shows the highest tested concentrations at which *Acidocella* spp. was observed to grow in this study, and the minimum inhibitory concentration (MIC) of each.

Table 7.4. Comparison of metal tolerances of *Acidocella* spp. as shown by minimum inhibitory concentrations (MIC). Values in parentheses indicate the highest tested concentrations that gave positive results.

	Bacterium					
Metal	PFBC	LGS-3	WJB-3	*Ac. facilis*	*Ac. aminolytica*	*Ac. aluminiidurans*
Fe^{2+*}	172 (89)	160 (70)	155 (60)	206 (104)	166 (100)	172 (108)
Ni^{2+}	200 (100)	3 (1)	200 (100)	3 (1)	3 (1)	3 (1)
Al^{3+}	300 (200)	400 (300)	300 (200)	400 (300)	400 (300)	600 (500)
Cu^{2+}	5 (3)	3 (1)	5 (3)	3 (1)	20 (15)	3 (1)
Zn^{2+}	300 (200)	300 (200)	300 (200)	200 (100)	200 (100)	100 (50)
Mn^{2+}	200 (100)	300 (200)	500 (400)	400 (300)	200 (100)	400 (300)

* Values were measured by Ferrozine assay after 7 days, as there was some precipitation.

All of the *Acidocella* spp. displayed similar tolerances to the metals tested except for nickel, where the three strains *Ac. aromatica* displayed a much greater tolerance than the other *Acidocella* spp. (MIC of 200 mM as opposed to 3-5 mM shown by the previously described spp.). All strains were also sensitive to copper, with *Ac. aminolytica* showing the greatest tolerance to this metal (MIC of 20 mM). All strains were also highly tolerant of aluminium, with *Ac. aluminiidurans* showing the highest tolerance of all the *Acidocella* spp. tested.

7.4. Determination of chemotaxonomic and molecular biological characteristics of isolate PFBC in comparison to described *Acidocella* spp.

7.4.1. Chemotaxonomic analysis of strain PFBC

7.4.1.1. Methods

DNA was extracted from biomass grown in fructose medium at 30°C by Blood and Cell Culture DNA Kit (QIAGEN), and the G + C content determined. This was carried out using reverse-phase HPLC (High performance liquid chromatography) using a Nucleosil 100–5 C18 column (Macherey–Nagel) and purified non-methylated DNA of lambda phage (Sigma) as a standard, by Dr. Olga Golyshina (Bangor University). Analyses of polar lipids, fatty acids and respiratory quinones were carried out (on fructose-grown biomass) by the Identification Service, Leibniz-Institute DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen*, Braunschweig, Germany).

7.4.1.2. Results

The chromosomal DNA content of strain PFBC was G+C 61.6%-63.9 mol%. This was similar to *Ac. facilis* (64.4 mol%; Kishimoto *et al.*, 1993), *Ac. aminolytica* (58.7-59.2 mol%; Kishimoto *et al.*, 1993) and *Ac. aluminiidurans* (65.6 mol%; Kimoto *et al.*, 2010). Table 7.5 shows that the major cellular fatty acids for strain PFBC differ from those of the other *Acidocella* spp. The major quinones of strain PFBC were ubiquinones Q-10 (92%) and Q-8 (8%). The major quinone reported for *Ac. facilis, Ac. aminolytica* and *Ac. aluminiidurans* was also ubiquinone-10 (Kishimoto *et al.*, 1993; Kishimoto *et al.*, 1995; Kimoto *et al.* 2010).The major polar lipids of strain PFBC were four different glycolipids, diphosphatidylglycerol, two aminolipids, phosphatidylglycerol phosphatidylethanolamine, and phosphatidylmonomethylethanolamine. Wichlacz *at al.*, (1986) reported four phospholipids and two aminolipids for *Ac. facilis*, and Kishimoto *et al.* (1993) reported two ornithine amide lipids for *Ac. aminolytica*.

Table 7.5. Cellular fatty acids in strain PFBC and comparison with described *Acidocella* spp.

	Strain PFBC[1]	*Ac. facilis*[2]	*Ac. aminolytica*[2]	*Ac. aluminiidurans*[2]
Cyclopropyl $C_{19:0}$	3.34	39.6	52.3	49.1
$C_{18:1}$	81.2	35.8	22.6	25.4
$C_{16:0}$	10.1	13.2	11.2	12.2
$C_{16:0}$ 2-OH	5.0	2.9	5.2	6.7
$C_{14:0}$ 3-OH	–[c]	5.8	6.1	4.3
$C_{18:0}$	2.0	2.0	1.8	2.2
Summed feature 2[c]	1.54	–	–	–

[1]Cultures grown in fructose medium (pH 3.5) for 2 days
[2]Data from Kimoto *et al.* (2010). Cultures grown in 1 % glucose medium (pH 5) for 7 days
[3]Summed features are groups (two or three) fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contains $C_{12:0}$, iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH

7.4.2 Phylogenetic analysis of strain PFBC

7.4.2.1. Methods

DNA was extracted from 2 mL of fructose-grown PFBC using the lysis method (section 2.7.1.2), the 16S rRNA gene amplified (section 2.7.2) and sequenced by Macrogen Inc., South Korea. The resulting gene sequence was analysed by NCBI BLAST software (http://ncbi.nlm.nih.gov/BLAST), and added to the GenBank database. It was then aligned with the 16S rRNA gene sequences of other obligately heterotrophic acidophilic bacteria using the software program ClustalX, trimmed so that all sequences were complete and bootstrapped (x1000) phylogenetic trees (alignment length; 1024 bp) generated using maximum parsimony, neighbour-joining and maximum-likelihood algorithms as detailed in section 2.7.6. Only the neighbour-joining tree is presented as all tree topologies generated by the methodologies were nearly identical,.

7.4.2.2. Results

The designated type strain of *Ac. aromatica*, isolate PFBC (GenBank accession number KC590088), was 99% related to the two other strains designated as isolates of the novel species *Ac. aromatica,* strains WJB-3 and LGS-3. These three strains of *Ac. aromatica* shared 98% 16S rRNA gene sequence similarity to *Ac. aluminiidurans* and 97% 16S rRNA gene sequence similarity to *Ac. facilis* and *Ac. aminolytica*. This data plus physiological differences between the three strains of *Ac. aromatica* (PFBC, WJB-3 and LGS-3) and previously described *Acidocella* spp. supports their placement as a distinct species. Fig. 7.4 shows the phylogenetic relationship between the proposed *Ac. aromatica* strains, and the previously described *Acidocella* spp..

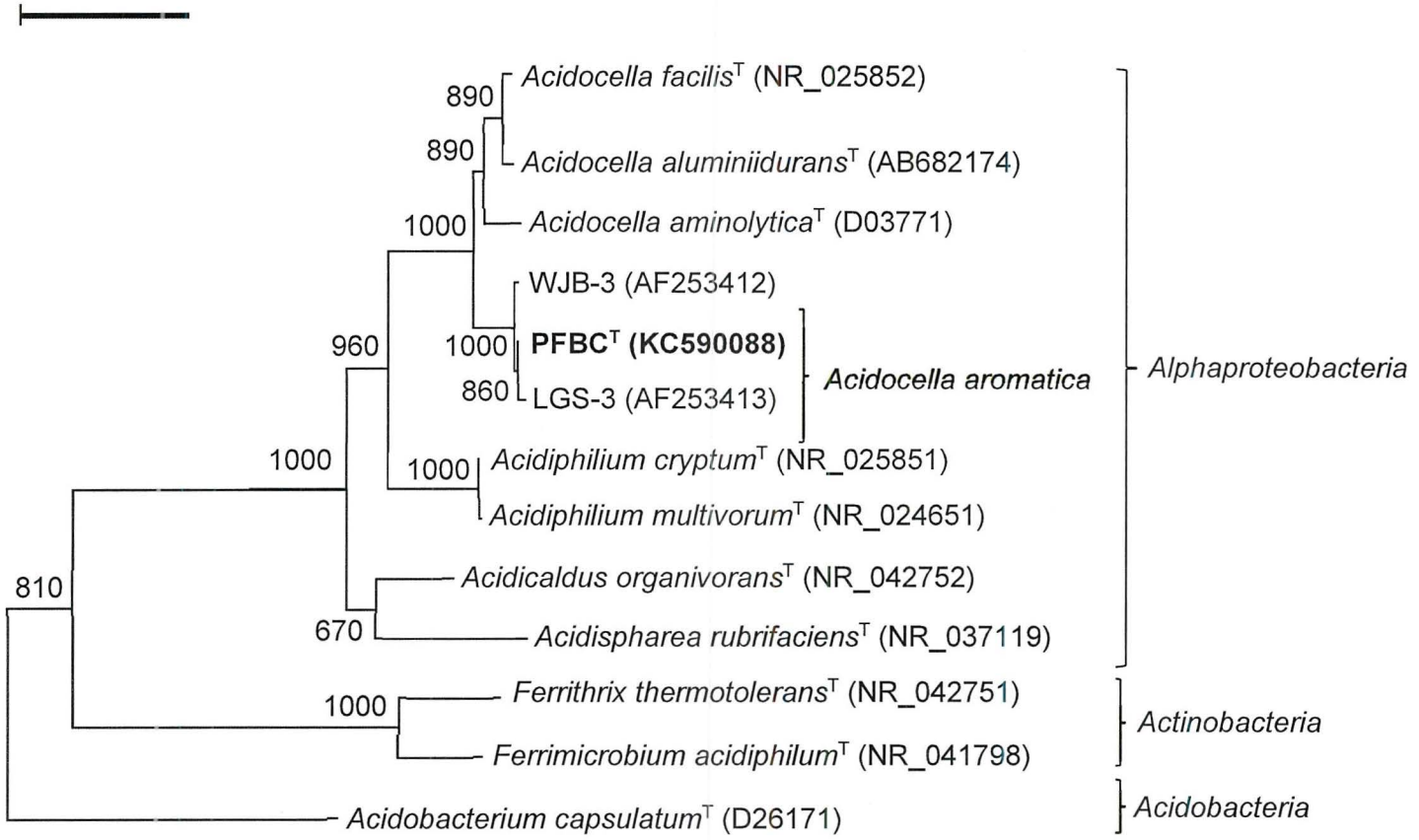


Figure 7.3. Neighbour joining phylogenetic tree showing the three strains of the proposed novel spp. *Ac. aromatica* (PFBC, LGS-3 and LGS-3) placed within the genus *Acidocella*, and in relation to other acidophilic heterotrophs of the class *Actinobacteria* and *Acidobacteria*. *Ferroplasma acidiphilum* (GenBank accession number AJ224936) is the tree root (not shown). Bootstrap values appear on their respective nodes. Values in parenthesis are GenBank accession numbers. Scale bar represents 0.05% sequence divergence.

7.4. Discussion

The proposed novel species *Acidocella aromatica* (type strain PFBC and strains WJB-3 and LGS-3), displayed unusual phenotypic traits for an acidophilic heterotroph. These differentiate the proposed *Acidocella* sp. from those previously reported.

The first of these differences is that the three strains of the proposed *Ac aromatica* did not use many of the organic substrates that support the growth of previously described *Acidocella* spp. and many other acidophilic heterotrophs such as *Acidiphilium* spp.. Strains of *Ac. aromatica* grew using fructose but no other monosaccharide examined, including glucose. Sucrose was metabolised, but the cell yield was comparable to that of cultures grown on equimolar amounts of fructose. This indicated that the fructose moiety of the disaccharide was metabolised, but not the glucose moiety. It was not determined whether the glycosidic bond of the disaccharide was hydrolysed in an intra- or extra-cellular fashion. The proposed type strain PFBC also did not metabolise alcohols, amino acids or complex substrates such as tryptone and yeast extract, but did use acetic acid. Strains LGS-3 and WJB-3 metabolised ethanol, and some organic compounds such as lactic acid.

Use of small-weight organic acids such as acetic acid and benzoic acid is unusual, as these substrates are normally toxic to acidophiles in micromolar quantities. Gemmell and Knowles (2000) had previously observed a pH dependency for growth of strain WJB-3 on acetic acid. The strength of an acid is defined as the ease by which it releases protons, or performs the forward reaction in equation [7.1].

$$[7.1] \qquad HA \rightleftharpoons H^+ + A^- \qquad [7.1]$$

The point at which reaction [7.1] is in equilibrium is termed its pK_a. Above this pK_a value, the forward reaction is dominant and the concentration of the dissociated form is higher than that of the associated form. Organic acids are mostly weak acids, with pK_a values that are similar to, or higher than, extreme acidic environments (pH 3 or below). This is an issue for acidophiles, particularly with small-weight mono-carboxylic acids, because the associated

(HA) form present in the environment passes into the cell through proton motive force where it dissociates in the higher pH. Uncontrolled movement of an organic acid into the cell can result in extreme acidification and cell death (Norris and Ingledew, 1992). Therefore, some organic acids can be particularly toxic to acidophiles in micro-molar concentrations.

For example, acetic acid has a single pK_a value of 4.7 (equation [7.2]) and benzoic acid a single pK_a value of 4.2 (equation [7.3]). This means that acetate/benzoate is the more relatively abundant form present above pH 4.7/4.2 while acetic/benzoic acid is more relatively abundant at pH of less than 4.7/4.2:

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+ \qquad [7.2]$$

$$Na^+ + CH_3COO^- + 2\ O_2 \rightleftharpoons 2\ CO_2 + Na^+ + OH^- + H_2O \qquad [7.3].$$

The actual amounts of undissociated and dissociated forms of an acid at any pH can be worked out using equation [7.4]:

$$pH = pKa + \log {}^{[A^-]} / {}_{[HA]} \qquad [7.4].$$

For example, at pH 3.0 and with a total concentration of acetic acid/acetate of 10 mM, it was calculated that the actual concentration of the undissociated acid was 9.84 mM and that of acetate was 0.16 mM. Similar calculations were carried out for both acetic and benzoic acids at the pH and concentrations used in bioreactor experiments (Table 7.7).

Table 7.7. Calculated concentrations of undissociated benzoic and acetic acids at the concentrations and pH values used in bioreactor experiments. Data in blue indicate cultures where growth was observed. ND indicates where data was not determined.

Total benzoate + benzoic acid (mM)				
	1.0	2.5	4.0	5.0
Undissociated benzoic acid (mM)				
pH 4.0	0.81	2.08	3.33	ND
pH 5.0	0.33	0.83	1.33	1.67
Total acetate + acetic acid (mM)				
	10		20	
Undissociated acetic acid (mM)				
pH 3.5	9.47		18.93	
pH 5.0	5.62		7.20	

Growth on acetate (equation [7.1]) and benzoate (equation [7.2]) at low pH causes the culture pH to increase (and therefore the equilibrium of dissociations to change in favour of the charged forms of these organic acids) in shake flask cultures. Therefore, only data from the fixed pH bioreactors could be used to determine accurately the concentrations of benzoic and acetic acid inhibited the growth of strain PFBC. These were evaluated as between 0.83 and 1.33 mM benzoic acid, and 10 and 19 mM acetic acid.

The ability of *Ac. aromatica* PFBC^T to tolerate high concentrations of acetic acid as well as metabolise this aliphatic acid means that this strain is far better able than other heterotrophic acidophiles to essentially detoxify environments that contain acetic acid, which would benefit other species that are much more sensitive. Tolerance of *Ac. aromatica* PFBC^T to acetic and is far greater than that reported for other *Acidocella* spp. (MIC calculated at between 9.5 mM

and 19 mM, as opposed to 0.25 mM previously reported; *Ac. aminolytica;* Kishimoto *et al.*, 1995).

Growth on benzoate and other aromatic compounds is unusual in acidophilic heterotrophs, but not unique. *Acidicaldus organivorans*, a moderately thermophilic, extremely acidophilic bacterium, was reported to use phenol below 5 mM (Johnson *et al.*, 2006). *Ac. aminolytica* was shown in the present study to grow on benzoate and all of the *Acidocella* spp. to metabolise benzyl alcohol.

Another interesting characteristic of *Ac. aromatica* strain PFBC is its respiratory physiology. *Acidocella* as a genus is described as obligately aerobic. Micro-aerobic growth of *Ac. aromatica*^{T} was confirmed in the present study, though the acidophiles did not grow under anaerobic conditions by ferric iron respiration (using schwertmannite ($Fe_8O_8(OH)_6SO_4$) as the ferric iron source since its pH growth minimum precluded use of ferric sulfate, as shown for *Acidiphilium* spp. (Coupland and Johnson, 2008). Other methods of anaerobic respiration investigated have been reported elsewhere (Table 7.8).

Table 7.8. Methods of anaerobic respiration reported for strain PFBC: – indicates no growth and + indicates growth.

Metabolism		Reference
Fermentation (fructose)	-	Kimura *et al.* (2006)
Acetoclastic reaction	+	Kimura *et al.* (2006); Rowe (2007)
Anaerobic respiration using the following election acceptors:		
NO_3^-	-	Kimura (2005)
$S_2O_3^{2-}$	-	Kimura (2005)
$S_4O_6^{2-}$	-	Kimura (2005)
$Fe_2(SO_4)_3$	-	Kimura (2005)
Fe^{3+} ($Fe_8O_8(OH)_6SO_4$)	-	Coupland and Johnson (2008); Kimura (2005); Rowe *et al.* (2007)
S^0	-	Kimura (2005)

The only pathway strain PFBC appears to grow anaerobically by is an acetoclastic reaction in co-culture with acid-tolerant sulfate reducing bacteria that produce acetic acid as a waste product and can use hydrogen as an electron donor (Kimura *et al.*, 2006). Kimura *et al.* (2006) noted that there was little accumulation of this aliphatic acid when the sulfate reducing bacterium was grown in mixed culture with *Acidocella* sp. strain PFBC, while acetic acid accumulated in a pure culture of *Desulfosporosinus* strain M1 (now *D. acididurans*^{T}). To explain these observations, Kimura *et al.* (2006) proposed that the relationship between strain PFBC and the sulfidogens under anaerobic conditions was syntrophic. The acetic acid metabolism of strain PFBC (equation [7.5]) produced hydrogen and carbon dioxide:

$$4\ CH_3COOH + 8\ H_2O \rightarrow 16\ H_2 + 8\ CO_2 \qquad [7.5]$$

The H_2 produced in equation [6.4] could be used by the *Desulfosporosinus* spp. to perform sulfate reduction coupled to the partial oxidation of glycerol (equation [7.6]):

$$4\ C_3H_8O_3 + 3\ SO_4^{2-} + 6\ H^+ \rightarrow 4\ CH_3COOH + 4\ CO_2 + 3\ H_2S + 8\ H_2O \quad [7.6]$$

This reaction could generate additional ATP for the sulfate reducer (other experiments confirmed that the acid-tolerant sulfate reducers could grow on hydrogen as sole electron donor). Reaction [7.5] would then result in low partial pressure of hydrogen by removing H^+, resulting in an energetically favourable system (ΔG = -199 kJ/mol at pH 4 as opposed to the ΔG = +82.00 kJ/mol at pH 4 equation [7.4] by itself; Johnson *et al.*, 2006; Kimura *et al.*, 2006). However, because strain PFBC uses acetate rather than acetic acid, equation [7.5] becomes:

$$4\ CH_3COO^+ + 16\ H_2O \rightarrow 16\ H_2 + 8\ HCO_3^- + 4\ H^+ \quad [7.7]$$

Reaction [7.6] is less energetically favourable than reaction [7.7] (ΔG = +104.50; Rowe, 2007). Therefore, the total energy of the acetoclastic reaction becomes -176.5 kJ/mol. However, this is still an energetically favourable reaction. Furthermore, Rowe (2007) found that *Acidocella* sp. strain PFBC could grow anaerobically in pure culture in the presence of activated palladium, which was thought to act as an abiotic sink for the hydrogen it produced. The result of these syntrophic interactions is reaction becoming exogonic and therefore thermodynamically viable because of the presence of an efficient sink for one or more of the reaction products (hydrogen in the present case). These syntrophic interactions appear more common among neutrophiles (Sieber *et al.*, 2012).

A high tolerance to metals often found in elevated concentrations in mine waters of pH below 2.5 (iron, manganese and aluminium) was expected as *Ac. aromatica* strains, like the other *Acidocella* spp. and extreme acidophiles in general, grow in extremely acidic environments that often contain elevated concentrations of soluble transition metals and aluminium. *Ac. aromatica* could also tolerate relatively high concentrations of nickel (MIC 200-300 mM), a trait shared with "*Ac. acidivorans*" only. All described *Acidocella* spp were found to be relatively sensitive to copper (MIC 3-20 mM).

The above characteristics, and substrate use in particular, make *Ac. aromatica* strains useful members of microbial consortia in some contexts (Kimura *et al.*, 2006; Johnson *et al.*, 2008; Ňancucheo and Johnson, 2010), and also as the underlay bacterium in low pH overlay solid medium (Kay *et al.*, 2013; Johnson and Hallberg, 2007; Ňancucheo and Johnson, 2010; this study). The strains of *Ac. aromatica* use substrates that are generally inhibitory to acidophiles, and do not compete for substrates, such as yeast extract) that other acidophilic heterotrophs (e.g. *Acidiphilium, Desulfosporosinus*) use. This may be an adaptive strategy by *Ac. aromatica*, allowing it to avoid competition with other acidophilic heterotrophs for scarce and commonly used substrates. Increased tolerance to toxic substrates is also a competitive advantage. These traits are similar to "*Ac. acidivorans*" and the other Trefriw *Acidocella* isolates. *Ac. aromatica* appears to have more in common with these bacteria than the other described *Acidocella* spp., despite being relatively distantly related (96% 16S rRNA gene sequence similarity). It is likely that the Trefriw Wells Spa isolates also possess these characteristics as a competitive advantage and may be used in the same way as *Ac. aromatica*. That is, as an underlayer organism and as a consortium component.

.

7.5 Description of *Acidocella aromatica* sp. nov.

Acidocella aromatica ([a.ro.ma'ti.ca]. L. fem. adj. aromatic fragrant, referring to its ability to degrade aromatic compounds)

Cells are rod-shaped, 1-2 µm long and 0.2-0.4 µm wide and motile. They are Gram-negative, and do not form endospores. Colonies are white, smooth, entire, domed and round or convex on acidic (pH3) non-overlay fructose solid medium. Mesophilic (optimum 30°C, maximum 37.5°C) and moderately acidophilic (optimum pH 3.8, minimum 2.5). Obligately heterotrophic, growing on fructose, acetate and benzoate but not glucose or complex organic substrates such as yeast extract. Strains also use some aromatic compounds and organic acids, including acetic acid, phenol and benzoic acid. Facultatively aerobic, capable of ferric iron reduction under microaerobic conditions, and anaerobic growth by acetic acid oxidation. No growth by fermentation or other anaerobic means. Quinones are Q-9 (8%) and Q-10 (92%). Major fatty acids of type strain PFBC are cyclo- $C_{18:1}$ ω7c and $C_{16:0}$. Its main polar lipids are: four different glycolipids; two aminolipids; diphosphatidylglycerol; phosphoglycerol; phosphatidylethanolamine and phosphatidylmonomethylethanolamine. Chromosomal DNA base composition of strain PFBC is 61.58 mol% G+C. 16S rRNA gene sequencing (GenBank accession number KC590088) places *Acidocella aromatica* in Class *Proteobacteria*, subclass *alpha-proteobacteria*. Type strain PFBC (= DSM 27026, = NCCB 100456) was isolated as contaminant of an anaerobic *Desulfosporosinus* PFB culture from Mynydd Parys, North Wales. Strain WJB-3 was isolated from acid mine drainage water in Cornwall (the Wheal Jane mine), and LGS-3 from an acidic geothermal site, Lower Gages, Montserrat.

Chapter 8. *Acidithrix ferrooxidans,* gen. nov. sp. nov.; an acidophilic, iron-oxidising, heterotrophic filamentous *Actinobacterium*

8.1. Introduction

The proposed novel genus and species "*Acidithrix ferrooxidans*" was identified at Trefriw Wells Spa (Hallberg *et* al., 2006; chapter 5) but not isolated in this study. A similar isolate was also isolated from Cae Coch, but subsequently lost (CCH7; Johnson *et al.*, 1992). A strain of this species (PY-F3) was isolated from acid streamers found in mine water draining the abandoned Mynydd Parys copper mine, North Wales, and so was used in this study. 16S rRNA gene sequence analysis (BLAST) showed *Fm. acidiphilum* as the closest described relative (92.4%-93% similarity; Kay *et al.* 2013). This distance was more than the proposed 97% 16S rRNA gene sequence threshold for novel species, and more than the proposed 95% threshold for defining a novel genus (Tindall *et al.*, 2011). According to these definitions, isolate PY-F3 appeared to be the first species of a novel genus of the phylum *Actinobacteria*.

The phylum *Actinobacteria* currently includes four approved genera and one proposed genus, each of which currently contains a single species. These are *Acidimicrobium, Aciditerrimonas, Ferrimicrobium, Ferrithrix* and *'Acidithiomicrobium'* (Clarke and Norris, 1996; Itoh *et al*, 2011; Johnson *et al.*, 2009; Norris *et al.*, 2011). Physiological characteristics vary across the genera. *Am. ferrooxidans, Fm. acidiphilum* and *Atn. ferrireducens* grow predominantly as single rods, while *Fx. thermotolerans* grows in entangled filamentous. *Am. ferrooxidans* and *Atn. ferrireducens* have been reported to grow by oxidation of hydrogen, and species of the genus *'Acidithiomicrobium'* have been reported to grow autotrophically by oxidation of sulfur. Three of these species reported a CO_2 fixing ability, while *Fx. thermotolerans* and *Fm. acidiphilum* have been described as obligately heterotrophic. All of

these extremely acidophilic actinobacteria can couple the oxidation of organic substrates to the reduction of ferric iron, and *Am. ferrooxidans* can also use hydrogen to reduce iron (Hedrich and Johnson, 2013). *Am. ferrooxidans, 'Acidithiomicrobium'*, *Fm. acidiphilum* and *Fx. thermotolerans* also catalyse the dissimilatory oxidation of ferrous iron (Johnson *et al.*, 2009, Itoh *et al*, 2011; Norris *et al.*, 2011). *Am. ferrooxidans*, *Atn. ferrireducens* and *Fx. thermotolerans* are moderately thermophilic (temperature optima 43-50°C), while *Fm. acidiphilum* is mesophilic (temperature optimum 30-32°C). They are all extremely acidophilic (pH optima for growth of 1.4 – 3.0).

This chapter details the physiology and phylogeny of isolate PY-F3, the type strain of the novel genus/species actinobacterium, *Acidithrix ferrooxidans.*

8.2. Isolation and maintenance of isolate PY-F3

Strain PY-F3 was isolated by Kay *et al.* (2013) from an acid streamer growing in the Afon Goch, a stream draining the abandoned Mynydd Parys copper mine in Anglesey, North Wales. Non-overlay FeTSB solid medium was inoculated with homogenised streamer sample. Colonies that had a distinctive "fried egg"-like morphology and consisted of filamentous bacteria were purified by repeated single colony isolation on the same medium. Isolate PY-F3 was maintained at the *Bangor Acidophile Culture Collection* in a liquid medium containing 10 mM ferrous iron and 0.02% yeast extract (pH 2.0), and cultures stored short-term (up to 6 months) at 4ºC in the same medium.

The 'fried-egg'-like colony morphology of strain PY-F3 (Fig. 8.1 (a)) is a typical characteristic of heterotrophic and mixotrophic iron-oxidising acidophiles. In liquid media with low iron concentrations (~0.1 mM), it formed macroscopic, aggregated, off-white coloured growths that disintegrated as the culture aged. In iron-rich media (up to 20 mM), schwertmannite-like ferric iron deposits were precipitated on the aggregated biomass of isolate PY-F3 as time progressed (Fig. 8.1 (b)). Isolate PY-F3 formed chains and filaments of cells that became

shorter as incubation time increased, and occasional motile rods were observed once cultures reached late stationary growth phase (Fig. 8.1 (c)). No endospores were observed at any time.

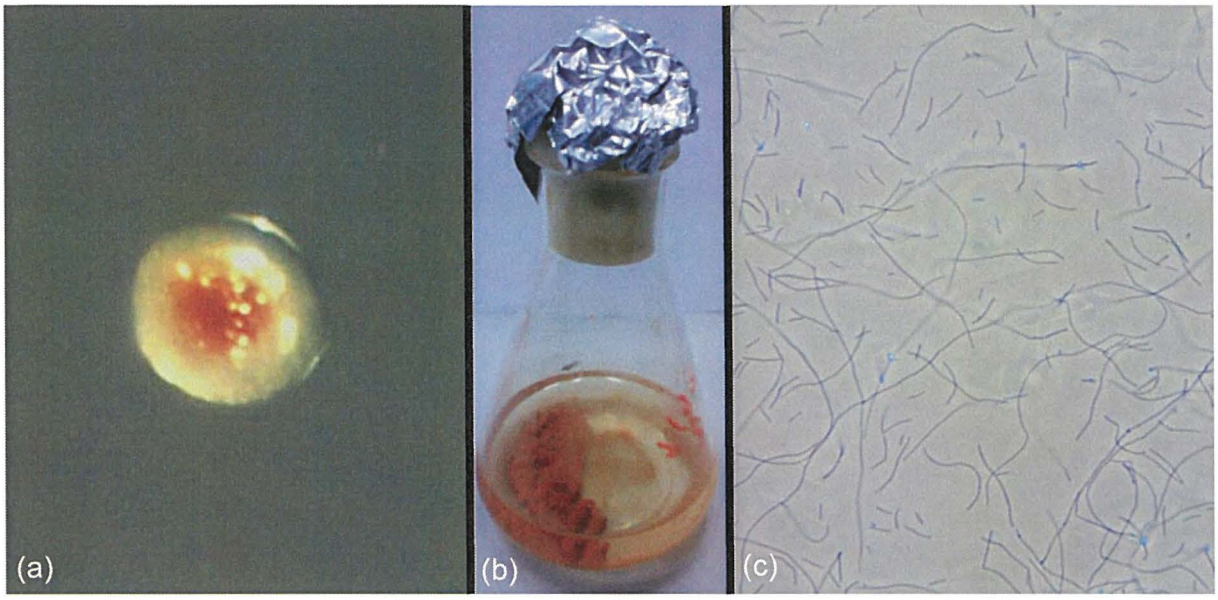


Figure 8.1. (a) Image of PY-F3 colonies grown on a FeTSBo/PFBC plate (x100 magnification). (b) Image of PY-F3 culture after 4 days grown in liquid medium containing basal salts #1, 0.01% yeast extract, 5 mM glucose and 10 mM iron. (c) Phase contrast microscope image of PY-F3 cells (x1000 magnification).

8.2.1. Optimization of isolate PY-F3 growth on solid media

8.2.1.1. Methods

Colony growth of isolate PY-F3 on non-overlay FeTSB solid medium was highly variable. High plating efficiencies are desirable for isolating bacteria from environmental samples, and uniform colony morphologies are useful to distinguish isolates. Therefore, growth of isolate PY-F3 on other solid media was tested. These were: YE3o/PFBC (0.02% yeast extract, basal salts #1, 0.1 mM Fe^{2+} and *Ac. acidocella*^{T} as the underlay organism), YEo, (0.02% yeast extract, basal salts #1, 0.1 mM Fe^{2+} and *Acidiphilium* SJH as the underlay organism), iFeo (basal salts #1, trace elements, 20 mM Fe^{2+}), FeSo (2.5 mM potassium tetrathionate,

0.025% tryptone soy broth, basal salts #2 and 20 mM Fe^{2+}), YE3 (non-overlay medium; 0.02% yeast extract, basal salts #1 and 0.1 mM Fe^{2+}) and a modified medium, designated FeTSBo/PFBC (0.025% tryptone soy broth, basal salts #2 and 1 mM Fe^{2+}). Duplicate plates of each solid medium were inoculated with lightly homogenized (Pro Scientific Multi-gen 7 homogenizer) cultures of isolate PY-F3, and incubated at 30°C for up to 9 days.

8.2.1.2. Results

Colonies of PY-F3 on FeTSBo/PFBC were more numerous and uniform (~2-4 mm diameter) than on the other solid media where growth was observed. No colonies were observed on YE3o, iFeo or FeSo solid media.

8.3. Physiological characteristics of isolate PY-F3

8.3.1. Iron oxidation coupled to growth

8.3.1.1. Methods

Conical flasks (250 mL) containing 100 mL of 5 mM glucose, 0.01% yeast extract, basal salts #1 and 20 mM Fe^{2+} medium (section 2.2.2.2) with an initial pH of 2.5, were inoculated with lightly homogenised PY-F3 culture. Inoculum was from a pure culture grown in the same medium as above, except that it contained 0.1 mM Fe^{2+} (section 2.2.2.2). These were incubated at 30°C for 11 days.

Duplicate flasks were removed for sampling each day. 10 mL of liquor was filter-sterilized (through Whatman 0.2 µm nitrocellulose membranes) then concentrations of ferrous iron, total iron (Ferrozine assay) and glucose (ion chromatography) were determined in addition to pH. For protein determination, the rest of the culture (90 mL) was shaken, homogenised for 10 sec and then 5 mL analysed using the Bradford assay (section 2.6.3). These factors were

combined in a 3-Y axis graph (drawn using R (Version 3.0.2, programme script in Appendix 2).

8.3.1.2. Results

Fig. 8.2 shows a clear relationship between iron, glucose and protein concentrations in these cultures at these conditions (30°C, pH 2.5, 5 mM glucose, 20 mM Fe^{2+}). Iron oxidation and protein concentrations appeared to be closely and positively correlated, while glucose concentrations decreased as iron oxidation progressed and protein concentrations increased. Growth and ferrous iron oxidation therefore appeared to be tightly coupled in cultures of isolate PY-F3.

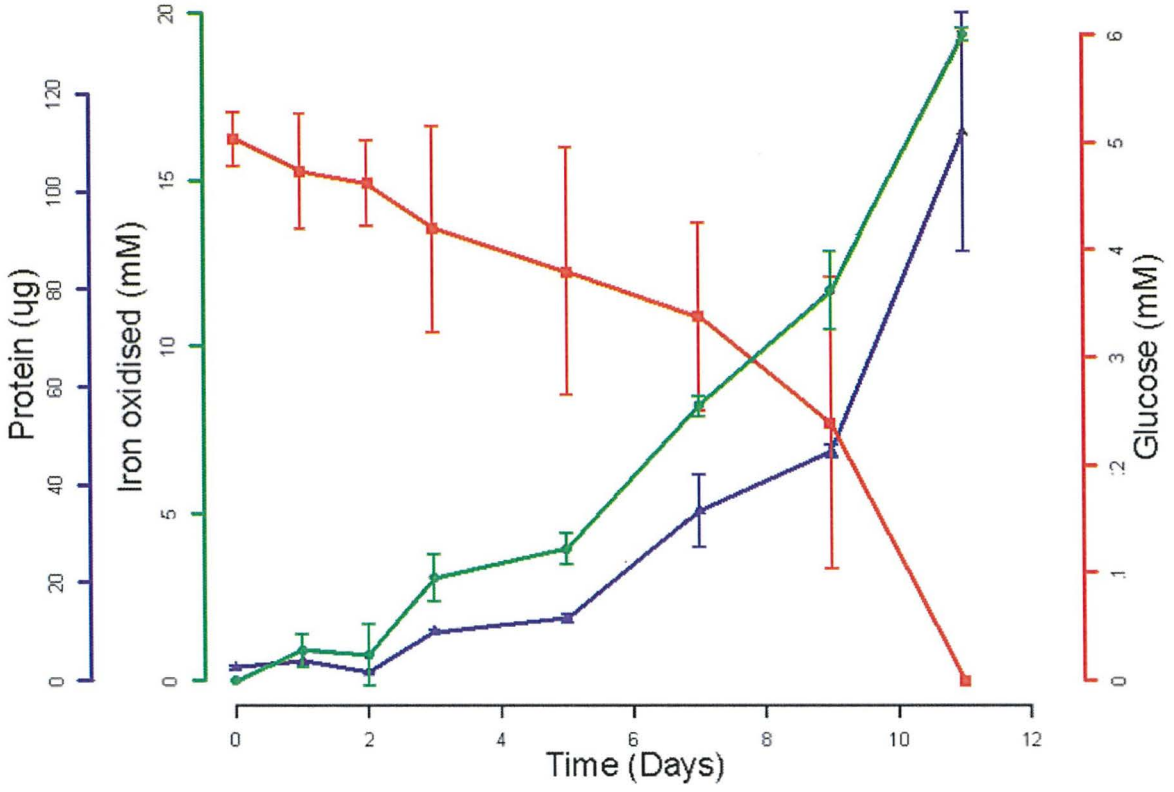


Figure 8.2. The effect of growth of isolate PY-F3 on iron oxidation, protein concentration and glucose concentration over time in flask cultures (error bars show standard deviation). Key: (■) glucose, (▲) protein, (●) ferrous iron.

8.3.2. Effect of pH and temperature on the growth of isolate PY-F3

8.3.2.1. Methods

The effect of pH on the growth of isolate PY-F3 was determined in a 2.5 L stirred bioreactor (section 6.3.1.1). This contained 1.5 L of 5 mM glucose, 0.01% yeast extract, basal salts #1 and 20 mM Fe^{2+} (section 2.2.2.2) and was maintained at 25°C at pH 2.7 - 3.8. The bioreactor was aerated at ~1 L air/min and stirred at 100 rpm.

Temperature minimum and optimum of isolate PY-F3 were determined in shake flask cultures. Triplicate 250 mL conical flasks containing 100 mL of 5 mM glucose, 0.01% yeast extract, basal salts #1 and 20 mM Fe^{2+}medium at pH 2.5 (section 2.2.2.2) were inoculated with lightly homogenised PY-F3 culture (grown in 5 mM glucose, 0.01% yeast extract, basal salts #1 and 0.1 mM Fe^{2+}medium at pH 2.5; section 2.2.2.2) and incubated at 8 - 38°C.

Samples were regularly removed from the bioreactor and flask cultures and their ferrous iron concentrations measured (Ferrozine assay). Semi-logarithmic plots of changes in ferrous iron concentration against time were prepared, and used to calculate mean generation times and growth rates. This approach of measuring growth by ferrous iron oxidation rather than turbidity, cell counts or protein analysis was necessary because methods of growth determination carried out on sub-samples of culture were inherently inaccurate due to biomass forming as streamer-like growths in a bioreactor (these adhered to the base and fittings) and as biomass aggregates in flask cultures. As growth and iron oxidation were tightly coupled (section 8.3.1), changes in ferrous iron concentrations were used to determine growth rates. This approach is also used with chemolithotrophic iron-oxidises such as *At. ferrooxidans*. However, this approach assumes that the linear relationship shown in section 8.3.1 is true at other conditions (e.g. pH and temperature). This was shown in section 8.3.3 to be untrue, though variation in temperature had less effect on ferrous iron concentration than pH. This approach could also not be used to determine growth rates accurately above pH 3.5 as abiotic oxidation of ferrous iron became increasingly significant

above this value. However, it was still considered the best option available to measure growth for the reasons stated above, with the caveat that the actual values may be somewhat different to the reported values.

8.3.2.2. Results

The optimum pH and temperature for the growth of isolate PY-F3 using this methodology were pH 3.0 and 25°C, respectively (Fig. 8.3). The minimum pH for growth was 2.0 and 10°C. It did not grow at 38°C and above. The highest pH it was observed growing in the bioreactor was pH 3.6, but it was observed growing at higher pH (pH 5.5) in "iron-free" liquid medium. Time scales were different between the pH and temperature growth curves as examination of the effect of pH on growth of isolate PY-F3 was conducted in a bioreactor, and temperature in flasks. This was done because it was not possible to cool the bioreactor below ~20ºC.

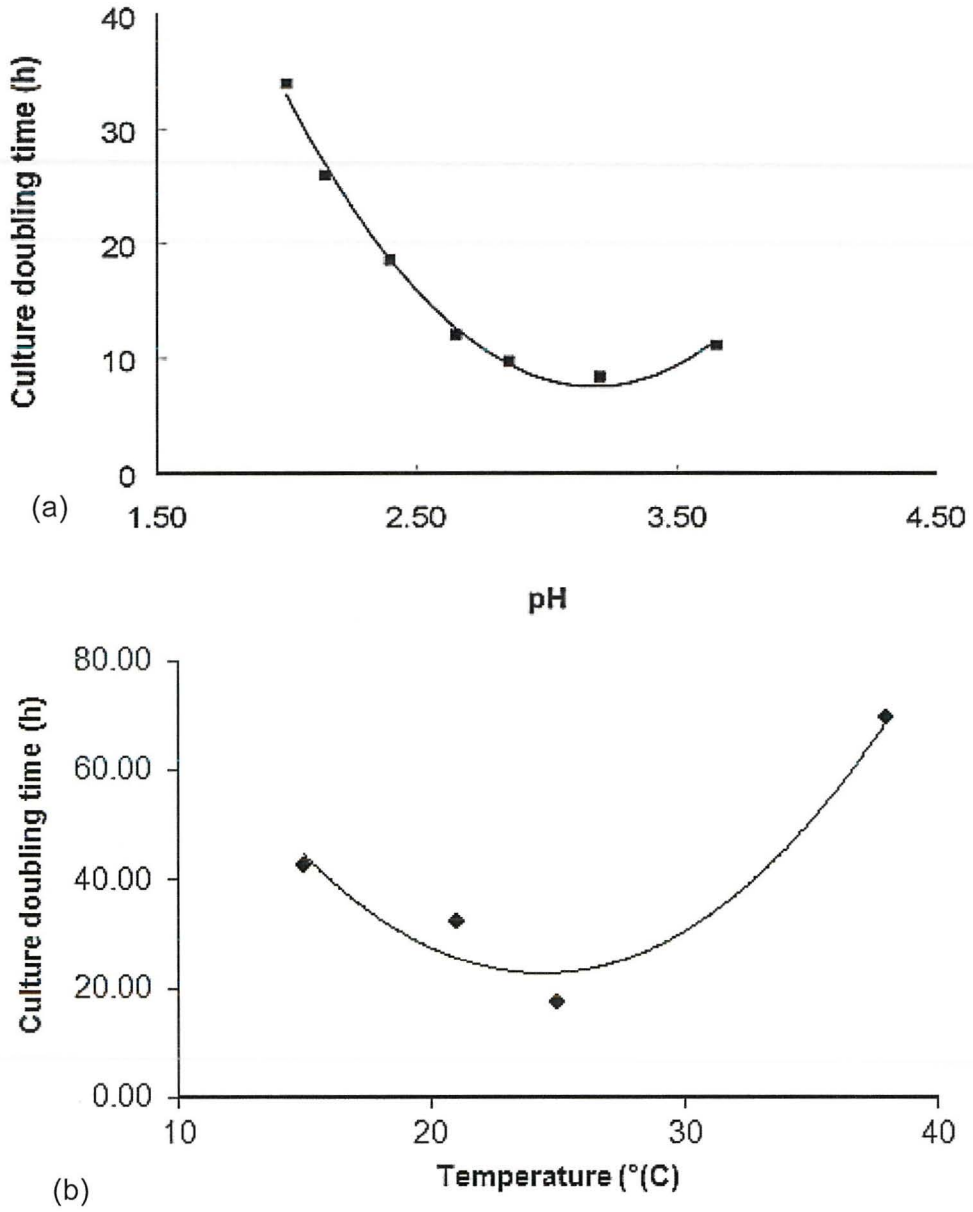


Figure 8.3. (a) Effect of pH on the culture doubling time of isolate PY-F3 grown in batch culture at 25°C in a pH- and temperature-controlled bioreactor. (b) Effect of temperature on the growth rate of isolate PY-F3 grown in flask cultures at pH 2.5.

8.3.3. Specific rates of iron oxidation

8.3.3.1. Methods

Biomass of isolate PY-F3 grown in low iron iron-containing liquid medium (5 mM glucose, 0.01% yeast extract, basal salts #1 and 0.1 mM Fe^{2+}medium, pH 2.5, incubated at 30°C for

7 days) was harvested and suspended in 50 mL sterile basal salts #1 (section 2.2.2.1). Biomass was also grown in the same medium, but containing 10 mM ferrous iron and an initial pH of 2.3. For each assay (carried out in triplicate), universal bottles containing 1 mM ferrous iron in 10 mL of basal salts #1 adjusted to pH 1.5 – 3.5 was placed in the water bath and allowed to come to the required temperature (0 - 40°C; ice was added to the water bath to maintain conditions below 22°C.). Air was bubbled through the suspensions at ~20 mL/min. The reaction was initiated by the addition of 1 mL of lightly-homogenised PY-F3 biomass. Protein concentrations of the homogenised biomass samples were determined using the Bradford assay. Samples were withdrawn at regular intervals to determine residual concentrations of ferrous iron (Ferrozine assay). Biomass-free controls were also set up.

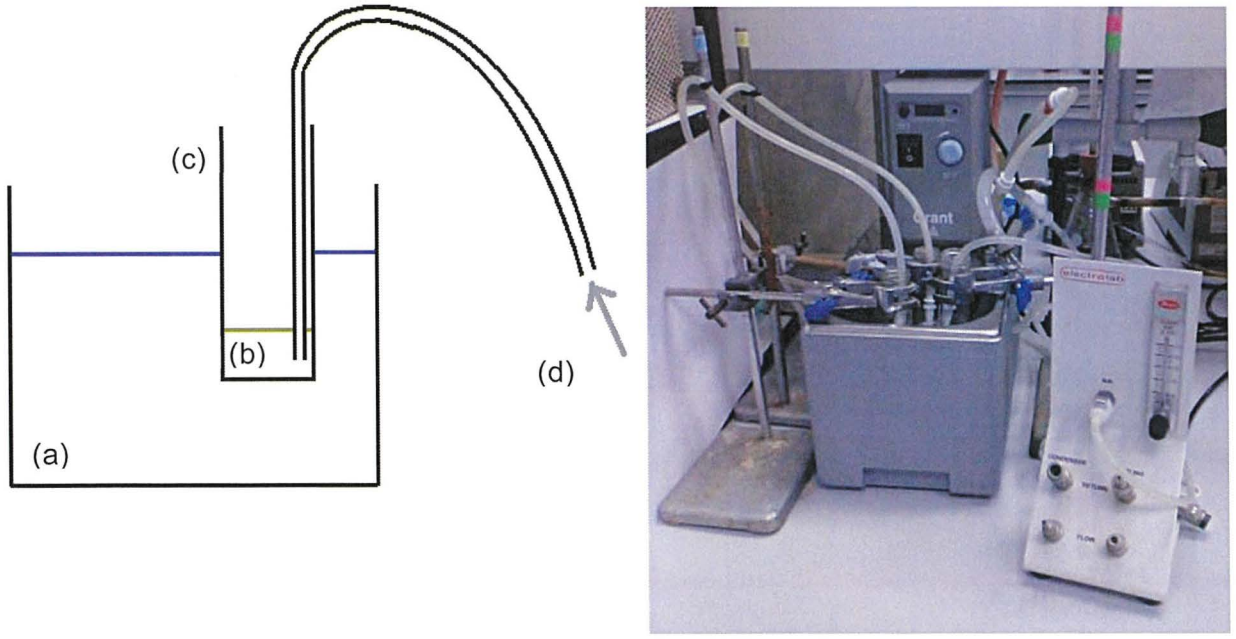
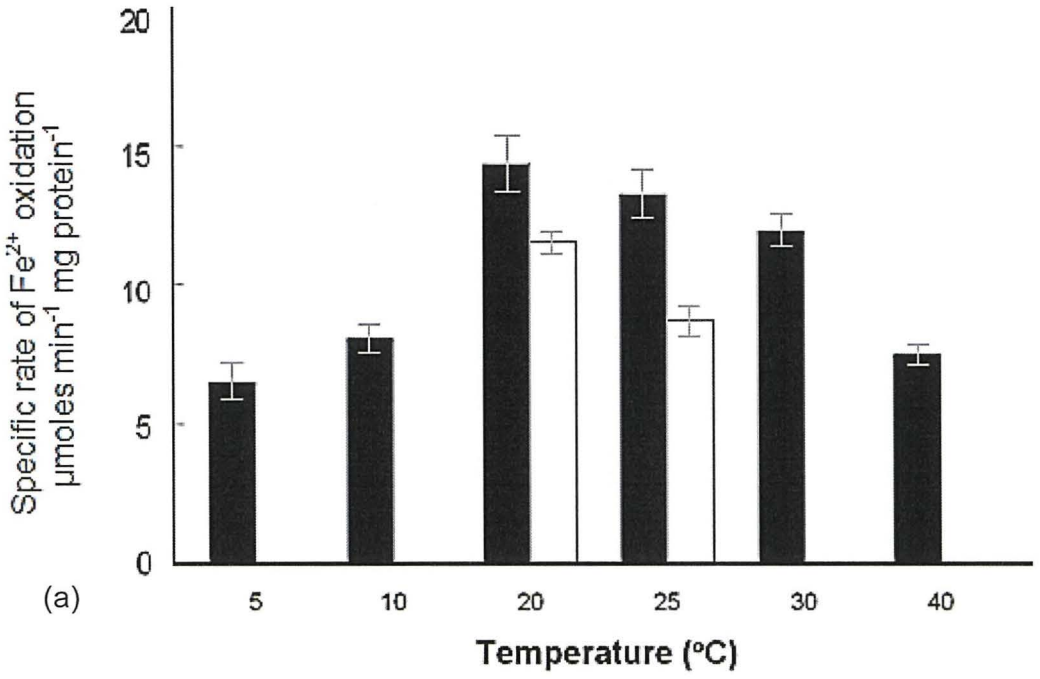


Figure 8.4. Diagram and image of system set-up for specific rates of ferrous iron oxidation. (a) water bath; (b) reaction mixture; (c) clamped universal bottle; (d) tubing connected to air-flow meter. Three universal bottles suspended in the water bath enabled triplicated analysis to be performed at the same time.

8.3.3.2. Results

The highest specific rates of ferrous iron oxidation by isolate PY-F3 were at between 20°C and 25°C, and between pH 2.0 and 2.5 (Fig. 8.5). The temperature range that iron oxidisation was observed was 5°C to 40°C; iron was not oxidised at 0°C. Ferrous iron was also oxidised at between pH 1.5 and 3.5 (higher pH was not tested because of abiotic oxidation of iron). These data showed that isolate PY-F3 could oxidise iron outside of its pH and temperature growth range. However, there was a noticeable decrease in rate of iron oxidation outside of the growth range (the pH 1.5 rate was 21% than pH 2.0 and the 5°C rate was 45% less than at 10°C).



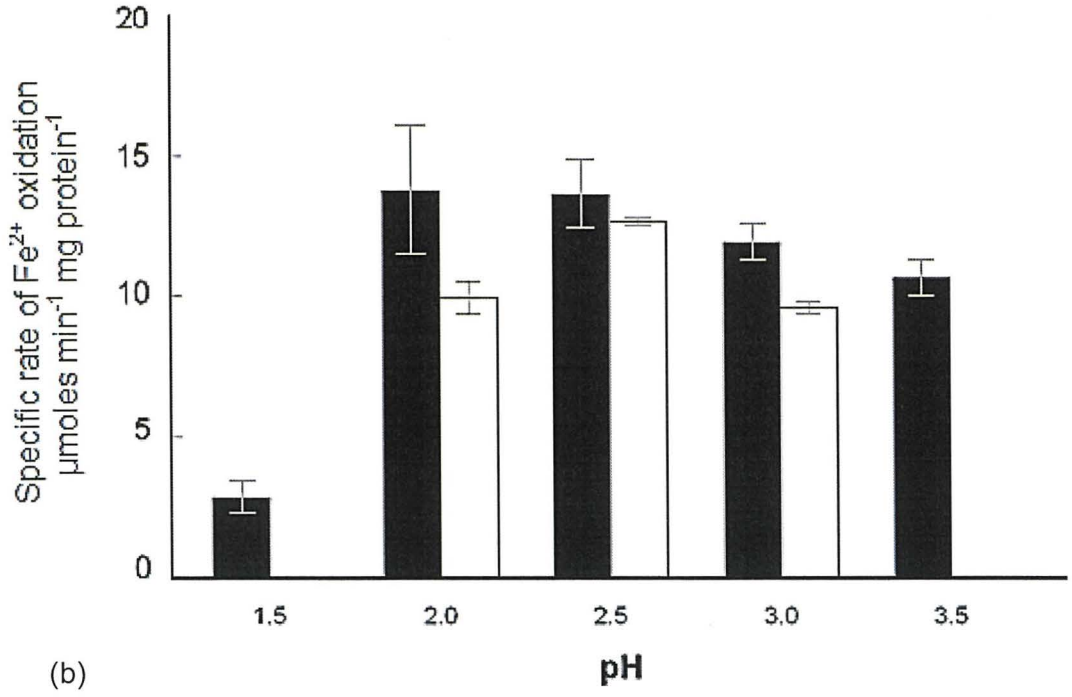


Figure 8.5. Specific rates of ferrous iron oxidation by isolate PY-F3 by cultures grown in medium at pH 2.5 containing 5 mM glucose, 0.01% yeast extract, basal salts #1 and either 10 mM Fe^{2+} (black bars, with error bars) or 0.1 mM Fe^{2+} (white bars, with error bars). (a) The effect of temperature on specific rates of iron and (b) the effect of pH on specific rates of iron oxidation.

8.3.4. Catalysis of dissimilatory iron reduction by isolate PY-F3

8.3.4.1. Methods

Isolate PY-F3 was grown under aerobic conditions in 5 mM glucose/0.01% yeast extract/5 mM ferrous iron (pH 2.2), and ferrous iron and total iron concentrations measured each day. On day 4, 5 mM additional glucose was added and the flasks shaken to disrupt the streamer-like growths. Aliquots (20 mL) of this suspension were added to sterile 100 mL flasks, three of which were placed in a sealed jar within which a micro-aerobic atmosphere was generated (section 2.2.3) and three others in a second jar within which an anaerobic atmosphere was generated (section 2.2.3). On days 4 and 8, the cultures were removed and ferrous iron and total iron concentrations measured (section 2.6.2).

8.3.4.2. Results

Isolate PY-F3 was shown to catalyse the dissimilatory reduction of ferric iron under both micro-aerobic and anaerobic conditions (Fig. 8.6). Iron added to the cultures was almost totally reduced (99%) under anaerobic conditions, and partially oxidised under micro-aerobic conditions (42%).

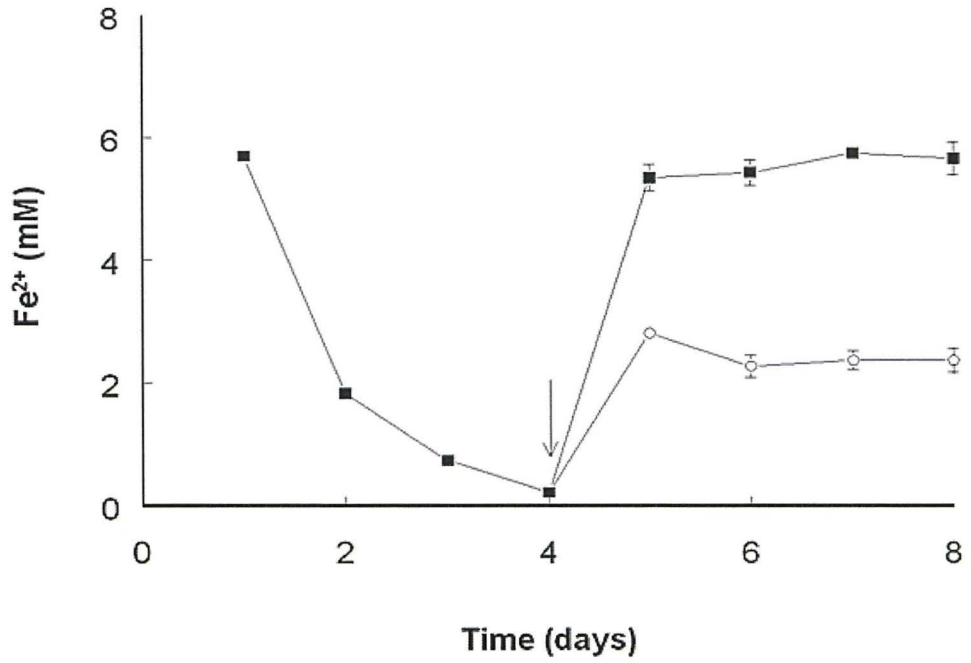


Figure 8.6. Oxido-reduction of iron by isolate PY-F3. Cultures of the acidophile were grown for four days under aerobic conditions in media containing 5 mM glucose, 0.01% yeast extract, basal salts #1 and 5 mM Fe^{2+} (section 2.2.2.2) at pH 2.2, and subsequently incubated under either micro-aerobic (○) or anaerobic (■) conditions.

8.3.5. Screening for growth of isolate PY-F3 under anaerobic and microaerobic conditions, and oxidation of hydrogen and sulfur

8.3.5.1. Methods

To screen for growth in the absence of an external electron acceptor (anaerobic fermentation) under micro-aerobic or anaerobic conditions, cultures of 10 mL of liquid medium containing 5 mM glucose, 0.01% yeast extract, 0.1 mM Fe^{2+} and basal salts #1, and Glu/YEo solid media (section 2.2.2.3), were inoculated with lightly-homogenised PY-F3 biomass. These were placed in 2.5 L sealed jars where either micro-aerobic or anaerobic atmospheres were generated (section 2.2.3), and incubated at 30°C.

To screen for growth via aerobic oxidation of hydrogen, isolate PY-F3 was placed in a jar where a hydrogen-enriched atmosphere was generated (section 2.2.3.4). To screen for the

ability to grow by dissimilatory sulfur oxidation, isolate PY-F3 was grown in medium containing 0.001% yeast extract, basal salts #1, 0.1 mM Fe^{2+} at pH 2.5 supplemented with 0.5% w/v sterile elemental sulfur at 30°C and 100 rpm shaking. pH of cultures was measured and compared against controls grown without sulfur or hydrogen.

8.3.5.2. Results

After 20 days, there was growth of isolate PY-F3 on solid and liquid media in microaerobic conditions but not anaerobic conditions. There was no additional growth observed in cultures grown in the presence of either hydrogen or elemental sulfur, compared to controls.

8.3.6. Use of organic substrates by isolate PY-F3

8.3.6.1. Methods

To examine which substrates isolate PY-F3 could use for growth, duplicate cultures containing 0.01% yeast extract, basal salts #1 and 0.1 mM Fe^{2+} were amended with the following substrates, in similar amounts of carbon-equivalents:

Carbohydrates: cellobiose, sucrose, maltose, trehalose, lactose, arabinose, galactose, glucose, glucosamine, fructose, fucose, mannose, sorbose; arabinose, rhamnose, ribose, xylose (all at 5 mM);

Organic/amino acids: butyric acid, citric acid, glucuronic acid, glutamic acid, proline (all at 5 mM), lactic acid, propanoic acid, glycine, asparginine, proline (all at 10 mM);

Alcohols: mannitol (10 mM), sorbitol (5 mM), glycerol (10 mM), ethanol (15 mM), methanol (20 mM);

Aromatics: phenol, benzoic acid, sodium benzoate (all at 5 mM);

Others: tryptone, tryptone soy broth, yeast extract (all at 0.02%, w/v); cellulose (0.05% w/v cellulose powder; Sigma-Aldrich)

The inoculation culture (PY-F3 biomass grown in 5 mM glucose, 0.01% w/v yeast extract, basal salts #1 and 0.1 mM Fe^{2+}) was lightly homogenised (Scientific Pro 200) and small amounts added to the media. Cultures were incubated with shaking for 7-14 days. They were then lightly homogenised, and OD_{600} values measured and compared with negative (containing 0.01% yeast extract but no other organic material) and positive (the same medium, but amended with 5 mM glucose) controls. Growth of *Am. ferrooxidans* on some of these substrates (Table 8.1) was also examined, as no information was available on substrate use by this acidophilic *Actinobacteria*.

8.3.6.2. Results

Strain PY-F3 grew on a limited range of substrates, including glucose, fructose, and glycine (Table 8.1). It also used the glucose dimer cellobiose (though not the glucose polymer, cellulose), and complex media (tryptone soy broth and yeast extract) though it did not use tryptone, peptone or any alcohols examined (Table 8.1). Substrate utilisation by strain PY-F3 differed from that of the other acidophilic *Actinobacteria* (Johnson *et al.*, 2009; Itoh *et al.*, 2011).

Table 8.1. Comparison of substrate use by isolate PY-F3 and other acidophilic *Actinobacteria.* +; positive growth, –; negative growth and blank; not determined.

Substrate	PY-F3	*Am. ferrooxidans*	[1]*Fm. acidiphilum*	[1]*Fx. thermotolerans*	[2]*Atm. ferrireducens*
Glucose	+	+	-	-	+
Galactose	-				+
Mannose	-				+
Fructose	-	+	-	-	+
Sorbose	-				
Ribose	-	+	-	-	+

Xylose	-				+
Arabinose	-				-
Rhamnose	-				
Fucose	-				
Glucosamine	-		-	-	
Glucuronic acid	-		-	-	
Cellobiose	+				
Maltose	-				+
Sucrose	+				+
Trehalose	-				
Lactose	+				+
Methanol	-				
Ethanol	-	+	-	+	
Glycerol	-	+	+	+	
Mannitol	-				
Sorbitol	-				
Citric acid	-	+	+	-	-
Lactic acid	-				-
Butyric acid	-				-
Propionic acid	-				
Glycine	-		-	-	
Glutamic acid	-		+	-	
Proline	-				
Asparagine	-				
Benzyl alcohol	-	-			

Benzoate	-				
Phenol	-	-			
Peptone	-				
Tryptone	-				
Tryptone soy broth	+				
Yeast extract	+	+	+	+	+

[1] Johnson *et al.*, 2009.[2] Itoh *et al.*, 2011.

8.3.7. Tolerance of isolate PY-F3 to metals, metalloids, sodium chloride and acetate.

8.3.7.1. Methods

Various metals were added to media containing 5 mM glucose/0.01% yeast extract/ 0.1 mM Fe^{2+}/basal salts #1 liquid medium, pH 2.5 (pH 2.3 for Fe^{2+} tests, to minimise precipitation) as sterile standard solutions to give final concentrations of the following metals: Al^{3+} (100-400 mM), Cu^{2+} (10-40 mM), Fe^{2+} (1-700 mM), Fe^{3+} (1-50 mM), Mn^{2+} (50-200 mM), Ni^{2+} (50-200 mM) and Zn^{2+} (50-200 mM) as sulfates. MoO_4^{2-} (1-10), arsenic (III) (as arsenate) and arsenic (V) (as arsenate) were added as sodium salts to give final concentrations of 0.1-1.0 mM. PY-F3 inoculum cultures were lightly homogenised (as above) before addition to the media. Cultures were incubated for 7 days, and then inspected for typical streamer-like growths of isolate PY-F3. OD_{600} values of homogenised cultures were also measured. Ferrozine assays were performed on iron cultures to determine final ferrous iron concentrations. Salt tolerance and acetic acid tolerance was performed as described above, with concentrations of 0-200 mM NaCl and 0.25-1 mM acetic acid added to the medium.

8.3.7.2. Results

Isolate PY-F3 was able to tolerate elevated concentrations of ferrous iron and aluminium and was also highly tolerant of other metals (Table 8.2). It displayed far greater sensitivity to the

metal/metalloid anions molybdate and arsenate. Arsenite would have been present mostly as undissociated (H_3AsO_3) at pH 3.0 (pK_a values 2.19, 6.94, 11.5), which may partly explain its increased toxicity relative to that of arsenate. Data for metal tolerances of the other acidophilic *Actinobacteria* are limited, but show that the isolate PY-F3 was apparently more tolerant to ferrous iron than the other species and that it was also less tolerant to copper than its closest relative, *Fm. acidiphilum*.

Table 8.2. Minimum inhibitory concentrations (MIC, in mM) of some metals and metalloids to isolate PY-F3 in comparison to those (where reported) of other acidophilic *Actinobacteria.* The highest observed concentrations at which the isolates grew shown in parentheses.

Metal/Metalloid	PY-F3	*Am. ferrooxidans*[1]	*Fm. acidiphilum*[2]	*Fx. thermotolerans*[2]
Al^{3+}	400 (300)			
As(III) (H_3AsO_3)	0.10 (0.00)			
As(V) ($H_2AsO_4^-$)	0.50 (0.10)			
Cu^{2+}	40 (20)	(9.4)	200 (150)	(200)
Fe^{2+}	673 (465)	(79)	(200)	(200)
Fe^{3+}	25 (10)		(200)	100
Mn^{2+}	150 (100)			
MoO_4^{2-}	10 (1)			
Ni^{2+}	100 (50)	(26)		
Zn^{2+}	100 (50)	(50)	50	(200)

[1]Watkin *et al.*, 2009 (values originally reported in g/L). [2]Johnson *et al.*, 2009.

Isolate PY-F3 tolerated up to 0.25 mM acetic acid. This is less than was reported for its closest relative *Fm. acidiphilum* (1 mM). It also tolerated up to 100 mM NaCl (MIC 150 mM).

8.3.8. Determination of the Gram reaction of isolate PY-F3

8.3.8.1. Methods

(i) Gram staining

A small amount of PY-F3 culture was added to a drop of distilled water on a slide and allowed to dry, facilitated by being occasionally passed quickly through a flame. Once dry, cells were fixed by passing the slide face down over a flame. The sample was flooded with 0.5% methyl violet for 20 s, washed with distilled water, flooded with Lugols iodine and left for 1 min to fix the colour. This was washed off, and the sample flooded with 95% ethanol for 10 sec, washed, flooded with 1% safranin for 1 sec and washed off. Most Gram-positive bacteria stain violet, and Gram-negative bacteria red, in this test.

(ii) Alternative method

Two drops of 3% KOH solution were placed on a microscope slide, an inoculating loop worth of fresh culture added and agitated on the slide. Gregersen (1978) reported that, in the case of Gram-negative bacteria, the solution became viscous due to cell lysis, while the solution did not change in consistency for Gram-positive bacteria. The Gram-positive control bacterium used in this test was *Micrococcus luteus*, and the Gram-negative control was *Chromobacterium violaceum*.

8.3.8.2. Results

Cells of isolate PY-F3 stained violet and resisted lysis with KOH, indicating that it was a Gram-positive bacterium.

8.4. Chemotaxonomic analysis of isolate PY-F3

8.4.1. Methods

Biomass of isolate PY-F3 was grown in a bioreactor containing 5 mM glucose, 0.01% yeast extract, basal salts #1 and 0.1 mM Fe^{2+} at pH 3 and 30°C, harvested (section 2.5.1). Its purity was confirmed by T-RFLP analysis (section 2.7.3). Freeze dried biomass was sent to the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ; Braunschweig, Germany) for analysis of peptidoglycan, respiratory quinones, fatty acids, and pelleted biomass sent for its chromosomal base (mol % G+C) composition.

8.4.2. Results

The chromosomal DNA of isolate PY-F3 contained 57.4 mol % G+C, which is comparable to most other acidophilic *Actinobacteria* (*Am. ferrooxidans*, 67-69%; *Fm. acidiphilum*, 54.9%; *Fx. thermotolerans*, 50.2%). It contained *meso*-diaminopimelic acid type A1γ, also reported in *Fm. acidiphilum* and *Fx. thermotolerans*. The major respiratory quinone of isolate PY-F3 was MK-9(H_4) (90%), with MK-9(H_6) (3%) and MK-9(H_4) (trace) also present. The dominant fatty acid was found to be iso-$C_{16:0}$, as for the other described acidophilic *Actinobacteria*. It contained significantly more $C_{15:0}$ and iso-$C_{16:1}$ than reported for other the acidophilic *Actinobacteria*.

8.5. Phylogenetic analysis of isolate PY-F3

8.5.1. Methods

DNA was extracted by lysis (section 2.7.1.2) from harvested PY-F3 biomass grown in glucose/yeast extract/0.1 mM Fe^{2+} media at 30°C and the 16S rRNA gene amplified (section 2.7.2). The amplified gene was sequenced by Macrogen (Korea) and the gene sequence

processed as described previously (6.4.2.1.). A BLAST sequence matrix was created by multiple BLAST alignments (NCBI BLAST software; http://ncbi.nlm.nih.gov/BLAST) of the 16S rRNA gene sequence of isolate PY-F3 and type strains of other acidophilic *Actinobacteria*. These sequences and those of other iron-oxidising acidophilic bacteria were trimmed so that all sequences were complete (length 1230 bp) and used to construct maximum liklehood, maximum parsimony and neighbour-joining phylogenetic trees as detailed in section 2.7.6 The archaeon *Ferroplasma acidiphilum* (accession number NR_028183) was used as the root (not shown). As all tree topographies were identical, only the Neighbour-joining tree is shown (Fig. 8.7).

8.5.2. Results

The 16S rRNA gene sequence of isolate PY-F3 was 91-93% related to the other acidophilic *Actinobacteria* (Table 8.3). A phylogenetic tree showing the placement of isolate PY-F3 in relation to other acidophilic genera within the phylum *Actinobacteria* is shown in Fig. 8.7. Both analyses distinguish between isolate PY-F3 and acidophilic genera that are closely related, indicating that isolate PY-F3 as a novel genus and species.

Table 8.3. BLAST sequence matrix of 16S rRNA gene sequence similarities (in %) between isolate PY-F3 and other acidophilic *Actinobacteria*.

	PY-F3	*Am. ferrooxidans*	*Fx. thermotolerans*	*Fm. acidiphilum*	*Atn. ferrireducens*
PY-F3	100	91	92	93	91
Am. ferrooxidans	91	100	91	93	93
Fx. thermotolerans	92	91	100	94	91
Fm. acidiphilum	92	93	94	100	92
Atn. Ferrireducens	91	93	91	92	100

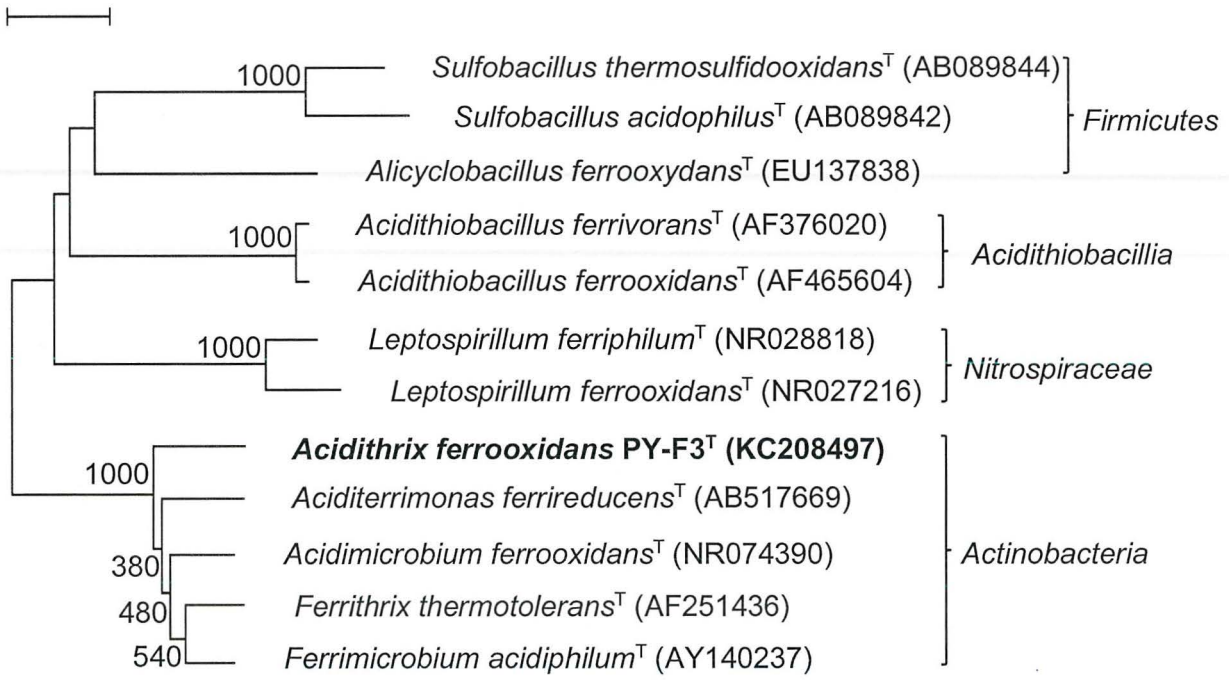


Figure 8.7. Neighbour joining phylogenetic tree showing the relationship of isolate PY-F3 to other species of acidophilic *Actinobacteria,* and other iron-oxidising acidophiles. Scale bar represents 0.05% sequence divergence. The tree was rooted with *Ferroplasma acidiphilium* (NR028183; not shown).

8.6. Screening of isolate PY-F3 for RuBiSCO and nifH genes

8.6.1. Methods

PY-F3 was screened for *nifH* genes to investigate if it had the potential to fix nitrogen. Purified DNA was extracted from collected biomass grown in 5 mM glucose, 0.01% yeast extract, basal salts #1 and 0.1 mM Fe^{2+} (section 2.2.2.2) using a MOBIO soil DNA kit and CTAB method (section 2.7.1.1). Samples were screened as described in section 2.7.4. Product from this analysis was sent to Macrogen (Korea) for sequencing.

Isolate PY-F3 was also screened for *cbbL* and *cbbM* genes (section 2.7.5), using DNA extracted by the methods described above. The type strain of *At. ferrooxidans* was used as the positive control in both analyses.

8.6.2. Results

Initial screening for *nifH* genes in isolate PY-F3 gave a positive result (a UV-labelled band in an electrophoresis gel). The amplified product was sequenced, which showed that it was not a partial *nifH* gene. This indicates that PY-F3 does not fix nitrogen, though this could not be confirmed experimentally because of an absolute requirement for yeast extract by isolate PY-F3 (to supply an unknown growth factor), which contains nitrogen.

Screening for RuBisCO gene forms I and II were both negative, indicating that it was unlikely to fix CO_2, an hypothesis that was supported by the inability of isolate PY-F3 to grow in the absence of an organic carbon source.

8.7. Discussion

The 16S rRNA gene sequence of isolate PY-F3 was 93% similar to its closest relative, *Fm. acidiphilum*. This is below the 16S rRNA gene sequence similarity threshold for distinguishing separate prokaryotic genera (95%; Tindall *et al.*, 2011). It was therefore proposed as a novel species of the novel genus *Acidithrix*, of the order *Acidimicrobiales* and phylum *Actinobacteria*. It shares a number of characteristics with the other acidophilic heterotrophic bacteria of the phylum *Actinobacteria*, such as catalysing dissimilatory iron oxidation and reduction, and obligate heterotrophy (in common with *Fm. acidiphilum* and *Fx. thermotolerans*).

Isolate PY-F3 has a filamentous morphology, similar to moderate thermophiles *Fx. thermotolerans* and *Am. ferrooxidans strain* TH3 (under some conditions) (Clark and Norris, 1996). It formed macroscopic flocs and streamers in liquid media, like *Fx. thermotolerans*. These flocs were found to be formed of entwined filaments, rather than cells embedded in an EPS matrix such as for "*Fv. myxofaciens*", another iron-oxidising streamer-forming bacterium that dominated streamer formations at Mynydd Parys, the location of the original isolation of isolate PY-F3 (Kay *et al.*, 2013). Isolate CCH7, from the Cae Coch abandoned sulfur mine near to Trefriw Wells Spa showed the same morphological characteristics, and similar

physiological characteristics (Johnson *et al.*, 1992). Their phylogenetic relationship could not be confirmed, as the isolate was lost before its 16S rRNA gene sequence could be extracted, but the physiological similarities between isolates PY-F3 and CCH7 suggest that they are related. Isolate CCH7 formed macroscopic streamers from entangled filaments, and was a mesophilic obligate heterotroph that oxidised iron and could conserve the energy generated. If the presumed relationship is correct, then this last ability may also be present in isolate PY-F3.

Isolate PY-F3 grew on a limited range of the substrates tested, though it always required yeast extract to supply an unknown growth factor. It used fewer substrates than *Atn. ferrireducens* (Itoh *et al.*, 2011), and did not use any small molecular weight alcohols, unlike *Fx. thermotolerans* and *Fm. acidiphilum* (Johnson *et al.*, 2009).

When supplied the same concentration of substrates, growth yields of some disaccharides such as lactose (a glucose and galactose dimer) and sucrose (a glucose-fructose dimer) were similar to that of the glucose monomer. This suggested that isolate PY-F3 could break the glycosidic bond between saccharide units and use the glucose moiety but not the galactose moiety, which substrate tests showed that isolate PY-F3 could not use. Isolate PY-F3 could also use cellobiose (which gave a growth yield of approximately double that of glucose), but not maltose and trehalose, despite all three being glucose dimers. Monomer units in lactose and cellobiose are linked by β(1-4) glycosidic bonds and by a α(1-2)β glycosidic bond in sucrose, while maltose and trehalose are linked by α-glycosidic bonds. While isolate PY-F3 did grow using cellobiose, it did not grow on cellulose. Cellobiose is the simplest unit of cellulose, a large, complex molecule of an estimated 3500-5000nm long (McFarlane *et al.*, 2014; O'Sullivan, 1997). It was therefore supposed that isolate PY-F3 hydrolyses the dimer bond intercellularly (where internal pH is likely to be circum-neutral, as in other acidophiles) and does not produce acid-stable cellulases, which would be required to hydrolyse cellulose extracellularly.

Isolate PY-F3 appeared to be an obligate heterotroph, as it showed no CO_2 fixing ability by growth the absence of an organic carbon source, and an apparent absence of *cbb*L and *cbb*M genes. *Fm. acidiphilum* and *Fx. thermotolerans* also do not fix CO_2 (Johnson *et al.*, 2009), though the other acidophilic *Actinobacteria* can fix CO_2 (Clark and Norris, 1996; Itoh *et al.*, 2011; Norris *et al.*, 2011). Since this study was completed, Eisen *et al.* (2015) published the draft genome of strain PY-F3, identifying a *cbb*L gene and genes coding for several enzymes involved in the CBB cycle. Neither *cbbL* nor *cbbM* genes were amplified in the present study. This could be because the primers used in this study did not correspond exactly to the *cbbL* gene in this organism. However, neither strain PY-F3 nor similar isolate CCH7 could be grown in the laboratory in the absence of organic carbon (yeast extract). It is also possible that isolate PY-F3 does fix CO_2 to some extent when provided with an unidentified growth factor (or factors), but further tests are required to confirm this. Tests were carried out on isolate CCH7 in the presence of $^{14}CO_2$, confirming that this iron-oxidising acidophile was an obligate heterotroph (Johnson *et al.*, 1992).

Isolate PY-F3 also did not appear to possess *nif*H genes, indicating that it is not diazotrophic. Verification of N_2 fixation inability in PY-F3 was not possible as there is nitrogen in yeast extract, which is an absolute requirement for growth of isolate PY-F3. The draft genome indicated that isolate PY-F3 used amino acids as sole nitrogen source, and confirmed that isolate PY-F3 did not possess genes responsible for nitrogen fixation (Eisen *et al.*, 2015).

Isolate PY-F3 was able to oxidise iron under aerobic conditions and to reduce Fe^{3+} under low oxygen (6%) or anoxic conditions. Iron reduction and oxidation are common characteristics in the acidophilic *Actinobacteria*, with the exception of *Atn. ferrireducens*, which cannot oxidise iron (Clark and Norris, 1996; Johnson *et al.*, 2009; Itoh *et al.*, 2011; Norris *et al.*, 2011). Isolate PY-F3 used ferric iron as a terminal electron acceptor under anaerobic conditions, but did not grow without oxygen. The threshold at which reduction takes over from oxidation could not be determined with the equipment available. The iron

oxidation ability of isolate PY-F3 was examined by producing specific rates of iron oxidation, over a range of pH and temperature values. PY-F3 displayed faster specific rate of iron oxidation when grown in the presence of relatively high ferrous concentration, which may be due to pre-activation of the genes coding for high iron oxidation or more proteins already present to deal with the elevated concentrations. These characteristics in particular may influence the Cae Coch sites as this species can contribute to the generation of ferric and ferrous iron.

The pH and temperature range at which harvested PY-F3 biomass was able to oxidise iron fell outside of those of its growth range. This may be due to the optimum conditions required of the unknown iron-oxidase system of isolate PY-F3, and may be only be a short term ability as no rate experiment was more than 2 h in duration. The archaean *Acidianus brierleyi* (Franzmann *et al.*, 2005) was reported to oxidise sulfur above its growth maximum, though the rate slowed considerably after 24 h. However, the ability to function outside of its growth range is a potentially useful ability, allowing energy generation under conditions outside its required conditions and a more robust organism. Other organisms identified at Mynydd Parys are psychrotolerant (Kay *et al.*, 2013), indicating that an ability to cope with low temperatures may be useful in that environment. Cae Coch sulfur mine and Trefriw are also low temperature (~10°C) environments.

In summary, all these characteristics showed that isolate PY-F3 was different from its nearest relatives, and was therefore proposed as *Acidithrix ferrooxidans* gen. nov., sp. nov.. Strains of this novel species were identified in both water bodies associated with the Cae Coch pyrite ore. That is the Trefriw Wells Spa and Cae Coch mine sites. These could not be isolated in the present study, so an isolate from Parys Mountain was described instead. This species likely contributes to iron cycling at the sites by oxidising and reducing conditions.

8.8. Description of *Acidithrix* gen. nov.

Acidithrix (A.ci.di.thr'ix. N.L. n. *acidum* (from L. adj. acidus, sour), an acid; Gr. fem. n. thrix, hair, thread; N.L. fem. n. *Acidithrix*, acid thread).

Grows as long (>50 μm) filaments. Individual cells are 2–3 μm long by ~0.2 μm wide, motile, and stain Gram-positive. Does not form endospores. Acidophilic, mesophilic and heterotrophic. Capable of both oxidizing ferrous iron and reducing ferric iron. The peptidoglycan contains *meso*-diaminopimelic acid. The predominant fatty acids are iso-$C_{16:0}$, $C_{15:0}$ and iso-$C_{16:1}H$, and the major respiratory quinone is MK-9(H_4). Phylogenetically affiliated (from comparative gene sequences) to the order *Acidimicrobiales* and the phylum *Actinobacteria*. Type species is *Acidithrix ferrooxidans*, and the description of the genus is based on the type species, as it is currently the only species ascribed to this genus.

8.9. Description of *Acidithrix ferrooxidans* sp. nov.

Acidithrix ferrooxidans fer.ro.ox.id.ans. L. n. *ferrum*, iron; Gr. adj. *oxus*, sharp, acid; N.L. v. oxido, to make acid, to oxidize; N.L. part. adj. *ferrooxidans* iron-oxidizing.

Morphological, cultural properties, chemotaxonomic and phylogenetic features are as described for the genus. Grows at 10–30 °C and at pH 2.0 to 4.4 (but not at pH 5.5).

The type strain is PY-F3 (=DSM 28176^T, =JCM 19728^T), which was isolated from macroscopic streamer growths in an acidic stream draining an abandoned copper mine (Mynydd Parys; Anglesey, Wales). The DNA G + C content of the type strain is 57.4 mol%.

Chapter 9. Development of a novel continuous flow microbiological reactor for oxidising ferrous iron in acidic mine waters

9.1. Introduction

As described in chapter 8, the iron-oxidising acidophile *Acidithrix ferrooxidans* was isolated from an acid streamer microbial community in Mynydd Parys, North Wales, where it accounts for ~5-15% of the microbial community (from semi-quantitative T-RFLP analysis; Kay *et al.*, 2013). This species is also found at Trefriw Wells spa and Cae Coch sites. A distinguishing feature of *Acidithrix ferrooxidans* was the formation of macroscopic streamer-like growths. A streamer is a macroscopic microbial growth, which attaches to a solid substrate and forms filamentous structures that freely oscillate in the surrounding fluid (Rusconi *et al.*, 2010).

Iron (as ferrous) is frequently the most abundant soluble metal present in acid mine drainage waters (Hallberg, 2010), including that of Cae Coch sulfur mine (Kimura *et al.*, 2011). Oxidation of ferrous to ferric iron facilitates its removal from solution, as ferric iron is less soluble than ferrous at the pH range of this section. Microbes catalyse this process, as discussed in previous chapters. A number of studies made use of this catalysis ability by creating systems to remediate AMD, which included a biological component in the form of a bioreactor containing iron-oxidising acidophiles (e.g. Koseoglu-Imer and Keskinler, 2013; Chowdhury and Ojumu, 2014; Hedrich and Johnson, 2012). However, a large proportion of the studies focused on *Acidithiobacillus ferrooxidans* as the single or main biological catalyst.

During investigations into the specific rate of oxidation by *Atx. ferrooxidans,* the type strain of this species was observed oxidising iron at pH and temperature values outside those of its growth range (pH growth minimum 2.0, iron oxidation recorded at pH 1.5; temperature

minimum 10°C, iron oxidation recorded at 5°C). This suggested that *Atx. ferrooxidans* might be a useful bacterium to use for accelerated iron oxidation in mine waters. As *Atx.* ferrooxidans is heterotrophic, it would also allow more rapid establishment of biomass in comparison to autotrophic species such as *At. ferrooxidans.* The requirement for support materials for biomass development could be largely eliminated because *Atx.* ferrooxidans forms streamer-like filamentous growth in flowing waters, maximising the working volume of the reactor vessel.

This section of the study had two aims. The first was to establish a ferrous iron-oxidising bioreactor (FOB) using *Atx. ferrooxidans,* and the second was to test this reactor using actual mine water to see (i) how the system performed with the addition of AMD and (ii) how the composition of the microbial community within the bioreactor changed over time. This system could then be applied to Cae Coch AMD amelioration. Parys Mountain AMD was used in this study because of ease of access and water collection in comparison to Cae Coch AMD, though this issue of iron remediation is also being considered for Parys Mountain (Younger and Potter, 2012).

9.2. Establishment of an *Atx. ferrooxidans* ferrous iron-oxidising bioreactor (FOB)

A continuous flow bioreactor was set up using a pure culture of *Atx. ferrooxidans* as the inoculum. This system was chosen as continuous liquid flow was considered to encourage streamer growth of *Atx. ferrooxidans,* washing out planktonic cells and retaining those that attached to surfaces within the reactor vessel. In this way, filamentous macroscopic growths would be encouraged and retained in the reactor.

9.2.1. Methods

A 2 L Nalgene bioreactor vessel fitted with an inflow and an outflow port was used in this experiment. No pH or temperature control was possible with this vessel, so the operating temperature was 21-25 ºC (room temperature). The bioreactor was operated with a working liquid volume of 1.33 L and variable flow rates (as described below). Sterile air was delivered (~0.25 L/min) via the inlet tube (Fig. 9.1) providing the only agitation in the vessel. Liquid medium was pumped in and out of the vessel using Ecoline peristaltic pumps (Ismatec, Germany) and calibrated peristaltic tubing (Elkay, U.K).

Liquid medium in the reactor vessel contained per L; 5 mM glucose, 0.01% yeast extract, 12.5 mL basal salts #1, 0.5 mM Fe^{2+}, at pH 2.5 as growth medium.

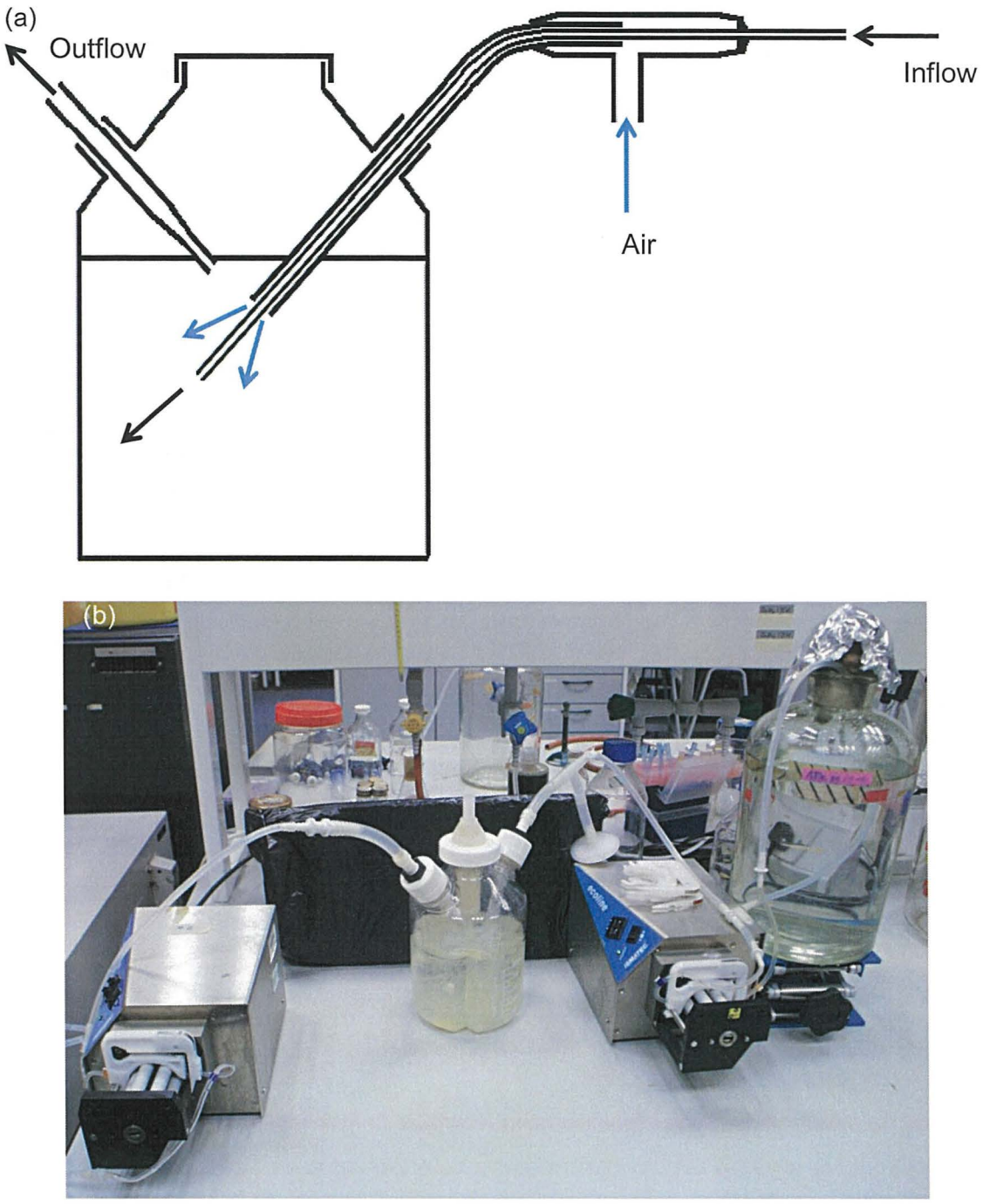


Figure 9.1. (a) Schematic representation of the continuous flow reactor. Black arrows represent media flow and blue arrows represent airflow. (b) Image of the bioreactor, feed and drain vessels, and pumps used to input and to drain the reactor vessel.

The sterilised reactor vessel was inoculated with an active culture of *Atx. ferrooxidans*. *Atx. ferrooxidans* biomass had accumulated in the bioreactor after 4 days, and continuous flow (F) of liquid medium (1 mM glucose, 0.001% yeast extract, half-strength basal salts #1, 0.5

mM Fe^{2+}, at pH 2.0-2.5) initiated at the rate of 66 mL/h (corresponding to a dilution rate (D) of 0.05 h^{-1} and a hydraulic retention time (HRT) of 20 h as:

$$D = {}^{F}/_{V}$$

$$HRT = {}^{1}/_{D}$$

Continuous flow continued for a further 10 days to further encourage filamentous biomass development within the bioreactor vessel (Fig. 9.2).

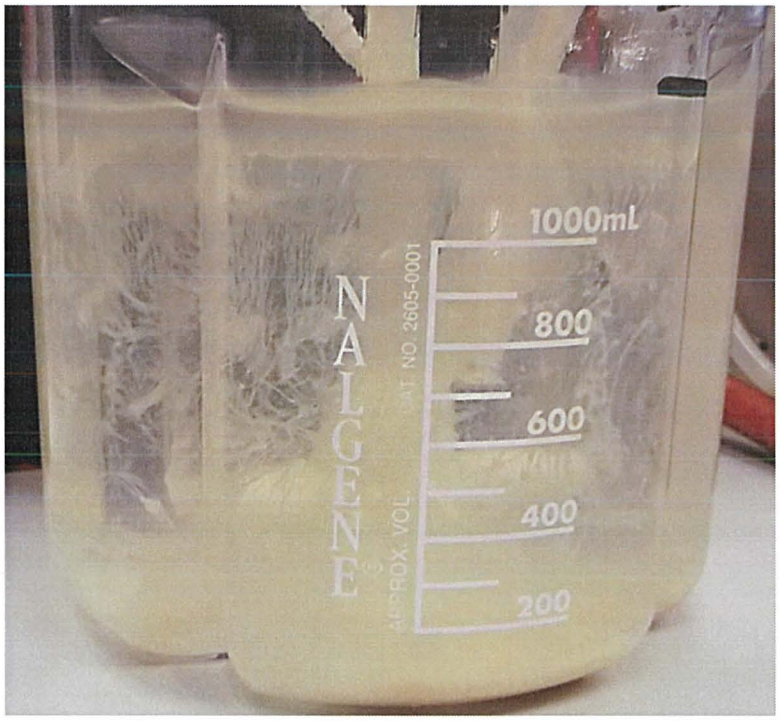


Figure 9.2. Established *Atx. ferrooxidans* biomass in the continuous flow bioreactor. White filaments are *Atx. ferrooxidans* streamers, attached to vessel walls.

The bioreactor was operated with gradually increasing flow rates after day 14, and changes in water chemistry recorded. These were: pH, E_H, concentrations of total and ferrous iron and ferrous (Ferrozine assay) in both influent and effluent liquors. For each flow rate, measurements were made regularly until the measured parameters had stabilised (when three consecutive samples were taken that had approximately the same concentrations in the effluent liquor, the system was considered to have reached equilibrium for that particular

flow rate). The pH of the influent liquor was 2.2 for the first 14 days of this experiment, and this was lowered to pH 2.0 for the following 3 days.

9.2.2. Results

Data from this experiment are shown in Table 9.1 and Fig. 9.3 (for pH 2.2 influent liquor) and Table 9.2 and Fig. 9.3 (for pH 2.0 influent liquor). Rates of ferrous iron oxidation were calculated from the difference between concentrations of Fe^{2+} in influent and effluent liquors and flow rates. This was then standardised for a 1 L bioreactor volume. Rates of ferric iron hydrolysis were calculated from differences in total iron concentrations in influent and effluent liquors, the assumption being that only ferric iron was hydrolysed and precipitated at the pH of the system.

The *Atx. ferrooxidans* FOB successfully facilitated the oxidation of ferrous iron (evidenced both by large differences in concentrations of Fe^{2+} in influent and effluent liquors, and increased E_H values of >200 mV). Fig. 9.3 shows that rates of iron oxidation increased as the flow rates were increased in the first experiment (pH 2.2 influent liquor). There was a strong positive correlation between these two parameters (r^2 =997). This was considered likely because of increasing accumulation of biomass in the reactor vessel with time, as the influent liquor contained some glucose and yeast extract, as well as ferrous iron. Between 93 and 98% of the ferrous iron in the feed liquor was oxidised. Concentrations of total iron in the effluent liquors were always slightly less than those in the influent liquors due to partial hydrolysis of the ferric iron produced. However, rates of ferrous iron oxidation were always far greater than those of ferric iron hydrolysis (by more than an order of magnitude). Given that oxidation of one ferrous iron consumes one proton (equation [1.2]) and ferric iron hydrolysis of one ferric iron generates three protons (equation [1.3]), the combined effect of these reactions should have been net consumption of protons and therefore a higher pH in

the effluent than the influent liquors. However, little change in pH values was observed in this experiment.

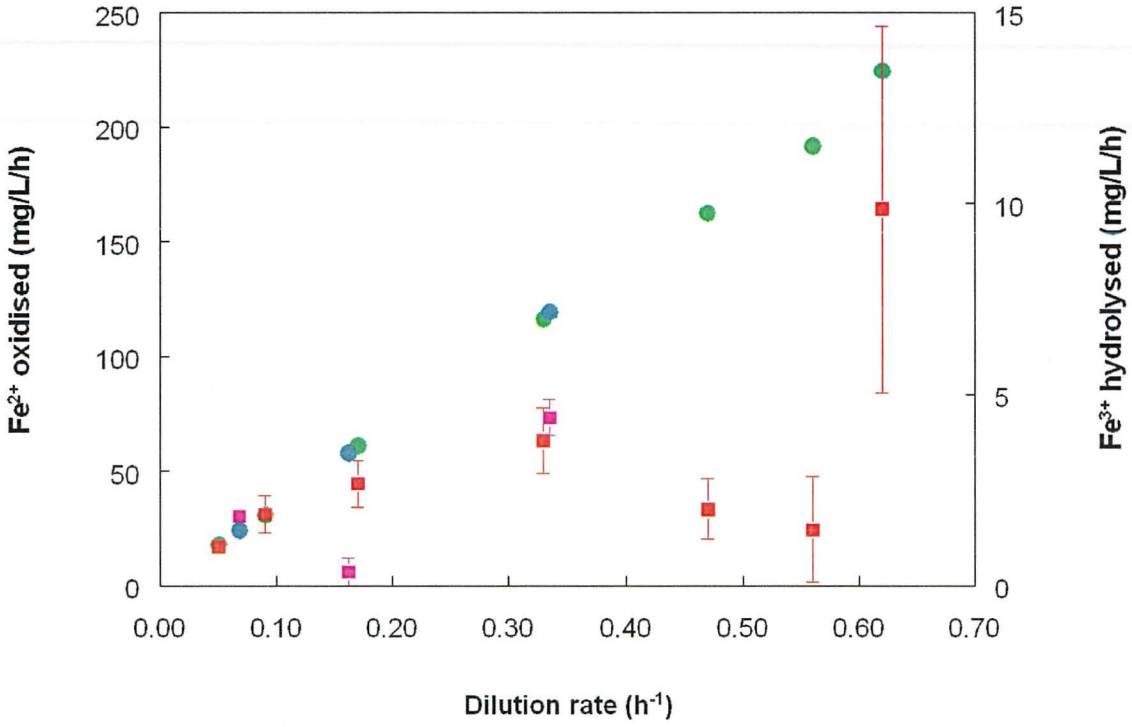


Figure 9.3. Scatter plots of rates of ferrous iron oxidation (●,●) and ferric iron hydrolysis (■, ■) for the FOB fed with pH 2.2 (●, ■) or pH 2.0 (●,■) influent liquor. Error bars (where visible) depict standard deviations.

Table 9.1. Hydraulic and chemical parameters of the *Atx. ferrooxidans* FOB operated at different flow rates with pH 2.2 influent liquor. All concentrations are mg/L. Rates of ferrous iron oxidation and ferric iron hydrolysis are means and standard deviations of triplicate analyses.

Date	F	D	HRT	Fe^{2+}			Fe_{total}		Fe^{3+}	E_H		pH
	mL/h	h^{-1}	H	influent	effluent	oxidised mg/L/h	Influent	Effluent	hydrolysed mg/L/h	influent	effluent	effluent
26 - 27/ 02/14	66	0.05	20	375	7.8 +/- 0.8	18.23 +/- 0.05	387	366 +/- 1.4	1.04 +/- 0.07	625	845 +/- 6	2.3 +/- 0.1
03 - 04/ 03/14	120	0.09	11	351	6.3 +/- 1.4	31.10 +/- 0.11	360	339 +/- 5.5	1.89 +/- 0.49	627	837 +/- 12	2.2 +/- 0.0
04 - 05/ 03/14	230	0.17	5.81	362	7.8 +/- 1.1	61.30 +/- 0.13	364	352 +/- 3.6	2.69 +/- 0.61	599	852 +/- 10	2.2 +/- 0.2
05 - 06/ 03/14	435	0.33	3.06	364	7.3 +/- 1.1	116.4 +/- 0.27	371	359 +/- 3.1	3.82 +/- 0.86	629	852 +/- 2	2.2 +/- 0.0
06/03/14	620	0.47	2.15	364	15.1 +/- 0.8	162.5 +/- 0.22	371	366 +/- 2.0	2.02 +/- 0.79	629	820 +/- 4	2.2 +/- 0.1
07/03/14	740	0.56	1.80	368	23.3 +/- 2.5	191.7 +/- 2.1	369	366 +/- 2.6	1.48 +/- 1.38	649	842 +/- 15	2.2 +/- 0.4
11/03/14	820	0.62	1.62	371	6.5 +/- 0.8	224.6 +/- 0.28	371	355 +/- 9.8	9.86 +/- 4.80	544	845 +/- 13	2.2 +/- 0.1

Table 9.2. Hydraulic and chemical parameters of the *Atx. ferrooxidans* FOB operated at different flow rates with pH 2.0 influent liquor. All concentrations are mg/L. Rates of ferrous iron oxidation and ferric iron hydrolysis are means and standard deviations of triplicate analyses.

Date	F	D	HRT	Fe^{2+}			Fe_{total}		Fe^{3+}	E_H		pH
	mL/h	h^{-1}	H	influent	effluent	oxidised mg/L/h	Influent	effluent	hydrolysed mg/L/h	influent	effluent	effluent
12/03/14	90	0.068	15	365	5.95 +/- 0.32	24.30 +/- 0.02	367	339 +/- 2.08	1.84 +/- 0.14	568	859 +/- 6	2.0 +/- 0.1
13/03/14	215	0.162	6	365	6.14 +/- 0.56	58.01 +/- 0.09	367	365 +/- 2.31	0.37 +/- 0.37	568	858 +/- 6	2.0 +/- 0.0
14/03/14	445	0.335	3	365	7.18 +/- 0.96	119.5 +/- 0.32	373	360 +/- 1.52	4.41 +/- 0.47	621	853 +/- 6	2.0 +/- 0.2

The behaviour of the FOB was very similar with pH 2.0 influent liquor. Similar rates of ferrous iron oxidation of the FOB were observed (Table 9.2 and Fig. 9.3) and there was a strong positive correlation (r^2 = 0.999) between flow rates and rates of iron oxidation. Ferric iron hydrolysis was also far less rapid than ferrous iron oxidation, and there was little change in the pH of the liquid that was processed.

On day 17 (14/03/14), yeast cells were observed in effluent liquor from the FOB. The contaminated bioreactor was therefore decommissioned and a new one set up, as described above.

9.3. Effect of influent liquor pH on ferrous iron oxidation by the *Atx. ferrooxidans* FOB

9.3.1 Methods

The newly-established *Atx. ferrooxidans* FOB was operated at a fixed flow rate (350 mL/h, corresponding to a dilution rate of 0.26 h^{-1} and a HRT of 3.8 h). The pH of the influent liquor (which had the same chemical composition as before; section 9.2) was gradually decreased from pH 2.10 to pH 1.37, because grown biomass was observed to oxidise ferrous iron at pH values as low as 1.5 (section 8.3.3) despite a growth minima of pH 2.0. Therefore, the possibility that the *Atx. ferrooxidans* FOB could operate effectively outside of the bacterial growth range was examined.

9.3.2. Results

Results are shown in Table 9.3 and Fig. 9.4. Ferrous iron oxidation occurred at all of the pH values tested (as evidenced by lower concentrations of Fe^{2+} in effluent liquors, and more positive E_H values). Rates of iron oxidation were negatively correlated with influent pH (r^2 = 0.94) while there was no apparent correlation between rates of ferric iron hydrolysis and pH. Even at pH 1.38 (the lowest effluent pH and therefore that within the bioreactor itself), the rate of ferrous iron oxidation was ~13% of that at pH 2.16. In most cases, the pH of the

effluent liquors was higher than those of the influent liquors, presumably as a result of proton-consuming ferrous iron oxidation.

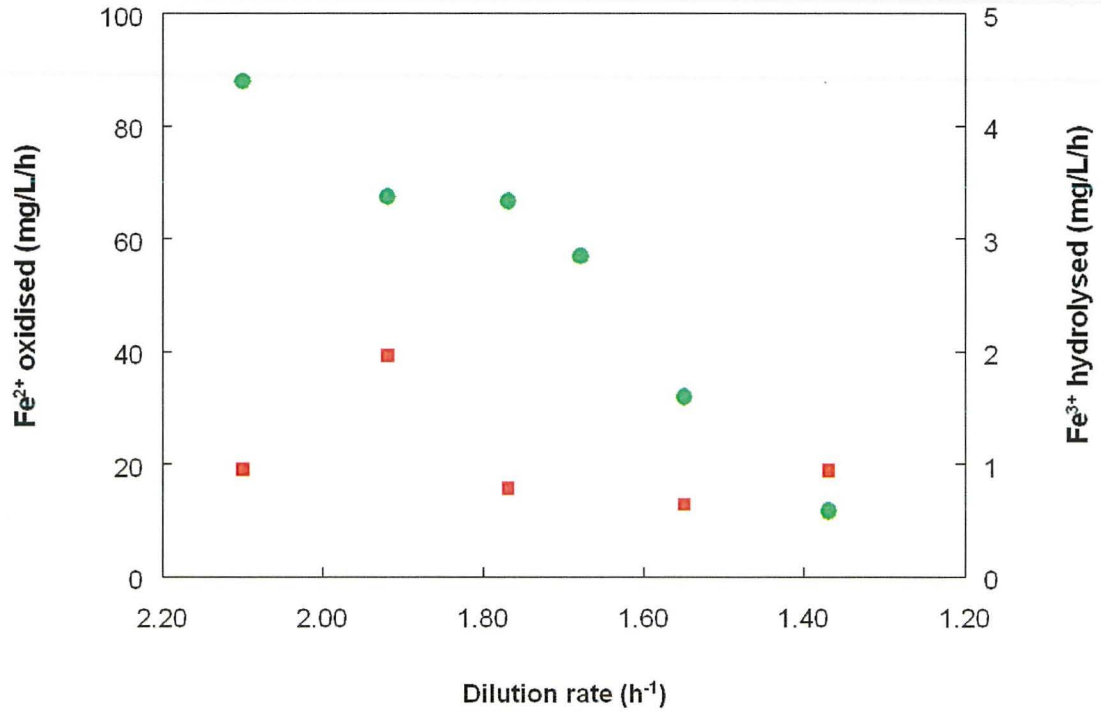


Figure 9.4. Scatter plots of rates of ferrous iron oxidation (●) and ferric iron hydrolysis (■) for the FOB fed with influent liquor with pH 1.37 – 2.10, at a constant flow rate of 350 mL/h, corresponding to a dilution rate of 0.26 h^{-1} and a HRT of 3.8.

Table 9.3. Hydraulic and chemical parameters of the *Atx. ferrooxidans* FOB operated at variable pH influent liquors, operated at a flow rate of 350 mL/h, corresponding to a dilution rate of 0.26 h^{-1} and a HRT of 3.8 h. All concentrations are mg/L. Rates of ferrous iron oxidation and ferric iron hydrolysis are means of duplicate analyses.

Date	pH		Fe^{2+}			Fe_{total}		Fe^{3+} hydrolysed mg/L/h	E_H	
	influent	effluent	influent	effluent	oxidised mg/L/h	influent	effluent		influent	effluent
20-21/ 03/14	2.10	2.16	367	32.0	88.0	371	367	1.13	601	793
28-29/ 03/14	1.92	1.99	366	109	67.5	376	368	1.97	599	759
29-30/ 03/14	1.77	1.82	372	118	66.7	383	380	0.79	612	780
02-03/ 04/14	1.68	1.60	349	132	57.0	367	366	0.26	605	682
05-06/ 04/14	1.55	1.66	370	248	32.1	374	372	0.65	609	712
16-17/ 04/14	1.37	1.38	384	338	12.1	395	391	1.10	604	661

9.4. Oxidation of ferrous iron present in acidic, metal-rich water draining the abandoned Mynydd Parys copper mine, using *Atx. ferrooxidans* FOBs

Having established that an *Atx. ferrooxidans* FOB could be used to accelerate the oxidation of ferrous iron in acidic liquors, it was decided to determine the performance of the bioreactor when challenged with actual mine water draining a metal mine site. This would involve three major differences in the operational parameters used in sections 9.2 and 9.3, namely: (i) *Atx. ferrooxidans* would be exposed to transition metals other than iron, some of which may inhibit its growth and/or iron oxidation capacity; (ii) the only organic carbon present in the feed liquor would be that in the mine water itself, not all of which would be likely to be metabolised by *Atx. ferrooxidans*; (iii) other bacteria, indigenous to the mine water and including species of autotrophic iron-oxidising acidophiles, would enter and possibly colonise the bioreactor, potentially displacing *Atx. ferrooxidans* as the dominant bacteria. The effects of the above parameters on the performance of two *Atx. ferrooxidans* FOBs were assessed in a similar way to that described in section 9.2 and 9.3. The composition of microbial populations in the effluent liquors over time and the biomass retained in the bioreactor was also investigated, using a combined molecular and cultivation-based approach.

9.4.1. Collection of mine water from Mynydd Parys

Large volume (20 – 60 L) acid mine drainage water samples were collected from the Afon Goch, an outflow drainage adit from the abandoned Mynydd Parys copper mine, North Wales (Grid ref. SH 43797 91236) on four separate occasions, between June 24 and July 14, 2014 (referred to below as "Collection batches 1 – 4"). Acid mine drainage water was collected as close as access allowed to the drain outflow pipe in large carboys (~50 m from the drainage adit), and transported to the Bangor University laboratory within 1 hour. Care was taken to exclude the macroscopic streamer growths that occupied much of the stream

bed. Collection batch 1 was filtered immediately on return to the laboratory through sterile 25 mm 0.2 µm pore size nitrocellulose membranes (Whatman) in Millipore filter holders. All mine water samples were put into a cold room (at 4°C) immediately on return to the laboratory, and removed from cold storage approximately 10-16 h before use so they could equilibrate to room temperature.

In addition to the large volume samples, approximately 250 mL of acid mine drainage was collected and filtered on site (through sterile Whatman 0.2 µm pore size membranes mounted in in 25 mm Millipore filter holders). These were also transported to the laboratory within 1 hour and stored at 4°C, with the addition of two drops of 1 M HCl to acidify the water and to retain metals in solution.

9.4.2. Analysis of Afon Goch water samples

9.4.2.1. Methods

The filtered and acidified mine water samples were analysed for transition metals (by ion chromatography; section 2.6.4), and dissolved organic carbon (DOC; using a LABTOC DOC analyser. Samples were sparged with nitrogen to remove any inorganic carbon and dissolved CO_2, followed by oxidation of the DOC to CO_2 using combined UV light exposure and addition of persulfate and nitric acid. The CO_2 evolved was measured using an infra-red detector. Standard solutions of potassium hydrogen phthalate (containing 0 – 25 mg C/L) were used for calibration.

9.4.2.2. Results

Table 9.4 shows the concentrations of the major transition metals present in the Afon Goch samples and DOC contents. In general, concentrations of the transition metals measured showed relatively little (<10%) variation. Concentrations of DOC were slightly more variable.

Table 9.4. Concentration of transition metals and DOC (mg/L) in the Afon Goch water samples.

Sample	Date	Cu^{2+}	Zn^{2+}	Mn^{2+}	Fe^{2+}	DOC
Collection batch 1	24/06/2014	39.1	57.7	14.9	442	4.39
Collection batch 2	01/07/2014	36.3	57.0	14.6	416	5.64
Collection batch 3	07/07/2014	37.5	51.0	14.5	399	5.80
Collection batch 4	14/07/2014	37.4	58.8	15.0	416	3.11
Mean values		37.2	56.2	14.5	414	4.74
Standard deviations		1.69	5.37	0.36	23.00	1.14

9.4.3. Bioreactor experiments

9.4.3.1. Methods

Two *Atx. ferrooxidans* FOBs were fed with water from the Afon Goch (initially filtered through sterile 0.2 μm membranes; Collection batch 1, but later unfiltered; Collection batches 2, 3 and 4). These were first tested sequentially (from the collection batch 1 sample) but later in parallel (i.e. both FOBs connected to the same AMD feed reservoir, for collection batches 2, 3 and 4), at different flow rates. pH, E_H, concentrations of ferrous and total iron were measured in influent and effluent liquors on a regular basis to assess the performance of the bioreactor in the same manner as sections 9.2 and 9.3. During periods (2 – 3 days) between feeding the bioreactors with different batches of Mynydd Parys AMD, they were put on 'stand-by'. For this mode of operation, the influent liquor contained: 1 mM glucose, 0.001% yeast extract, 6.25 mL basal salts #1, 0.5 mM Fe^{2+} per L, at pH 2.5. This was pumped into each FOB at the rate of ~120 mL/h for reactor 1 (equivalent to dilution rate of 0.09 h^{-1}) and 90 mL/h for reactor 1 (equivalent to dilution rate of 0.07 h^{-1}).

9.4.3.2. Results

A large proportion of the ferrous iron was removed from the AMD by being passed through the FOBs, as with the FOB containing a pure culture of *Atx. ferrooxidans* (Tables 9.5 – 9.7 and Figs. 9.5 – 9.8). This was evident in the lower concentrations of ferrous iron and increased E_H values in the effluent in comparison the influent liquor. The higher pH of the Mynydd Parys AMD in comparison to the previous experiments meant that rates of ferric iron hydrolysis were often higher than that of the previous experiments. Variable rates of iron in the inflow were a consequence of the presence of microorganisms in the untreated AMD, despite efforts to minimise oxidation of ferrous iron prior to introduction to the FOBs. Concentrations of ferrous iron in those samples that were filtered on site and acid-preserved were more consistent.

Rates of ferrous iron oxidation tended to increase as flow rates (and dilution rates) increased in both FOBs, though the percentage of total ferrous iron oxidised decreased above a D value of ~0.4 h^{-1} (Figs. 9.5 and 9.6). This was considered related to the decrease in HRT values, as time spent in the reactor vessel would have an impact on the amount of ferrous iron that could be oxidised. At a D value of 0.4 h^{-1} (corresponding to a HRT of 2.5 hours), the rate of ferrous iron oxidation was ~200 mg/L/h, and ~90% of ferrous iron in the Mynydd Parys feed was oxidised to ferric. A comparison of ferrous iron oxidation at the same flow rates (Figs. 9.7 and 9.8) showed that in general these increased with time, a factor that was possibly related to the evolution of the microbial communities in the FOBs.

There were also differences between the ferrous iron oxidation rates of the FOBs, despite receiving the same AMD samples. Rates and extents of ferrous iron oxidation rate tended to be more variable in FOB 2 than in FOB 1, though FOB 2 was a more efficient bioreactor. The relationship between rates of ferrous iron oxidation and bioreactor dilution rates was more strongly correlated in FOB 2 (r^2=0.841) than in FOB 1 (r^2=0.647), possibly due the

composition of the microbial communities that developed in the reactors as they were fed with untreated Mynydd Parys AMD (section 9.6).

Table 9.5. Hydraulic and chemical parameters of the *Atx. ferrooxidans* FOB operated at different flow rates with filtered AMD liquor (Collection batch 1). All concentrations are mg/L.

Date (2014)	F mL/h	D h^{-1}	HRT h	Fe^{2+}				Fe_{total}		Fe^{3+} hydrolysed mg/L/h*	E_H		pH	
				influent	effluent	oxidised		Influent	effluent		influent	effluent	influent	effluent
						mg/L/h*	%							
FOB 1														
24/06	120	0.09	11.1	377	44.7	30.0	88	761	442	28.7	751	808	2.56	2.38
25/06	275	0.21	4.76	344	128	44.7	63	785	772	2.69	743	784	2.60	2.61
FOB 2														
26/06	275	0.21	4.76	395	85.2	63.8	78	786	752	7.03	745	797	2.58	2.63
26/06	410	0.31	3.23	396	124	84.8	69	786	778	2.47	745	782	2.58	2.62

*mean values of duplicate samples

Table 9.6. Hydraulic and chemical parameters of the *Atx. ferrooxidans* FOB 1 operated at different flow rates with unfiltered AMD liquor (collection batches 2, 3 and 4). All concentrations are mg/L.

Date (2014)	F mL/h	D h^{-1}	HRT h	Fe^{2+}				Fe_{total}		Fe^{3+}	E_H		pH	
				influent	effluent	oxidised		Influent	effluent	hydrolysed	influent	effluent	influent	effluent
						mg/L/h*	%			mg/L/h*				
Collection batch 2														
02/07	295	0.22	4.54	420	21.7	88.3	95	782	772	2.21	737	832	2.61	2.63
03/07	380	0.29	3.45	449	32.9	119	93	788	739	14.0	735	832	2.61	2.63
04/07	460	0.35	2.86	445	18.2	147	96	789	730	20.4	739	845	2.62	2.63
	500	0.38	2.63	442	27.9	155	94	788	726	23.3	736	845	2.61	2.62
Collection batch 3														
08/07	430	0.32	3.13	440	47.7	127	89	795	711	27.1	738	810	2.62	2.57
09/07	475	0.36	2.78	597	60.9	191	90	799	727	25.7	738	801	2.63	2.58
10/07	570	0.43	2.33	470	57.8	176	88	797	739	24.8	732	795	2.62	2.58
Collection batch 4														
15/07	430	0.32	3.13	491	74.0	135	85	823	742	26.1	719	792	2.62	2.56
16/07	505	0.38	2.63	495	92.1	153	81	842	767	28.4	715	798	2.59	2.56
	630	0.47	2.13	492	102	184	79	825	745	37.8	715	770	2.60	2.58
17/07	920	0.69	1.45	468	182	197	61	807	788	13.1	721	758	2.62	2.60
	1200	0.90	1.11	514	282	209	45	817	799	16.2	710	736	2.59	2.60
	430	0.32	3.13	478	29.6	145	94	818	728	29.1	712	800	2.58	2.52

*mean values of duplicate samples

Table 9.7. Hydraulic and chemical parameters of the *Atx. ferrooxidans* FOB 2 operated at different flow rates with unfiltered AMD liquor (Collection batches 2, 3 and 4). All concentrations are mg/L.

Date (2014)	F mL/h	D h^{-1}	HRT h	Fe^{2+}				Fe_{total}		Fe^{3+}	E_H		pH	
				influent	effluent	oxidised		Influent	effluent	hydrolysed	influent	effluent	influent	effluent
						mg/L/h*	%			mg/L/h*				
Collection batch 2														
02/07	325	0.24	4.17	420	114	74.8	73	782	778	0.98	737	788	2.61	2.65
03/07	400	0.30	3.33	449	120	98.9	73	788	744	13.2	735	805	2.61	2.63
04/07	485	0.37	2.70	445	110	122	75	789	737	18.9	739	784	2.62	2.61
	510	0.38	2.63	442	85	137	81	788	738	19.2	736	784	2.61	2.61
Collection batch 3														
08/07	430	0.32	3.13	440	41.3	129	91	795	719	24.6	738	809	2.62	2.54
09/07	450	0.34	2.94	597	47.5	186	92	799	714	28.8	738	802	2.63	2.56
10/07	510	0.38	2.63	470	55.3	159	88	797	729	26.1	732	793	2.62	2.58
Collection batch 4														
15/07	430	0.32	2.23	491	18.4	151	96	823	733	29.0	719	814	2.62	2.54
16/07	460	0.35	2.86	495	24.0	163	95	842	732	38.0	715	820	2.59	2.55
	570	0.43	2.33	492	38.0	194	92	825	745	34.3	715	790	2.60	2.55
17/07	840	0.63	1.59	468	75.3	248	84	807	751	35.4	721	781	2.62	2.58
	990	0.75	1.33	514	150	270	71	817	770	35.0	736	755	2.59	2.59
	430	0.32	2.23	478	16.8	149	96	818	739	25.5	712	818	2.58	2.50

*mean values of duplicate samples

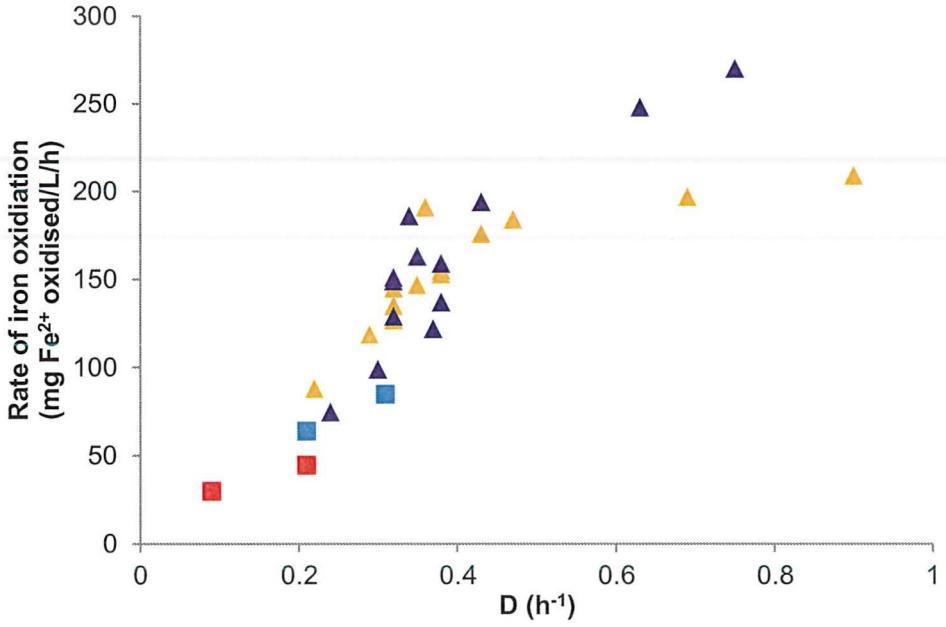


Figure 9.5. Relationship between rates of ferrous iron oxidation (Mynydd Parys AMD) and bioreactor dilution rates for FOB 1 (■, ▲) and FOB 2 (■, ▲). Tests using filtered mine waters are indicated by square symbols, and those with unfiltered mine water by triangles.

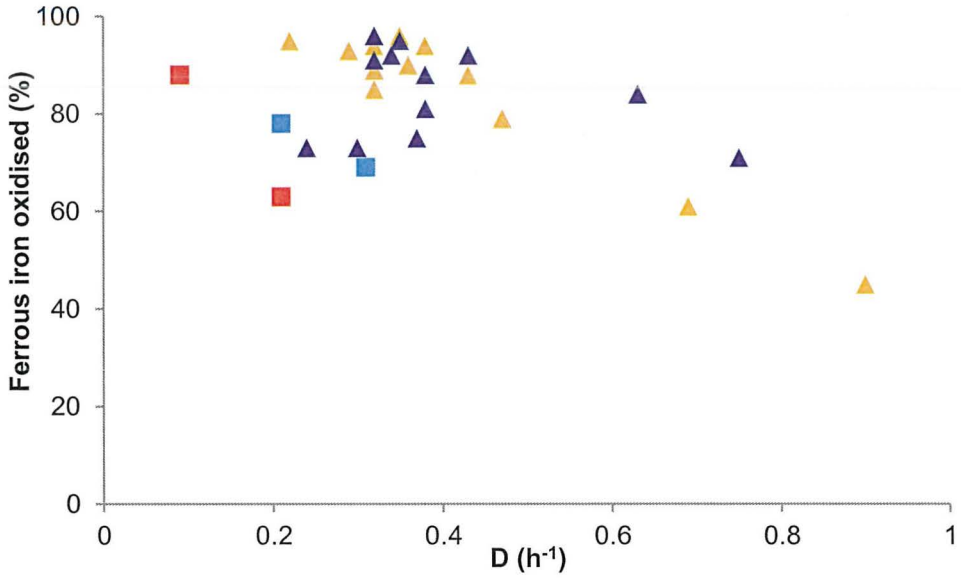


Figure 9.6. Relationship between percentage of ferrous iron oxidation (Mynydd Parys AMD) and bioreactor dilution rates for FOB 1 (■, ▲) and FOB 2 (■, ▲). Tests using filtered mine waters are indicated by square symbols, and those with unfiltered mine water by triangles.

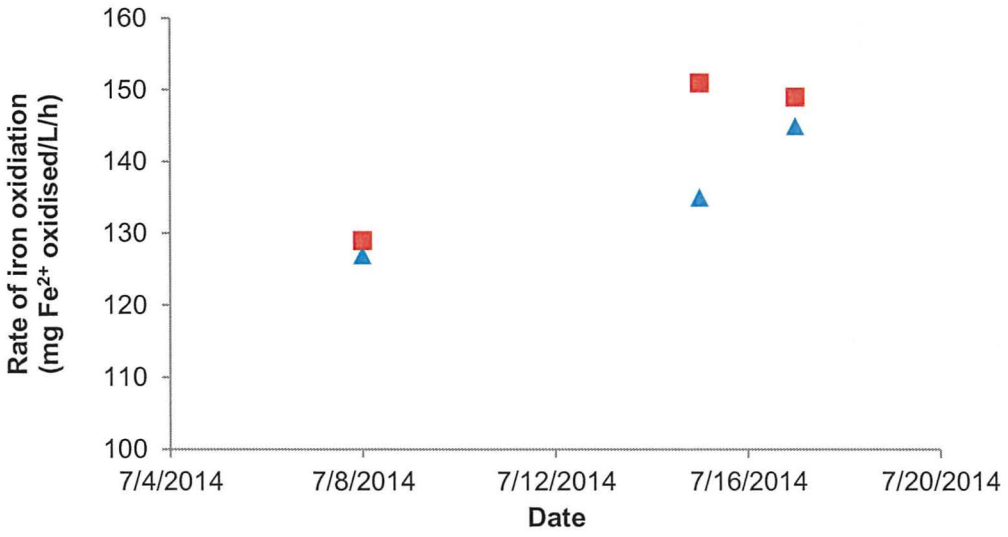


Figure 9.7. Comparison of time-related changes of rates of ferrous iron oxidation (Mynydd Parys AMD) by the FOBs, operated at a flow rate of 430 mL/h (equivalent to a dilution rate of 0.32 h^{-1}, and an HRT of 3.1 hours). Key: (▲) FOB 1; (■) FOB 2.

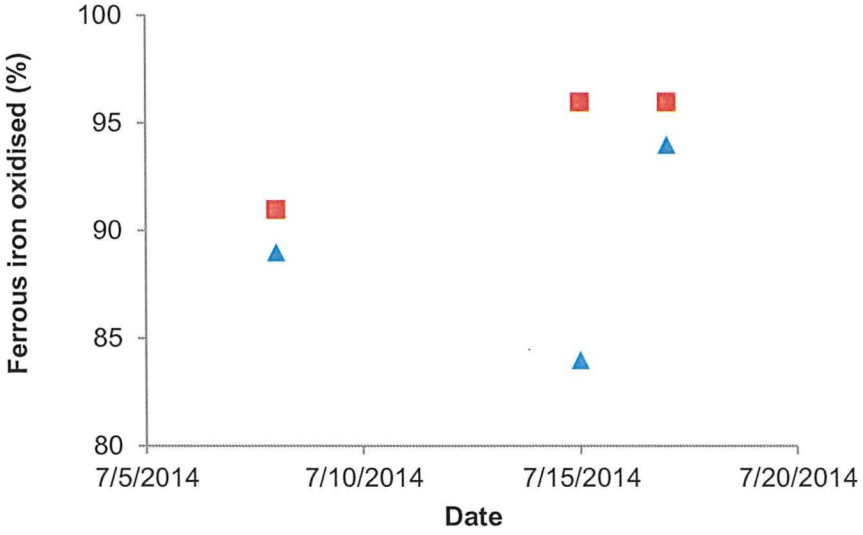


Figure 9.8. Comparison of time-related changes of percentage of ferrous iron oxidation (Mynydd Parys AMD) by the FOBs, operated at a flow rate of 430 mL/h (equivalent to a dilution rate of 0.32 h^{-1}, and an HRT of 3.1 hours). Key: (▲) FOB 1; (■) FOB 2.

9.5. Evolution of microbial communities in iron-oxidising bioreactors processing Mynydd Parys AMD

The introduction of ~200L unfiltered Mynydd Parys AMD over time to each FOB was anticipated to introduce native microorganisms into the vessels, potentially affecting the amount of ferrous iron oxidised. Microbial community development in the FOBs was monitored by T-RFLP analysis, and solid media cultivation of the bioreactor effluents. Planktonic bacteria in the effluent was analysed during the experiment and biomass analysed only on termination because removal was considered too disruptive.

9.5.1. Methods

During the experiment samples of Mynydd Parys AMD water, water draining the FOBs after the end of each collection batch and water draining the FOBs after each period on standby mode were filtered using 0.2 µm (pore size) membranes to collect biomass. DNA was extracted from these by MOBIO Soil DNA kits and community profiles determined for each sample by T-RFLP analysis (section 2.7.3).

Samples of each FOB effluent were also inoculated onto iFeo, FeSo, and YE3o solid media (section 2.2.2.3) and incubated aerobically for 14 - 21 days. Colonies were categorised by their colour and morphology (e.g. whether ferric iron-encrusted or not), DNA extracted from a representative colony of each and identified by amplification of their 16S rRNA gene sequences.

At the end of the experiment, biomass was removed from the vessels by using a sterile cell scraper. By this time, the biomass was heavily encrusted with ferric iron precipitates (Fig. 9.9 shows the reactor vessels darkening over time with increasing ferric iron encrustation and Fig. 9.10 shows the heavily encrusted biomass at the end of the experiment), requiring several washes with 50 mM oxalic acid and finally TE buffer (section 2.7.1.1.) to remove.

DNA was extracted from this by MOBIO Soil DNA kit biomass and treated as for the filtered samples above. Disrupted biomass that had not been washed with oxalic acid was streak-inoculated onto iFeo, FeSo, and YE3o solid media (section 2.2.2.3).

Figure 9.9. Images of the reactors after (a) collection batch 2 Mynydd Parys water (b) collection batch 3 Mynydd Parys water and (c) collection batch 4 Mynydd Parys water was passed through.

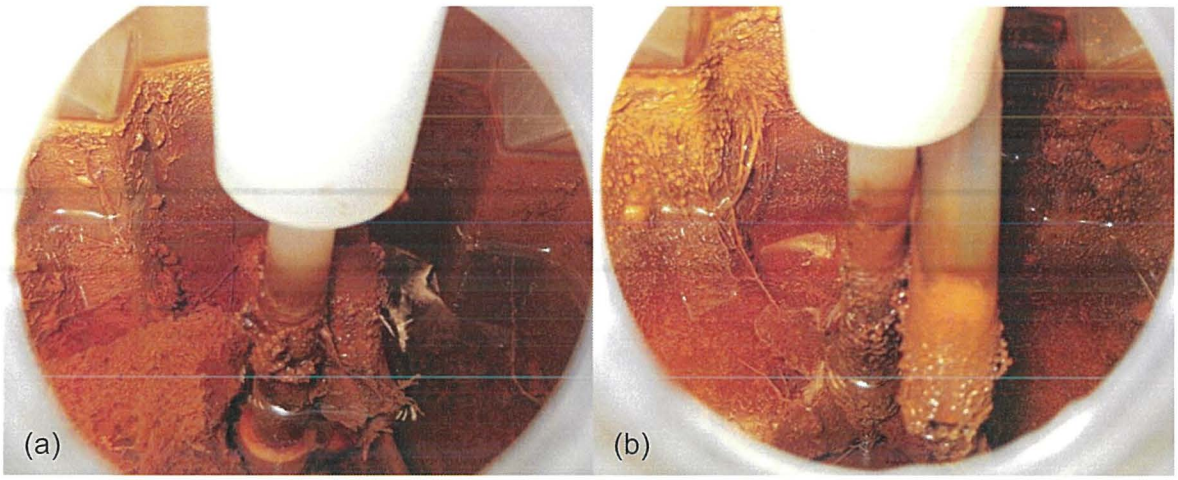


Figure 9.10. Images of the inside of the (partially drained) bioreactors showing ferric iron precipitate encrusted streamer growth. (a) reactor 1 and (b) reactor 2.

9.5.2. Results

T-RFLP profiles presented in Figs 9.11 – 9.14 were obtained with AluI digests only (AluI and CfoI digests were also used as comparisons). Digests using this enzyme gave the best T-RF species separation of the acidophilic bacteria encountered in the study (out of enzymes AluI, CfoI and HaeIII).

Fig. 9.11 shows the T-RFs identified from the bacterial populations present in Mynydd Parys mine water at the four sampling times.

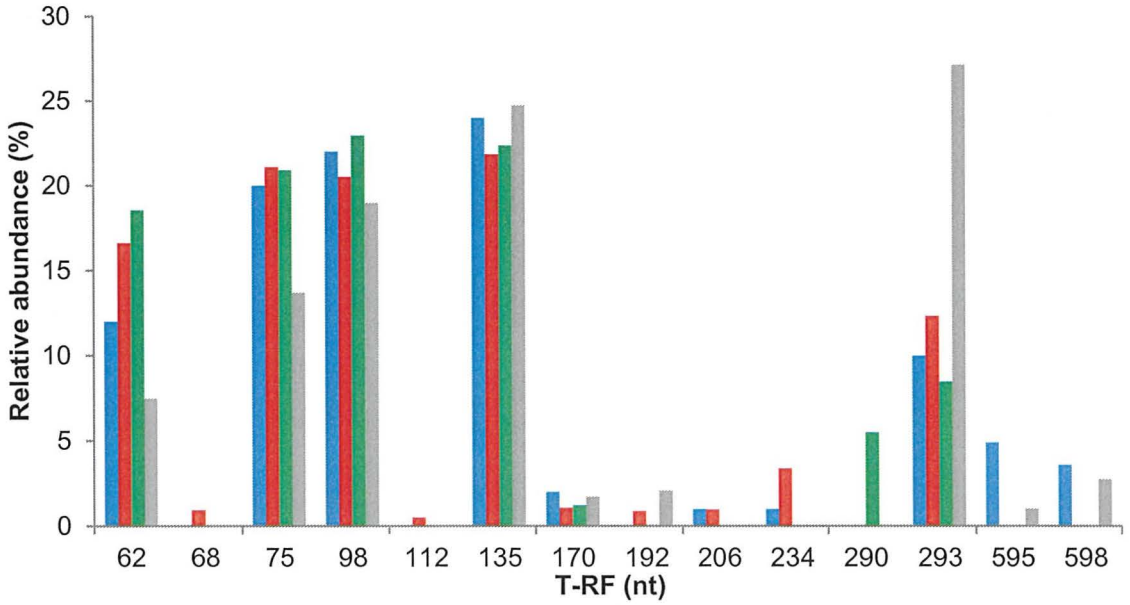


Figure 9.11. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracted from Mynydd Parys AMD and digested by the restriction enzyme AluI. Key: (■) collection batch 1; (■) collection batch 2; (■) collection batch 3, (■) collection batch 4.

There were five major T-RFs in AluI digests on all sampling occasions, but only one could be identified from the BART database. This was T-RF 135 nt, corresponding to either the iron-reducing *Proteobacterium Metallibacterium scheffleri* or the iron-oxidizing *Actinobacteria Fm. acidiphilum*. T-RFs obtained from CfoI and HaeIII digests suggested that the identity of T-RF was more likely to be *Metallibacterium scheffleri*. Several of the lesser abundant bacteria were identified from their T-RF lengths: *Atx. ferrooxidans* (68 nt), "*Fv. myxofaciens*" (170 nt), "*Ac. acidivorans*" (206 nt) and *At. ferrivorans* (234 nt).

T-RFLP profiles of *Atx. ferrooxidans* T-RFs only was obtained from the FOBs prior to the addition of Mynydd Parys AMD as influent liquor, confirming initial culture purity in the reactors. T-RFLP profiles showed T-RFs corresponding to *Atx. ferrooxidans* present ~50% relative abundance after the addition of filtered AMD from collection batch 1. The presence of other bacteria in in the FOBs after the addition of filtered AMD was confirmed by microscopic examination of effluent and plating of effluent samples, meaning that the filtration process had not been completely effective. T-RFs corresponding to *Atx.*

ferrooxidans became considerably less relatively abundant after the introduction of unfiltered AMD to the FOBs, with other T-RFs becoming more relatively abundant.

The T-RFLP profiles showed differences between the bacterial communities in the FOBs. T-RFs of 70, 98, 134/135, 170, 233/234, 292 and 294/295 nt length were present in both bioreactors after the addition of unfiltered AMD whereas those of 61/62, 74, 90 and 100 nt length were evident only in the T-RFLP profiles of FOB #2 (Figs 9.12 and 9.13). Although some of these T-RFs were present in the Mynydd Parys AMD profiles (61/62, 98, 135, 170, 234 and 293 nt) others were not (e.g. the 70 nt length T-RF). The T-RF corresponding to "*Fv. myxofaciens*" was present in both FOBs, but particularly abundant in FOB #1. Other bacteria identified from their T-RF lengths were *At. ferrivorans* (233/234 nt), *Ac. aromatica* (208 nt) and *Metallibacterium scheffleri/Fm. acidiphilum* (135 nt). Chapter 5 discusses limitations of T-RFLP analysis.

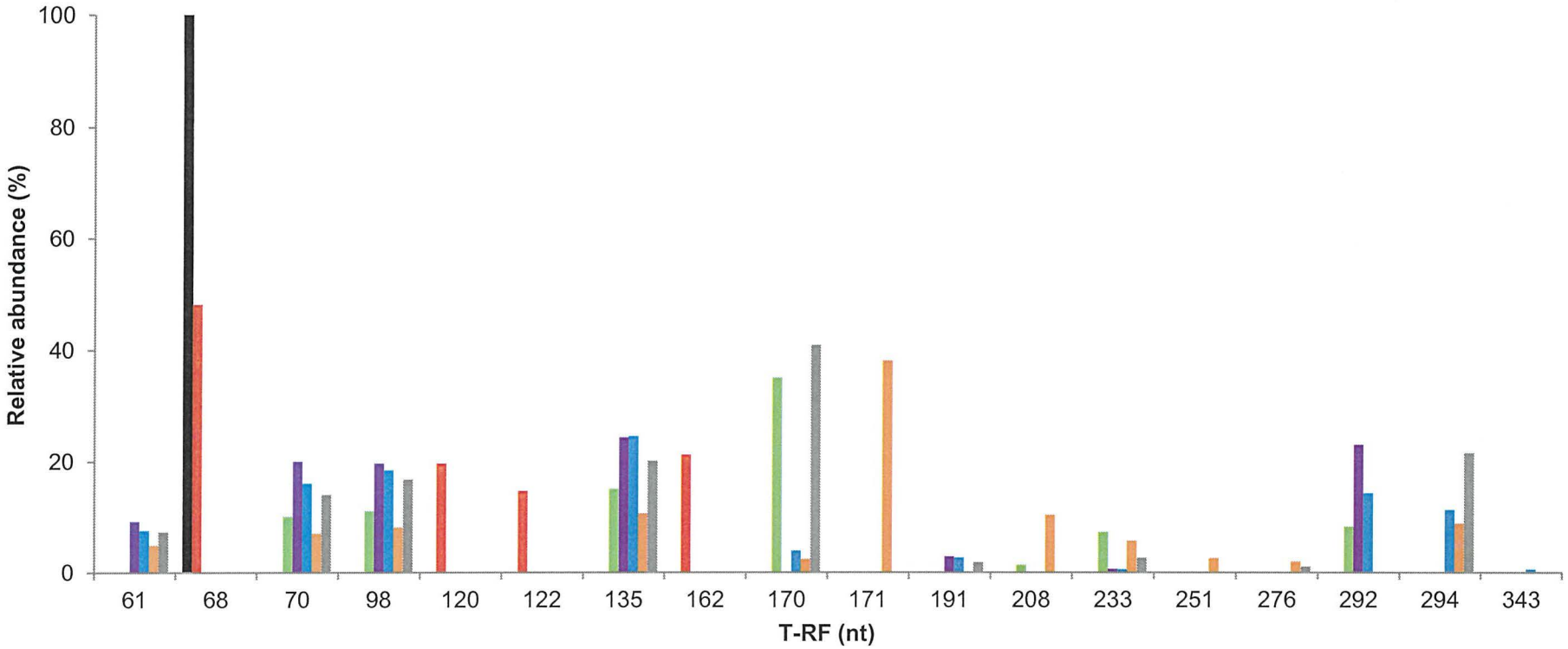


Figure 9.12. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracted from mine water draining FOB #1, and digested with the restriction enzyme AluI. Key: (■) prior to Mynydd Parys AMD as influent liquor; (■) after processing collection batch 1 (filtered mine water); (■) after processing collection batch 2; (■) prior to processing collection batch 3; (■) after processing collection batch 3; (■) prior to processing collection batch 3; (■) after processing collection batch 4.

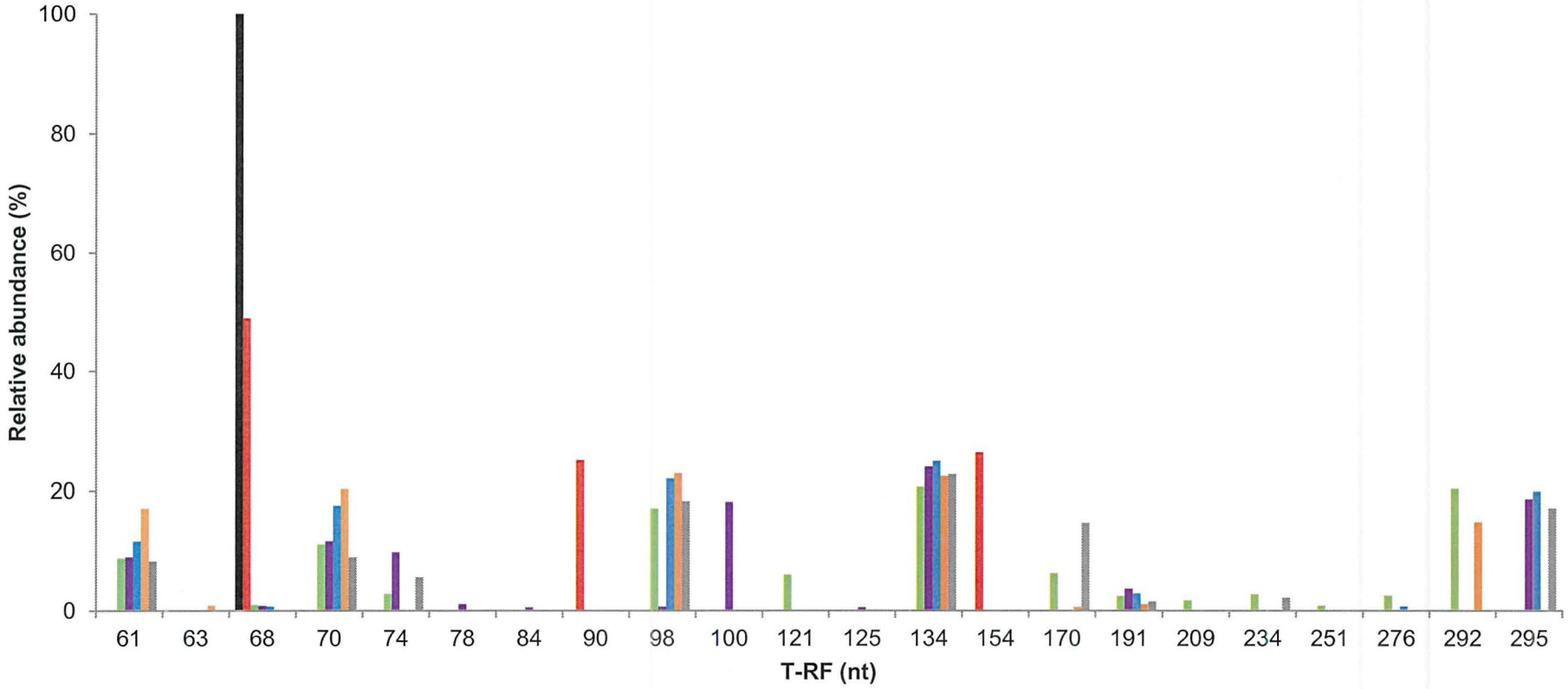


Figure 9.13. T-RFLP profiles of bacterial 16S rRNA genes amplified from DNA extracted from mine water draining FOB #2, and digested with the restriction enzyme AluI. Key: (■) prior to Mynydd Parys AMD as influent liquor; (■) after processing collection batch 1 (filtered mine water); (■) after processing collection batch 2; (■) prior to processing collection batch 3; (■) after processing collection batch 3; (■) prior to processing collection batch 3; (■) after processing collection batch 4.

The T-RFLP profiles of ferric iron-encrusted biomass taken from the FOBs at the end of the experiment (after ~200 L of AMD had flowed through the FOBs) are shown in Fig. 9.14.

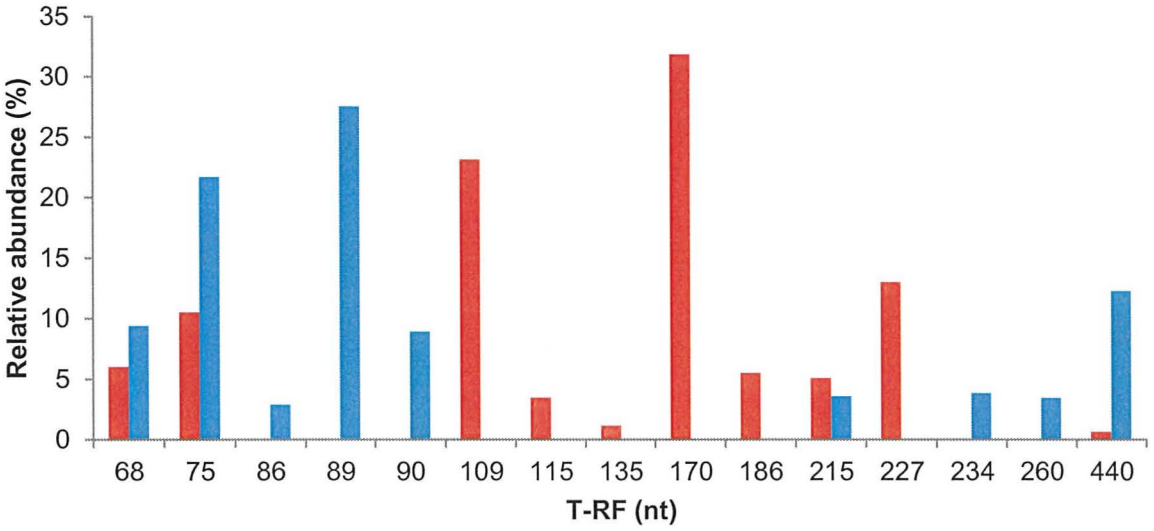


Figure 9.14. T-RFLP profiles of amplified 16S rRNA genes of biomass taken from FOB #1 (■) and FOB #2 (■), digested with the restriction enzyme AluI, from passage of ~200 L of Mynydd Parys mine water through reach bioreactor.

T-RFs common to both FOBs were: *Atx. ferrooxidans* (T-RF 68; ~6-9% relative abundance), T-RF 75 nt (also identified in the Mynydd Parys AMD) and a T-RF at 215 nt, which was tentatively identified as *L. ferrooxidans*. However, while FOB #1 biomass had the streamer-forming iron-oxidising acidophile "*Fv. myxofaciens*" as a major member of the microbial community, its signature T-RF was not found in FOB #2. Major T-RFs of 89 and 90 nt length were found in the FOB #2 profile but not that of FOB #1.

Relatively few different species of acidophilic bacteria were isolated from the bioreactor effluents (Table 9.8). These were two species of iron-oxidising autotrophs that were isolated on a regular basis ("*Fv. myxofaciens*" and *At. ferrivorans*) and two species of iron-reducing heterotrophs that were isolated more sporadically ("*Ac. acidivorans*" and *A. cryptum*).

Table 9.8. Identity of bacteria isolated from effluents of the FOBs at the end of testing with each of the four "collection batches" of AMD used.

(+) indicates that the bacterial species was isolated at that time, and (-) that it was not.

Isolate identity*	Solid medium	Collection batch 1		Collection batch 2		Collection batch 3		Collection batch 4	
		Reactor 1	Reactor 2	Reactor 1	Reactor 2	Reactor 1	Reactor 2	Reactor 1	Reactor 2
"Fv. myxofaciens"	iFeo	+	+	+	+	+	+	+	+
At. ferrivorans	So	+	+	+	+	+	+	+	+
"Ac. acidivorans"	YE3o	+	+	+	-	+	+	+	+
A. cryptum		-	+	-	+	-	-	-	-

9.4. Discussion

Results from these experiments demonstrated that *Atx. ferrooxidans* can be used successfully for oxidising ferrous iron present in acidic liquors in continuous-flow bioreactors. This was demonstrated by using a low pH ferrous iron solution amended with glucose and yeast extract (to support the bacterial streamer growths) and AMD from the abandoned Mynydd Parys copper mine. In principle, this system could also be used for other sites such as that of Cae Coch mine, provided the conditions present were initially suitable for *Atx. ferrooxidans*.

Various types of iron-oxidising bioreactors have been described in the literature, designed for use in different contexts. One of these is the generation of ferric iron-rich lixiviants for indirect oxidation of sulfide minerals (ferric iron-generating bioreactors, e.g. Kaksonen *et al.*, 2014). In this investigation, the aim was to maximise the generation of ferric iron, requiring solution pH values of 2 or below. Systems of this type require prokaryotes used for oxidising the ferrous iron to be tolerant of both extremely acidity and very elevated concentrations of ferric iron. Such prokaryotes include the bacterium *L. ferriphilum* and the archaeon *Ferroplasma acidiphilum*. The most commonly used prokaryote in this context is *At. ferrooxidans*, though the complex and changing taxonomy of this species means that the strains used may no longer be *At. ferrooxidans*. In addition, it is not always clear from study reports whether pure cultures or consortia of strains were used. Widely differing rates of ferrous iron oxidation have been claimed for these bioreactors (Table 9.9), ranging from <1 to 78 g Fe^{2+} oxidised/L/hour, though most reports are for <10 g Fe^{2+} oxidised/L/hour.

Table 9.9. Examples of different types of FOBs reported in the literature.

Bioreactor type	Application	Support material	Culture used	pH T (°C)	Fe^{2+} oxidation (g/L/h)	Reference
Rotating biological contactor	AMD treatment	polyethylene	not specified	2.6-3.2 9-12	0.34	Olem and Unz, 1977
Inverse fluidised bed	not specified	perlite-covered polystyrene	*At. ferrooxidans*	1.3-2.2 13-38	1.68	Karamenev and Nikolov, 1988
Circulating bed	not specified	polyurethane foam	*At. ferrooxidans*	2.3 28	1.56	Armentia and Webb, 1992
Agitated reactor	not specified	none	*L. ferrooxidans*	1.1-1.7 30-40	1.17	Breed and Hansford, 1999
Fluidised bed	mineral leaching	activated carbon	*Sb. thermosulfido-oxidans* consortium	1.5-1.8 60	0.5	Kinnumen *et al.*, 2003
Airlift	H_2S removal	honeycombe ceramics	*At. ferrooxidans* *L.. ferrooxidans*	1.8 ambient	0.80	Park *et al.*, 2005
Flooded packed bed	AMD treatment	porous glass beads	various	2.0-2.2 20	1.45-1.60	Rowe *et al.*, 2007
Continuous flow	AMD treatment	none	"*Fv. myxofaciens*"	2.1 21-23	0.13	Hedrich and Johnson, 2012
Airlift	Fe removal	jarosite	mixed culture	1.5 23	0.75	Kaksonen *et al.*, 2014

The highest specific rates of iron oxidation achieved in this study by the pure culture *Atx. ferrooxidans* bioreactor system (0.24 g Fe^{2+} oxidised/L/h at a D value of 0.62 h^{-1}) was low in comparison to others reported in the literature. However, systems used elsewhere have used pure or mixed cultures of autotrophic iron-oxidising bacteria, which tend to have higher specific rate of iron oxidation than heterotrophic iron-oxidising acidophiles (Johnson *et al.*, 2012). However, the main objective in this investigation was to establish a functioning FOB as quickly as possible (hence use of a heterotrophic bacterium), and to allow the iron-oxidising microbial community to evolve (presumably to one where autotrophic species eventually dominated) with time. Interestingly, rates of iron oxidation for the *Atx. ferrooxidans* pure culture FOB was greater than those reported for "*Fv. myxofaciens*" FOB of similar design and operating conditions (Hedrich and Johnson, 2012). Neither of these systems was optimised for rates of iron oxidation, however. Further optimisation might include the encouragement of additional biomass accumulation in the excess available reactor volume, and the importance of having sufficient oxygen available in the liquor, particularly at accelerated rates of iron oxidation (Hedrich and Johnson, 2013). Another consideration might be the balance between rates and extents of iron oxidation. As shown in the present study, increasing flow rates/dilution rates can often result increased rates of iron oxidation but also a smaller percentage of ferrous iron oxidised. A major objective with the present FOBs was to maximise the amount of iron oxidised as water flowed through the reactor vessels, rather than to optimise rates of iron oxidised.

Ferrous iron oxidation is only the first stage of the remediation process required to remove soluble iron from acidic mine waters. Ferric iron generated by this first stage is more readily removed by hydrolysis and precipitation, which requires a pH of above ~3 to be effective. Oxidised mine waters often equilibrate at between pH 2.3 and 2.5 because hydrolysis of iron generates protons (equation [1.8]). At this pH some ferric iron remains in solution, as seen in the present study. The system described by Hedrich and Johnson (2012) for the selective removal of iron from acidic mine waters involved three stages: (i) bacterial oxidation of

ferrous iron to ferric on a "*Fv. myxofaciens*"-dominated bioreactor; (ii) precipitation of ferric iron as the mineral schwertmannite in a pH-controlled (pH 3.5) second vessel; (iii) removal of residual soluble iron in a fixed-bed biofilm reactor. The *Atx. ferrooxidans* FOB developed and tested in the present study may be a useful alternative to the first of these modules, particularly as a newly-commissioned bioreactor unit.

T-RFs confirmed the presence of *Atx. ferrooxidans* in the FOBs when Mynydd Parys AMD was introduced. This was expected as this isolate (PY-F3) was originally isolated from acid streamers growing in the Dyffryn Adda, a stream that drains the Mynydd Parys mine site (Kay *et al.*, 2013). *Atx. ferrooxidans* is similar to "*Fv. myxofaciens*" (Hedrich and Johnson, 2012) in its streamer-forming growth habit and like "*Fv. myxofaciens*", requires no additional support materials for the development and retention of biomass. This results a maximum volume available for the bacterial biomass to occupy. However, a major difference between *Atx. ferrooxidans* and *"Fv. myxofaciens"* is that the former is heterotrophic and the latter autotrophic. This means that colonisation and accumulation of biomass can be faster in the heterotroph-containing reactor, as autotrophic species must divert much of the energy derived from the oxidation of Fe^{2+} (which is less than that derived from other inorganic electron donors such as sulfur and hydrogen; Johnson *et al.*, 2012) to fix CO_2, while heterotrophic species do not. Once AMD was introduced to the FOBs, other prokaryotes were anticipated to become more important members of the bioreactor microbial communities. This was because ferrous iron concentrations were ~400 mg/L in the Mynydd Parys AMD (which acts as an electron donor (energy source) for many species of acidophilic bacteria), in comparison to relatively small concentrations (<5 mg/L) of DOC for the growth of *Atx. ferrooxidans*. Growth of *Atx. ferrooxidans* was expected to be more limited than with the glucose/yeast extract-containing start-up medium because of this.

Having established two FOBs using synthetic glucose/yeast extract-containing solutions, these were then tested with actual mine water. Over the experiment period (once Mynydd Parys AMD was introduced), the bacterial communities in the two FOBs evolved into

different communities, despite both starting as pure cultures of *Atx. ferrooxidans* and having the same through-flow liquor (Mynydd Parys AMD). The reason for this is not known. During the experiment only bacteria in the effluent liquors were examined, as biomass sampling from within the FOBs was deemed invasive and potentially destructive. At the end of the experiment, residual streamer growth from the two FOBs were also shown have different bacterial communities. Nevertheless, both FOBs have proved to be effective at oxidising ferrous iron in Mynydd Parys AMD throughout the test period.

One final interesting point raised by this investigation concerned the bacteria identified in the AMD form Mynydd Parys. It was necessary to determine, in so far as is possible, the identity of the species of bacteria present in the water before its addition to the FOBs, and the main technique used was T-RFLP. The T-RFLP profiles obtained were different from those reported previously. A study by Kay *et al.* (2013) had focused mainly on the acid streamer community in the Afon Goch over a nine year period, but included T-RFLP profiles of the mine water bacterial community on samples collected in November 2008. Comparison of one of those profiles with one from the present study (Fig. 9.15) shows that only one of the T-RFs (70/71 nt, which corresponds to *At. ferrivorans* in HaeIII digests) was common to both. The 2014 profile suggests a much more diverse bacterial community, though few of these could be identified from the BART database, and currently are not known. One reason for the difference is that the Kay *et al.* (2013) profile was obtained six years before the present study, and the bacterial community might have changed over that time (in contrast to the acid streamer community, which showed very little change over the nine year study period; Kay *et al.*, 2013). Another reason could be the time of year at which the mine waters were sampled (mid-summer and late autumn). Although the temperature of the Afon Goch shows little seasonal variation (because of its origin as the overflow of a large subterranean water body), greater algal productivity (*Euglena* etc.) would be expected over the summer months due to prolonged solar irradiation of increased strength. This would increase primary production and therefore the amount of organic carbon entering the mine water as exudates

and lysates from the indigenous acidophilic algae. This in turn would promote the growth of heterotrophic acidophiles (Ňancucheo and Johnson, 2012). Other studies of terrestrial AMD sites with more heterotrophic bacteria than chemolithotrophic bacteria have also identified seasonal effects (Edwards *et al.*, 1999; Hao *et al.*, 2012; Streten-Joyce, 2013), with parameters such as increasing temperature and light reported as having an effect on the indigenous microbial communities.

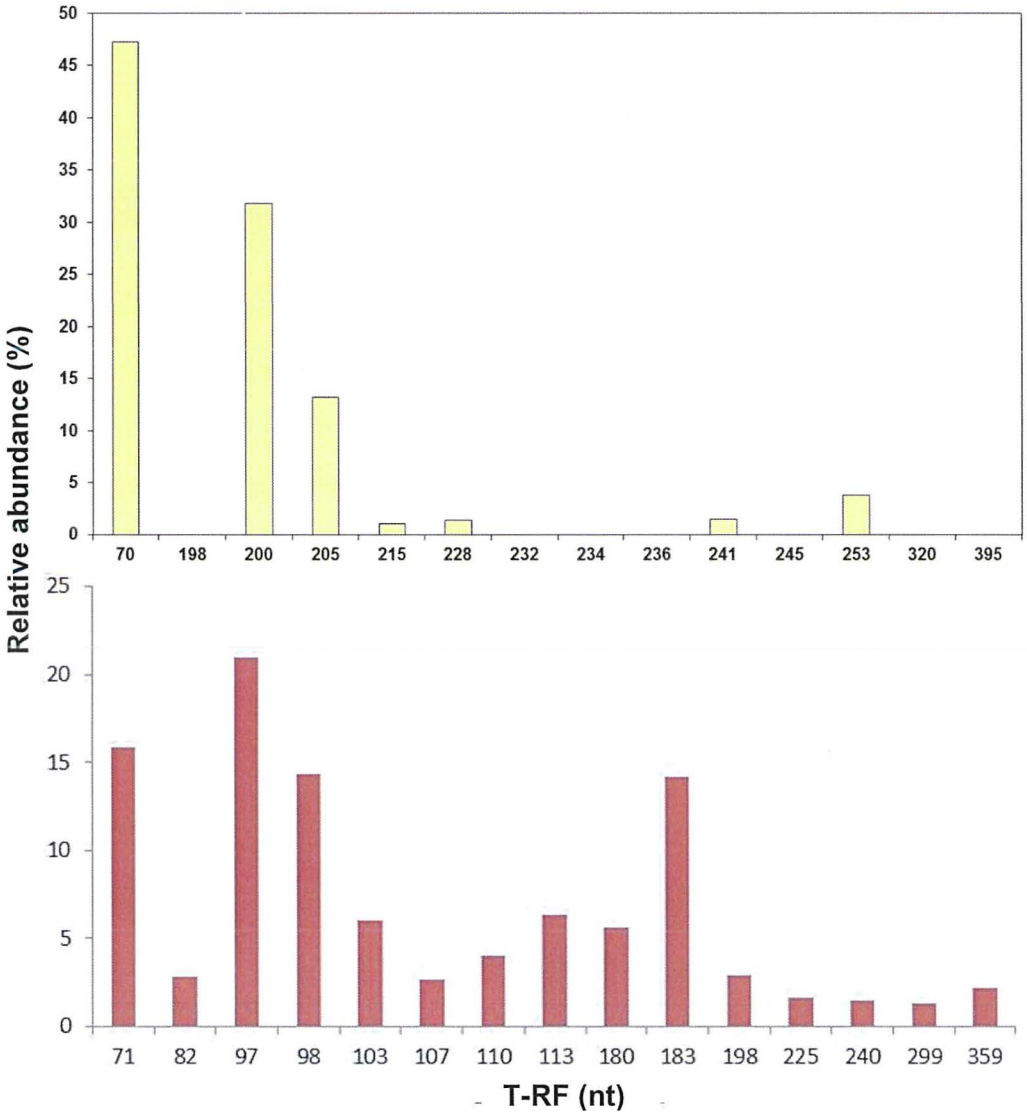


Figure 9.15. Comparison of T-RFLP profiles of amplified 16S rRNA genes, digested with the restriction enzyme HaeIII in November 2008 (Kay et al., 2012) and June 2014 (present study).

Furthermore, the sampling dates coincided with an extended period of above average sun and temperature, meaning that this difference in bacterial community may not be representative of summer sampling generally. However, the suggestion that unknown and possibly novel species of acidophiles inhabit the AMD at Mynydd Parys merits further investigation, but was beyond the scope of the present study.

An approximate estimate can be made of the size of an *Atx. ferrooxidans*-based bioreactor that would be required to oxidise the ferrous iron in Mynydd Parys AMD as a component of a remediation system. *Natural Resources Wales* estimates a discharge rate of ~ 10 L AMD/sec into the Afon Goch. Assuming a ferrous iron concentration of ~400 mg/L and a ferrous iron oxidation rate of 250 mg/L/h, an FOB of ~60 m^3 would be required. A second module to precipitate the ferric iron (preferably as schwertmannite) and a third polishing module, as suggested by Hedrich and Johnson (2012), would then follow this.

This same scaling calculation cannot be made of Cae Coch, as the discharge rate for the four adits is not available. Other considerations when adapting this system to the Cae Coch site includes a lower pH (pH 2.25) and variation in dissolved metals concentrations. These latter are generally lower than in Parys AMD, except for ferric iron, which is ~70% more (1340 mg/L Cae Coch (Kimura *et al.*, 2011) and 391 mg/L Parys Mountain, this study). *Atx. ferrooxidans* has been identified at Cae Coch and conditions appear within acceptable physiological parameters, so the proposed system is likely to be applicable. In any case, as bacteria from the site colonised the vessels, the community would become more adapted to the present conditions.

Therefore, this study showed that the iron-oxidising heterotroph *Atx. ferrooxidans* could be used successfully in a bioreactor designed to ameliorate waters with a high dissolved ferrous iron content such as those of Cae Coch (and Parys Mountain), by oxidising the ferrous iron to ferric iron.

Chapter 10. General discussion

The Cae Coch pyrite body is responsible for two iron-rich waters; acid mine drainage from the Cae Coch sulfur mine and Trefriw Wells Spa water. Both are naturally extremely low pH with elevated concentrations of ferrous iron and ferric iron in some instances, and no other significant quantities of dissolved metals (in comparison to other AMD). These qualities have resulted in the promotion of Trefriw Wells Spa water as a "natural" remedy for well over 100 years. The general aims of this research programme were to study biogeochemical issues related to the formation of the waters and to examine a variety of bacterial species identified in the low pH water.

The first general observation on the results of this study was that both bioreactor experiments involved the catalysis of iron cycling reactions such as oxidation by extremely acidophilic bacteria, but the purpose of the systems were different. Both systems also offered insight into iron cycling resulting from the bio-weathering of Cae Coch pyrite ore body. The purpose of chapter 3 was to add dissolved ferrous iron to liquor (a section of work of interest to the company partner, Nelsons Ltd.) and the purpose of chapter 9 was to gauge the effectiveness of the removal of ferrous iron. The low pH (2.5–3.0) ferrous iron-rich water at the Trefriw Wells Spa site is considered commercially valuable and the Cae Coch water (and the Parys Mountain water used in the study) as an environmentally damaging wastewater, yet the same general biotic and abiotic process occurs at all sites. A number of species have also been identified as common to all sites (Chapters 5, 9; Hallberg *et al.*, 2006; Kay *et al.,* 2013; Kimura *et* al., 2011). One major difference between site waters is the metals content in each. Trefriw Wells Spa water contains fewer dissolved metals other than ferrous iron (including ferric iron) than the Cae Coch mine water, which in turn contains considerably fewer dissolved metals than the Mynydd Parys water (Chapters 5, 9; Hallberg *et al.*, 2006; Kay *et al.,* 2013). The difference between Parys Mountain and Cae Coch pyrite body sites is mainly due to different geology. The water from the Cae Coch abandoned

sulfur mine is also more acidic (pH ~2.3-1.8; Kimura *et al.*, 2011) than the water from the Trefriw and Parys sites. Other insights into iron cycling at the sites include evidence for the proposed model of Trefriw Spa Water generation. That is, iron speciation is dependent on oxygen availability and the ability of some bacteria to catalyse iron oxidation and reduction. These sections of work also showed that degradation of pyrite and the collective abilities of the present community may have a noticeable effect on the concentration and speciation of dissolved iron in the waters of Cae Coch and Trefriw Wells Spa sites. These results concur with previous reports at both sites (Hallberg *et al.*, 2006; Kimura *et al.*, 2011), and for other sites (e.g. Parys Mountain; Kay *et al.*, 2013).

Both reactor systems included in this study require further work to become viable processes. The system described in chapter 3 was successful in achieving its aim of demonstrating that a synthetic Spatone concentrate could be made from the dissolution of pyrite by biological catalysis of iron cycling processes, including the control of iron speciation by generating oxidative or reductive conditions. However, the described system would require further development before it could be added to the Spatone manufacturing process. Exactly how this might be done would depend on the amount of synthetic Spatone required, but might include operations such as scaling up from a 2 L reactor or changing the system from a batch to a modular continuous flow system with possible fixed beds. The second bioreactor system, designed to alleviate the high ferrous iron content of Mynydd Parys AMD and by extension the Cae Coch AMD, would also require scaling up to perform its intended function. Factors such as metal content and the effect of native bacterial community establishment in the vessel would likely affect the effectiveness of the iron-oxidation capacity of the system, especially if it were used to ameliorate ferrous iron in AMD from other sites. When considered in the context of applying this system to the Cae Coch AMD, these factors are not anticipated as major issues, as conditions of the site liquor are within *Atx. ferrooxidans* tolerances, and strains of this genus have been detected previously at the site (Hallberg *et al.*, 2006; Kimura *et al.*, 2011). As discussed in chapter 9, other AMD iron-oxidation systems

have been studied, but most of these included autotrophic-iron oxidising bacteria rather than the heterotrophic iron-oxidising acidophilic bacteria used in this study. It was also demonstrated in chapters 8 and 9 that *Atx. ferrooxidans* can oxidise iron below its pH and temperature growth minima for appreciable amounts of time. This ability could confer an advantage in that the acidophilic bacteria concerned could continue to function for a time when conditions were not favourable for it, including in a bioreactor system and *in situ* at Cae Coch and Trefriw Wells spa. This has not been reported for other extremely acidophilic bacteria, possibly because this characteristic was not considered. Further work is required to confirm this.

The T-RFLP profiles of the bacterial communities at Trefriw Wells Spa and Parys Mountain sampling sites showed a number of interesting characteristics. Similar species were identified in in this study in T-RF profiles at Trefriw Wells Spa and Parys Mountain, which were iron-rich waters of pH ~2.5-3. Species identified at these sites were also identified in previously conducted studies at Cae Coch mine (Hallberg *et al.* 2006, Kimura, 2007; Kay *et al.*, 2013; Kimura *et al.*, 2011). These included iron oxidising bacteria (e.g. *Fv. myxofaciens* and *Atx. ferrooxidans*) and iron reducing bacteria (e.g *Acidocella* spp). It was also shown that there were many species present in samples from all studied sites that could not be identified by the methods used in this study. These unidentified bacteria were present in relatively large abundance in some instances. The problem with unknown bacteria and those of relatively large abundance in particular, is that a possibly significant amount of information on a bacterial community is missing, such including the roles of such unknown bacteria in the environment, and whether the peak in question represents a single or (in some instances) multiple species. The difficulties of identifying unknown acidophilic bacteria are discussed in section 1.2, and limitations of T-RFLP analysis in section 5.8. Different approaches than those attempted in this study might identify these unknown bacteria such as a genomic library and other cultivation independent molecular biology methods. This is a proposed subject for further study.

Another trend that emerged from this study was the ability of members of the genus *Acidocella* to tolerate and metabolise some aromatic compounds and organic acids. *Ac. aromatica* (chapter 6), "*Ac. acidivorans*" (chapter 7) and the Trefriw Wells Spa isolates (chapter 5) were reported to tolerate millimolar concentrations of select small-weight organic acids in this study, which was at least an order of magnitude more than previously reported for extremely acidophilic bacteria. This characteristic is noteworthy as extremely acidophilic bacteria are generally extremely sensitive to small-weight organic acids. Studies have connected this problem with issues in the isolation of novel bacteria (section 1.2) and biotechnological applications (e.g. Hallberg *et al.*, 1999; Kimura *et al.*, 2006). One possible solution to the problem of inhibitory compounds is the inclusion of heterotrophic bacteria to remove organic carbon sources from cultures to support growth of (often commercially important) autotrophic bacteria (e.g. Ňancucheo and Johnson, 2010). The *Acidocella* spp. reported here (in particular, *Ac. aromatica*; Kimura *et al.*, 2006; Johnson *et al.*, 2008; Ňancucheo and Johnson, 2010) are particularly suitable for this application as they do not metabolise yeast extract, and so do not compete for this commonly used substrate. They are therefore considered as suitable for use with facultative and obligate heterotrophs. *Ac. aromatica*^{T} was previously isolated from Parys Mountain and described in this study because; (a) *Acidocella* species were known as present at the Trefriw Wells Spa site, (b) it showed similar characteristics to those of *Acidocella* spp. isolated from the sites and (c) it was the most suitable available *Acidocella* isolate at the time ("*Ac. acidivorans*" was sucessefully isolated later in the study). Questions raised by this aspect of the study include further possible biotechnological applications of the novel *Acidocella* spp reported here. A further point of note concerning this general trend is that all *Acidocella* spp. isolates from Trefriw Wells Spa, including those that were identified as strains of *Ac. facilis,* could metabolise these problematic organic acids to a greater extent than reported previously (with the exception of those described in this study). Previous reports have also identified bacteria (mainly by cultivation-independent methods) at Cae Coch mine that are not usually identified

in extremely acidic environments (Kimura *et al.*, 2011). This suggests the possibility that identification and characterisation isolates from these two sites may yield more bacterial spp. and strains with novel and possibly useful characteristics.

In conclusion, the purpose of this study was to investigate biogeochemical issues related to the waters formed from the Cae Coch ore body and the Wells Spa water in particular. These aims were accomplished by the establishment of two bioreactors, and the study of bacteria isolated from the Trefriw Wells Spa site (or their closest available relatives). Results from these investigations included a system for generating concentrated synthetic Trefriw Wells Spa water, a continuous flow bioreactor to ameliorate dissolved ferrous iron containing waters such as those of the Cae Coch AMD, and the identification of bacteria found at the site, including characterisation of three novel acidophilic heterotrophic species.

Subjects for further investigation highlighted by this study include:

(i) Further development of the synthetic Spatone reactor with the aim of possible integration into the Spatone production system.

(ii) The possible ability of some acidophiles to oxidise iron outside their growth range, as shown by *Atx. ferrooxidans* in chapters 8 and 9.

(iii) Potential uses of the ability of *Acidocella* spp. to metabolise some organic acids and aromatic compounds, and whether this is a characteristic that is definitive of the genus.

(iv) Potential uses of the continuous flow bioreactor system in chapter 9 in ameliorating waters containing high concentrations of dissolved ferrous iron.

(v) Attempting to identify the unknown T-RFs shown in the community profiles, and the possibility of seasonal changes in community structure at Mynydd Parys.

(vi) Further attempts to identify and isolate novel species and strains from Cae Coch and Trefriw Wells Spa sites.

References

Aguilera, A. (2013). Eukaryotic organisms in extreme acidic environments, the Río Tinto case. *Life,* **3**, 363-374.

Albuquerque, L., Rainey, F. A., Chung, A. P., Sunna, A., Norbe, M. F., Grote, R., Antranikan, G., Costa, M. S. (2000). *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of São Miguel in the Azores. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 451-457.

Alfreider, A., Vogt, C., Hoffman, D., Babel, W. (2003). Diversity of ribulose 1,5-bisphosphate carboxylase/oxygenase large-subunit genes from groundwater and aquifer microorganisms. *Microbial Ecology*, **45**, 317–328.

Amann, R. I., Ludwig, W., Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143-169.

Amaral-Zettler, L. A. (2012). Eukaryotic diversity at pH extremes. *Frontiers in Microbiology*, **3**, 441.

Amouric, A., Brochier-Armanet, C., Johnson, D. B., Bonnefoy, V., Hallberg, K. B. (2011). Phylogenetic and genetic variation among Fe(II)-oxidizing acidithiobacilli supports the view that these comprise multiple species with different ferrous iron oxidation pathways. *Microbiology*, **157**, 111–122.

Armentia, H., Webb, C. (1992). Ferrous sulphate oxidation using *Thiobacillus ferrooxidans* cells immobilized in polyurethane foam support particles. *Applied Microbiology and Biotechnology*, **36**, 697-700.

Arsène-Ploetze, F., Koechler, S., Marchal, M., Coppée, J-Y., Chandler, M., Bonnefoy, V., Brochier-Armanet, C., Barakat, M., Barbe, V., Battaglia-Brunet, F., Bruneel, O., Bryan, C. G., Cleiss-Arnold, J., Cruveiller, S., Erhardt, M., Heinrich-Salmeron, A., Hommais, F., Joulian,

C., Krin, E., Lieutaud, A., Lièvremont, D., Michel, C., Muller, D., Ortet, P., Proux, C., Siguier, P., Roche, D., Salvignol, G., Slyemi, D., Talla, E., Weiss, S., Weissenback, J., Médigue, C., Bertin, P. N.. (2010). Structure, function, and evolution of the *Thiomonas* spp. genome. *PLOS Genetics*, **6**, doi: 10.1371/journal.pgen.1000859.

Auld, R. R., Myre, M., Mykytczuk, N. C. S., Leduc, L. G., Meritt, T. J. S. (2013). Characterization of the microbial acid mine drainage microbial community using culturing and direct sequencing techniques. *Journal of Microbial Methods,* **93**, 108-115.

Bacelar-Nicolau, P., Johnson, D.B. (1999). Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures. *Applied and Environmental Microbiology*, **65**, 585-590.

Barns, S. M., Takala, S. L., Kuske, C. R. (1999). Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. *Applied and Environmental Microbiology,* **65**, 1731–1737.

Baldi, F., Clark, T., Pollack, S.S, Olson, G. S. (1992). Leaching of pyrites of various reactivities by ***Thiobacillus ferrooxidans. A****pplied and Environmental Microbiology*, **58**, 1853–1856.

Ball, T. K., Bland, D. J. (1985). The Cae Coch massive sulphide deposit, Trefriw, North Wales. *Journal of the Geological Society, London,* **142**, 889-898.

Baker, B.J., Banfield, J.F. (2003). Microbial communities in acid mine drainage. *Current Microbiology*, **44**, 139-152.

Baker, B. J., Comolli, L. R., Dick, G. J., Hauser, L. J., Hyatt, D., Dill, B. D., Land, M. L., VerBerkmoes, N. C., Hettich, R. L., Banfield, J. F.. (2010). Enigmatic, ultrasmall, uncultivated Archaea. *Proceedings of the National Academy of Sciences*, **107**, 8806–8811.

Bellenberg, S., Bathen, R., Vera, M., Guilian, N., Sand, W. (2013). Biofilm formation, communication and interactions of mesophilic leaching bacteria during pyrite oxidation. *Advanced Materials Research*, **825**, 107-110.

Bennett, J., Vernon, R. W. (1997). *Mines of the Gwydyr Forest. Part 7*. Gwydyr Mines Publications.

Bhattacharyya, S., Chakrabaty, B. K., Das, A., Kundu, P.N., Banjeree, P.C. (1991). *Acidiphilium symbioticum* sp. nov., an acidophilic heterotrophic bacterium from *Thiobacillus ferrooxidans* cultures isolated from Indian mines. *Canadian Journal of Microbiology*, **37**, 78-85.

Bird, L.J., Bonnefoy, V., Newmann, D. K. (2011). Bioenergetic challenges of microbial iron metabolisms. *Trends in Microbiology*, **19,** 330-340.

Bini, E. (2010). Archaeal transformation of metals in the environment. *FEMS Microbiology Ecology*, **73**, 1–16.

Bogdanova, T. I., Tsaplina, I. A., Kondrateva, T. F., Duda, V. I., Susina, N. E., Melamud, V. S., Tourova, T. P., Karavaiko, G. I.. (2006). *Sulfobacillus thermotolerans* sp. nov., a thermotolerant, chemolithotrophic bacterium. *International Journal Systematic and Evolutionary Microbiology*, **56**, 1039-1042.

Bond, P.L., Banfield, J. F. (2001). Design and performance of rRNA targeted oligonucleotide probes for *in situ* detection and phylogenetic identification of microorganisms inhabiting acid mine drainage environments. *Microbial Ecology*, **41**, 149-161.

Bond, P. L., Smirga, S. P., Banfield, J. F. (2000). Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Applied and environmental Microbiology*, **66**, 3842–3849.

Botterell, S. H., Morton, D. B. (1992). A reinterpretation of the genesis of the Cae Coch pyrite deposit, North Wales. *Journal of the Geological Society*, **149**, 581-584.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.

Breed, A.W., Hansford, G.S. (1999). Effect of pH on ferrous-iron oxidation kinetics of *Leptospirillum ferrooxidans* in continuous culture. *Biochemical Engineering Journal*, **3,** 193-201.

Brierley, J. A. (1978). Thermophilic iron-oxidizing bacteria found in copper leaching dumps. A*pplied and Environmental Microbiology*, **36**, 523-525.

Brønstead, J. N. (1923). Some Remarks on the Concept of Acids and Bases. Recueil des Travaux Chimiques des Pays-Bas, **42**, 718-728.

Cantrell, J., Rylance A. (1992). *Sarn Helen. Walking a Roman road through Wales*. Cicerone press.

Cardenas, E., Tiedje, J.M. (2008). New tools for discovering and characterizing microbial diversity. *Current opinion in Biotechnology*, **19**, 544-549.

Chowdhury, F., Ojumu, T. V. (2014). Investigation of ferrous-iron biooxidation kinetics by *Leptospirillum ferriphilum* in a novel packed-column bioreactor: Effects of temperature and jarosite accumulation. *Hydrometallurgy*, **141**, 36-41.

Clark, D. A., Norris, P. D. (1996). *Acidimicrobium ferrooxidans* gen. nov., sp. nov.: mixed-culture ferrous iron oxidation with *Sulfobacillus* species. *Microbiology*, **142**, 785-790.

Clum, A., Nolan, M., Lang, E., Del Rio, T. G., Tice, H., Copeland, A., Cheng, J-F., Lucas, S., Chen, F., Bruce, D., Goodwin, L., Pitluck, S., Ivanova, N., Mavrommatis, K., Mikhailova, N., Pati, A., Chen, A., Palaniappan, K., Göker, M., Spring, S., Land, M., Hauser, L., Chang, Y-J., Jeffries, C. C., Chain, P., Bristow, J., Eisen, J. A., Markowitz, V., Hugenholtz, P., Kyrpides, N. O., Klenk, H-P., Lapidus, A.. (2009). Complete genome sequence of *Acidimicrobium ferrooxidans* type strain (ICP^T). *Standards in Genomic Sciences*, **1**, 38-45.

Coram, N. J., Rawlings, D. E. (2002). Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks that operate at 40°C. *Applied and Environmental Microbiology*, **68**, 838-845.

Cotter, P. D., Hill, C. (2003). Surviving the acid test: Responses of Gram-positive bacteria to low pH. *Microbiology and Molecular Ecology Reviews*, **67**, 429-435.

Coupland, K., Johnson, D.B. (2008). Evidence that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria. *FEMS Microbiology Letters*, **279**, 30-35.

Darland, G., T. Brock. (1971). *Bacillus acidocaldarius* sp. nov., an acidophilic thermophilic spore-forming bacterium. *Journal of General Microbiology*, **67**, 9-15.

Delavat, F., Lett, M-C., Lièvremont, D. (2012). Novel and unexpected bacterial diversity in an arsenic-rich ecosystem revealed by culture-dependent approaches. *Biology Direct*, **7**, 28.

Denef, V. J., Mueller, R. S., Banfield, J. F. (2010). AMD biofilms: using model communities to study microbial evolution and ecological complexity in nature. *International Society for Microbial Ecology*, **4**, 599–610.

De Vos, P., Trüper, H. G. (2000). Judicial commission of the international committee on systematic bacteriology. IXth International (IUMS) congress of bacteriology and applied microbiology. Minutes of the meetings, 14, 15 and 18 August 1999, Sydney, Australia. *International Journal of Systematic and Evolutionary Microbiology,* **50**, 2239-2244.

Dietrich, L. E. P., Tice, M. M., Newman, D. K. (2006). The co-evolution of life and Earth. *Current Biology*, **16**, 11.

Drobner E, Huber H, Stetter, K.O. (1990). *Thiobacillus ferrooxidans,* a facultative hydrogen oxidizer. *Applied and Environmental Microbiology*, **56**, 2922-2923.

Edwards, K., Gihring, T. M., Banfield, J. F. (1999). Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment. *Applied and Environmental Microbiology*, **65**, 3627–3632.

Eisen, S., Poehlein, A., Johnson, D.B., Daniel, R., Schlömann, M., Mühling, M. (2015). Genome sequence of the acidophilic ferrous iron-oxidizing isolate *Acidithrix ferrooxidans* strain Py-F3, the proposed type strain of the novel *Actinobacterial* genus *Acidithrix*. *Genome Announcements*, **3**, e00382-15. doi:10.1128/genomeA.00382-15.

Euzéby, J.P., Tindall, B.J. (2001). Nomenclatural type of orders: corrections necessary according to Rules 15 and 21a of the Bacteriological Code (1990 Revision) and designation of appropriate nomenclatural types of classes and subclasses. Request for an opinion. *International Journal of Systematic and Evolutionary Microbiology,* **51**, 725-727.

Euzéby, J. P., Tindall, B. J. (2004). Status of strains that contravene Rules 27(3) and 30 of the Bacteriological Code. Request for an opinion. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 293-301.

Exley, C., Birchall, J.D. (1992). The cellular toxicity of aluminium. *Journal of Theoretical Biology*, **159**, 83-98.

Falagán, C., Johnson, D. B. (2014). *Acidibacter ferrireducens* gen. nov., sp. nov.: an acidophilic ferric iron-reducing *gammaproteobacterium*. *Extremophiles,* **18**, 1067–1073.

Fierer, N., Jackson, R.B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences USA*, **103**, 626-631.

Franzmann, P.D., Haddad, C.M., Hawkes, R.B., Robertson W.J., Plumb, J.J. (2005). Effects of temperature on the rates of iron and sulfur oxidation by selected bioleaching Bacteria and Archaea: Application of the Ratkowsky equation. *Minerals Engineering*, **18**, 1304–1314.

Fredriksson, N.J., Hermansson, M., Wilén, B-M. (2014). Tools for T-RFLP data analysis using Excel. *BMC Bioinformatics*, **15**, 361.

Galand, P. E., Potvn, M., Casamayor, E. O., Lovejoy, C. (2010). Hydrography shapes bacterial biogeography of the deep Arctic Ocean. *The International Society for Microbial Ecology Journal,* **4**, 564–576.

Gamoclija, M., Garrel, L., Berthon, J., López-García, P. (2004). Bio-signatures and bacterial diversity in hydrothermal deposits of Solfatara crater, Italy. *Geomicrobiology Journal*, **21**, 529–541.

Gao, W., Zhang, W., Meldrum, D. R. (2011). RT-qPCR based quantitative analysis of gene expression in single bacterial cells. *Journal of Microbiological Methods*, **85**, 221-227.

García-Moyano, A., González-Toril, E., Aguilera, Á., Amils, R. (2012). Comparative microbial ecology study of the sediments and the water column of the Río Tinto, an extreme acidic environment. *FEMS Microbiology Ecology*, **81**, 303-314.

Gemmell, R., Knowles, C. (2000). Utilisation of aliphatic compounds by acidophilic heterotrophic bacteria. The potential for bioremediation of acidic wastewaters contaminated with toxic organic compounds and heavy metals. *FEMS Microbiology Letters*, **192**, 185-190.

Ghauri, M., A., Okibe, N., Johnson, D. B. (2007). Attachment of acidophilic bacteria to solid surfaces: The significance of species and strain variations. *Hydrometallurgy*, **85**, 72–80.

Golyshina, O. V., Pivivarova, T. A., Karavaiko, G. I., Kondratéva, T.F., Moore, E. R., Lünsdorf, H., Timmis, K.N., Yakimov, M.M., Golyshin, P.N. (2000). *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. nov., comprising a distinct lineage of the Archaea. *International Journal of Systematic and Evolutionary Micrbiology*, **3**, 997-1006.

Golovacheva, R. S., Golyshina, O. V., Karavaiko, G. I., Dorofeev, A. G., Pivovarova, T. A., Chernykh, N. A. (1992). *Leptospirillum thermoferrooxidans* sp. nov., a new iron oxidizing bacterium. *Microbiology*, **61**, 744–750.

Golovacheva, R. S., Karavaiko, G. I. (1978). *Sulfobacillus*, a new genus of thermophilic sporeforming bacteria. *Mikrobiologiya*, **47**, 815-822.

Goltsman, D. S. A., Dasari, M., Thomas, B. C., Shah, M. B., VerBerkmoes, N. C., Hettich, R. L., Banfield, J. F. (2013). New group in the *Leptospirillum* clade: Cultivation-independent community genomics, proteomics, and transcriptomics of the new species "*Leptospirillum* Group IV UBA BS". *Applied and Environmental Microbiology*, **79**, 5384 –5393.

Goltsman, D.S., Denef, V.J., Singer, S.W., VerBerkmoes, N.C., Lefsrud, M., Mueller, R.S., Dick, G.J., Sun, C.L., Wheeler, K.E., Zemla, A., Baker, B.J., Hauser, L., Land, M., Shah, M.B., Thelen, M.P., Hettich, R.L., Banfield, J.F. (2009). Community genomic and proteomic analyses of chemoautotrophic iron-oxidizing "*Leptospirillum rubarum*" (Group II) and "*Leptospirillum ferrodiazotrophum*" (Group III) bacteria in acid mine drainage biofilms. *Applied and Environmental Microbiology*, **75**, 4599-615.

González-Toril, E., Martínez-Frías, M., Gómez, G., Rull, F., Amils, R.. (2005). Iron meteorites can support the growth of acidophilic chemolithoautotrophic microorganisms. *Astrobiology*, **5**, No. 3.

Goto, K., Matsubara, H., Mochida, K., Matsumura, T., Hara, Y., Niwa, M., Yamasato, K. (2002). *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing ω-cycloheptane fatty acids, isolated from herbal tea. *International Journal of Systematic and Evolutionary Microbiology,* **52**, 109-113.

Goto, K., Mochida, K., Asahara, M., Suzuki, M., Kasai, H., Yokota, A.. (2003). *Alicyclobacillus pomorum* sp. nov., a novel thermo-acidophilic, endospore-forming bacterium that does not possess ω-alicyclic fatty acids, and emended description of the genus *Alicyclobacillus. International Journal of Systematic and Evolutionary Microbiology,* **53**, 1537-1544.

Goto, K., Mochida, K., Kato, Y., Asahara, M., Fujita, R., An, S. Y., Kasai, H., Yokota, A. (2007). Proposal of six species of moderately thermophilic, acidophilic, endospore-forming

bacteria: *Alicyclobacillus contaminans* sp. nov., *Alicyclobacillus fastidiosus* sp. nov., *Alicyclobacillus kakegawensis* sp. nov., *Alicyclobacillus macrosporangiidus* sp. nov., *Alicyclobacillus sacchari* sp. nov. and *Alicyclobacillus shizuokensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **57**, 1276-1285.

Gregersen, T. (1978). Rapid method for distinction of gram-negative from gram-positive bacteria. *European journal of applied microbiology and biotechnology*, **5**, 123-127.

Griffiths, R. I., Thompson, B. C., James, P., Bell, P., Bailey, M., Whiteley, A. S. (2011). The bacterial biogeography of British soils. *Environmental Bacteriology*, **13**, 1642-1654.

Guo, X., You, X. Y., Liu, L. J., Zhang, J. Y., Liu, S. J., Jiang, C. Y.(2009). *Alicyclobacillus aeris* sp. nov., a novel ferrous-and sulfur-oxidizing bacterium isolated from a copper mine. *International Journal of Systematic and Evolutionary Microbiology*, **59**, 2415-2420.

Haas, B. J, Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Ginnaoukos, G., Clulla, D., Tabbaa, D., Highlander, S. K., Sodergren, E., Methé, B., DeSantis, T. Z., The Human Microbiome Consortium, Petrosino, J. F., Knight, R., Birren, B. W. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research*, **21**, 494-504.

Halksworth, G., Moseley, L., Carter, K., Worwood, M. (2003). Iron absorption from Spatone (a natural mineral water) for prevention of iron deficiency in pregnancy. *Clinical and Laboratory Haematology*, **25**, 227-231.

Harrison, Jr., A. P. (1981). *Acidiphilium cryptum* gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. *International Journal of Systematic Bacteriology*, **31**, 327-332.

Harrison, A. P. Jr. (1983). Genomic and physiological comparisons between heterotrophic thiobacilli and *Acidiphilium cryptum*, *Thiobacillus versutus* sp. nov., and *Thiobacillus*

acidophilus nom. rev. *International Journal of Systematic and Evolutionary Microbiology,* **33**, 211-217.

Harrison, A.P. Jr. (1984). The acidophilic *Thiobacilli* and other acidophilic bacteria that share their habitat. *Annual Review of Microbiology*, **38**, 265–292.

Hallberg, K. B. (2010). New perspectives in acid mine drainage microbiology. *Hydrometallurgy*, **3**, 448–453.

Hallberg, K. B., Coupland, K., Kimura, S., Johnson, D. B. (2006). Macroscopic streamer growths in acidic, metal-rich mine waters in north Wales consist of novel and remarkably simple bacterial communities. *Applied and Environmental Microbiology*, **72**, 2022-2030.

Hallberg, B. K., Gonzáles-Toril, E., Johnson, D. B. (2010). *Acidithiobacillus ferrivorans*, sp. nov.; facultatively anaerobic, psychrotolerant iron-, and sulfur-oxidizing acidophiles isolated from metal mine-impacted environments. *Extremophiles*, **14**, 9–19.

Hallberg, K. B., Hedrich, S., Johnson, D. B. (2011). *Acidiferrobacter thiooxydans*, gen. nov. sp. nov.; an acidophilic, thermo-tolerant, facultatively anaerobic iron- and sulfur-oxidizer of the family *Ectothiorhodospiraceae*. *Extremophiles*, **15**, 271–279.

Hallberg, K.B., Kolmert, Å.K., Johnson, D.B., Williams, P.A. (1999). A novel metabolic phenotype among acidophilic bacteria: aromatic degradation and the potential use of these organisms for the treatment of wastewater containing organic and inorganic pollutants. In: Amils, R., Ballester, A. (Eds.). *Biohydrometallurgy and the environment toward the mining of the 21st century*. Part A. Elsevier, 719 – 728.

Hallberg, K. B., Lindstrom, E. B. (1994). Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. *Microbiology,* **140**, 3451-3456.

Hao, C., Wang, L., Gao, Y., Zhang, L., Dong, H. (2010). Microbial diversity in acid mine drainage of Xiang Mountain sulfide mine, Anhui Province, China. *Extremophiles*, 14, 465-474.

Hao, C., Zhang, L., Wang, L., Li, S., Dong, H. (2012). Microbial community composition in acid mine drainage lake of Xiang Mountain sulfide mine in Anhui Province, China. *Geomicrobiology Journal*, **29**, 886–895.

Harneit, K., Göksel, A., Kock, D., Klock, H., Gehrke, T., Sand, W. (2006). Adhesion to metal sulfide surfaces by cells of *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferrooxidans*. *Hydrometallurgy*, **83**, 245–254.

Hartikainen, T., Ruuskanen, J., Räty, K., von Wright, A., Martikainen, P. J. (2000). Physiology and taxonomy of *Thiobacillus* strain TJ330, which oxidizes carbon disulphide (CS2). *Journal of Applied Microbiology*, **89**, 580-586.

Hayward, J. W. (1881). ***Guide to Trefriw and the Vale of Conway spa*. Adam Holden.**

He, Z., Xie, X. H., He, Z. G., Xiao, S. M., Liu, J. S. (2007). Microbial diversity of mine water at Zhong Tiaoshan copper mine, China. *Journal of Basic Microbiology*, **47**, 485–495.

Hedrich, S., Johnson, D. B. (2013). *Acidithiobacillus ferridurans* sp. nov., an acidophilic iron, sulfur- and hydrogen-metabolizing chemolithotrophic gamma-proteobacterium. *International Journal of Systematic and Evolutionary Microbiology*, **63**, 4018–4025.

Hedrich, S., Johnson, D. B. (2012). A modular continuous flow reactor system for the selective bio-oxidation of iron and precipitation of schwertmannite from mine-impacted waters. *Bioresource Technology*, **106,** 44-49.

Hedrich, S., Johnson, D. B. (2013). Aerobic and anaerobic oxidation of hydrogen by acidophilic bacteria. *FEMS Microbiology Letters*, **349**, 40-45.

Hedrich, S., Schlomann, M., Johnson D. B. (2011). The iron-oxidizing proteobacteria. *Microbiology*, **157**, 1551–1564.

Hippe, H. (2000). *Leptospirillum* gen. nov. (*ex* Markosyan 1972), nom. rev., including *Leptospirillum ferrooxidans* sp. nov. (*ex* Markosyan 1972), nom. rev. and *Leptospirillum*

thermoferrooxidans sp. nov. (Golovacheva *et al.* 1992). *International Journal of Systematic and Evolutionary Microbiology,* **50**, 501–503.

Hirashi, A., Matzusawa, Y., Kanbe, T., Wakao, N. (2000). *Acidisphaera rubrifaciens* gen. nov., sp. nov., an aerobic bacteriochlorophyll-containing bacterium isolated from acidic environments. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 1539–1546.

Hiriashi, A., Nagashima, K. V. P., Matsuura, K., Shimada, K., Takaichi, S., Wakao, N., Katayama, Y. (1998). Phylogeny and photosynthetic features of *Thiobacillus acidophilus* and related acidophilic bacteria: Its transfer to the genus *Acidiphilium* as *Acidiphilium acidophilum* comb. nov. *International Journal of Systematic Bacteriology*, **48**, 1389-1398.

Hirashi, A., Shimada, K. (2001). Aerobic anoxygenic photosynthetic bacteria with zinc-bacteriochlorophyll. *Journal of General Applied Microbiology*, **47**, 168-180.

Inagaki, K., Ueno, N., Tamura, T., Tanaka, H. (2001). Purification and characterization of an acid trehalase from *Acidobacterium capsulatum*. *Journal of Bioscience and Bioengineering*, **91**, 141-146.

Itoh, T., Yamanoi, K., Kudo, T., Ohkuma, M., Takashina, T. (2011). *Aciditerrimonas ferrireducens* gen. nov., sp. nov., an iron-reducing thermos-acidophilic actinobacterium isolated from a solfataric field. *International Journal of Systematic and Evolutionary Microbiology*, **61**, 1281-1285.

Jiang, C. Y., Liu, Y., Liu, Y. Y., You, X. Y., Guo, X., Liu, S. J. (2008). *Alicyclobacillus ferrooxydans* sp. nov., a ferrous-oxidizing bacterium from solfataric soil. *International Journal of Systematic and Evolutionary Microbiology*, **58**, 2898-2903.

Johnson, D. B. (1998). Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbiology Ecology,* **27**, 307-317.

Johnson, D.B. (2012). Geomicrobiology of extremely acidic subsurface environments. *FEMS Microbiology Ecology*, **18**, 2-12.

Johnson, D. B., Bacelar-Nicolau, P., Okibe, N., Thomas, A., Hallberg, K. B. (2009). *Ferrimicrobium acidiphilum* gen. nov., sp. nov. and *Ferrithrix thermotolerans* gen. nov., sp. nov.: heterotrophic, iron-oxidizing, extremely acidophilic actinobacteria. *International Journal of systematic and evolutionary Microbiology*, **59**, 1082-1089.

Johnson, D.B., Ghauri, M.A., Said, M.F. (1992). Isolation and characterization of an acidophilic, heterotrophic bacterium capable of oxidizing ferrous iron. *Applied and Environmental Microbiology*, **58**, 1423-1428.

Johnson, D.B., Hallberg, K.B. (2005). Acid mine drainage remediation options: a review. *Science of the Total Environment*, **338**, 3-14.

Johnson, D. B., Hallberg, K. B. (2007). Techniques for detecting and identifying acidophilic mineral-oxidizing microorganisms. In: Rawlings, D. E., Johnson, D. B. (Eds.) *Biomining.* Springer-Verlag, Berlin . 237-261.

Johnson, D. B., Hallberg, K. B., Hedrich, S. (2014). Uncovering a microbial enigma: Isolation and characterization of the streamer-generating, iron-oxidizing, acidophilic bacterium "***Ferrovum myxofaciens***". *Applied and Environmental Microbiology*, **80**, 672-680.

Johnson, D. B., Joulan, C., D'Hugues, P. Hallberg, K. B. (2008). *Sulfobacillus benefaciens* sp. nov., an acidophilic facultative anaerobic *Firmicute* isolated from mineral bioleaching operations. *Extremophiles*, **12**, 789-798.

Johnson, D. B., Kanao, T., Hedrich, S. (2012). Redox transformations of iron at extremely low pH: fundamental and applied aspects. *Frontiers in Mirobiology*, **3**, 96.

Johnson, D. B., Roberto, F. F. (1997). Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals. In: Rawlings, D. E. (Ed.). *Biomining: Theory, Microbes and Industrial Processes*. Springer, 259–280.

Johnson, D.B., Sen, A.M., Kimura, S., Rowe, O.F., Hallberg, K., B. (2006). Novel biosulfidogenic system for selective recovery of metals from acidic leach liquors and waste streams. *Mineral Processing and Extractive Metallurgy*, **115**, 19-24.

Johnson, D. B., Stallwood, B., Kimura, S., Hallberg, K. B. (2006). Isolation and characterization of ***Acidicaldus organivorus***, gen. nov., sp. nov.: a novel sulfur-oxidizing, ferric iron-reducing thermo-acidophilic heterotrophic ***Proteobacterium.*** *Archives of Microbiology*, **185**, 212-221.

Johnson, D. C., Dean, D. R., Smith, A. D., Johnson, M. K. (2005). Structure, function and formation of biological iron-sulfur clusters. *Annual Reviews in Biochemistry*, **74**, 247-281.

Jones, M.. (1879). *Hanes Trefriw, fely bu ac fel y mae*. W. J. Roberts.

Jones, J. G. (1986). Iron transformations by freshwater bacteria. In Marshall, E. C. (Ed.). *Advances in Microbial Ecology, vol. 9*. Plenum Press, New York.

Jones, D. S., Albrecht, H. L., Dawson, K. S., Schaperdoth, I., Freeman, K. H., Pi, Y., Pearson, A., Maclady, J. L. (2012). Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm. *ISME Journal*, **6**, 158–170.

Jones, R.M., Hedrich, S. and Johnson, D. B. (2013). *Acidocella aromatica* sp. nov.: an acidophilic heterotrophic alphaproteobacterium with unusual phenotypic traits. *Extremophiles,* **17**, 841-850.

Jones, D. S., Kohl, C., Grettenberger, C., Larson, L. N., Burgos, W. D., Macalady. (2014). Geochemical niches of iron-oxidizing acidophiles in acidic coal mine drainage. *Applied and Environmental Microbiology*, **81**, 1242-1250.

Kaksonen, A.H., Morris, C., Hilaro, F., Rea, S.M., Li, J., Usher, K.M., Wylie, J., Ginige, M.P., Cheng, K.Y., Plessis, C. (2014). Iron oxidation and jarosite precipitation in a two-stage airlift bioreactor. *Hydrometallurgy*, **150**, 227-235.

Kannenberg, E.L., Poralla, K. (1999). Hopanoid biosynthesis and function in bacteria. *Naturwissenschaften,* **86**,168–176.

Kamagata, Y., Tamaki, H. (2005). Cultivation of uncultured fastidious microbes. *Microbes Environment*, **20**, 85–91.

Karamanev, D.G., Nikolov, L. N. (1988). Influence of some physicochemical parameters on bacterial activity of biofilm: ferrous iron oxidation by *Thiobacillus ferrooxidans. Biotechnology and Bioengineering*, **31**, 295-299.

Karavaiko, G. I., Bogdanova, T. I., Tourova, T. P., Kondrateva, T. F., Tsaplina, I. A., Egorova, M. A., Krasilnova, E. N., Zakharchuk, L. M. (2005). Reclassification of '*Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans*' strain K1 as *Alicyclobacillus tolerans* sp. nov. and *Sulfobacillus disulfidooxidans* Dufresne *et al.* 1996 as *Alicyclobacillus disulfidooxidans* comb. nov., and emended description of the genus *Alicyclobacillus. International Journal of Systematic and Evolutionary Microbiology*, **55**, 941-947.

Kay, C. M., Rowe, O. F., Rochetti, L., Coupland, K., Hallberg, K. B., Johnson, D. B. (2013). Evolution of microbial "streamer" growths in an acidic, metal contaminated stream draining an abandoned coper mine. *Life*, **3**, 189-210.

Kelly, D. P. & Wood, A. P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov.. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 511–516.

Kelly, D. P. & Wood, A. P. (2005). Genus I. *Acidithiobacillus*. In: D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. (Eds.). *Bergey's Manual of Systematic Bacteriology, 2nd ed, vol. 2 (The Proteobacteria), part B (The Gammaproteobacteria)*. Springer, 60–62.

Kelly, D. P. & Wood, A. P. (2005). Genus I. Acidithiobacillus. In: Brenner, D. J., Krieg, J. T., Staley, G. M., Garrity, G. M.. *Bergey's Manual of Systematic Bacteriology, 2nd Edn., Vol. 2 (The Proteobacteria), part B (The Gammaproteobacteria).* New York: Springer, 60–62.

Kelly, D. P. & Wood, A. P. (2014). The family *Acidithiobacillaceae*. In: Rosenberg, E., DeLong, E., Lory, S., Stackebrandt, E., Thompson, F. (Eds). *The Prokaryotes*; *Gammaproteobacteria*. Springer, 15-25.

Kim, S. B., Nedashkovskaya, O. I., Mikhailov, V. V., Han., S. K., Kim, K-O., Rhee, M-S., Bae, K. S. (2004). *Kocuria marina* sp. nov., a novel *Actinobacterium* isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 1617–1620.

Kimoto, K., Aizawa, T., Urai, M., Ve, N. B., Suzuki, K., Nakajima, M., Sunairi, M. (2010). *Acidocella aluminiidurans* sp. nov., an aluminium tolerant bacterium isolated from *Panicum repens* grown in a highly acidic swamp in actual acid sulfate soil area of Vietnam. *International Journal of Systematic and Evolutionary Microbiology*, **60**, 764-768.

Kimura, S. (2005). *Microbial communities and interactions in extremely acidic environments.* Ph.D. thesis, University of Wales.

Kimura, S., Bryan, C., Hallberg, K. B., Johnson, D. B. (2011). Biodiversity and geochemistry of an extremely acidic, low-temperature subterranean environment sustained by chemolithotrophy. *Environmental Microbiology*, **13**, 2092–2104.

Kimura, S., Hallberg, K.B., Johnson, D.B. (2006). Sulfidogenesis in low pH (3.8–4.2) media by a mixed population of acidophilic bacteria. *Biodegradation*, **17**, 159-167.

Kimura, S., Johnson, D.B. (2004). Sulfate reduction at low pH by a defined mixed culture of acidophilic bacteria. In: Vestraete, W. (Ed.) *European Symposium on Environmental Biotechnology*. Taylor and Francis, 165-168.

Kinnunen, P. H-M., Robertson, W.J., Plumb, J.J., Gibson, J.A.E., Nichols, P.D., Franzmann, P.D., Puhakka, J.A. (2003). The isolation and use of iron-oxidizing, moderately thermophilic acidophiles from the Collie coal mine for the generation of ferric iron leaching solution. *Applied Microbiology and Biotechnology*, **60**, 748-753.

Kinzler, K., Gehrke, T., Telegdi, J., Sand, W. (2001). Bioleaching —result of interfacial processes caused by extracellular polymeric substances (EPS). In: Ciminelli, V.S.T., Garcia Jr., O. (Eds.). *Biohydrometallurgy: Fundamentals, Technology and Sustainable Development*. Process Metallurgy, vol. 11A. Elsevier, Amsterdam, 191–197.

Kishimoto, N., Inagaki, K., Sugio, T., Tano, T. (1990). Growth inhibition of *Acidiphilium* species by organic acids contained in yeast extract. *Journal of Fermentative Bioengineering,* **70**, 7–10.

Kishimoto, N., Kosako, Y., Tano, T. (1991). *Acidobacterium capsulatum* gen. nov., sp. nov. An acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. *Current Microbiology*, **22**, 1-7.

Kishimoto, N., Kosako, Y., Tano, T. (1993). *Acidiphilium aminolytica* sp. nov. An acidophilic chemoorganotrophic bacterium Isolated from acidic mineral environment. *Current Microbiology*, **27**, 131-136.

Kishimoto, N., Kosako, Y., Wakao, N., Tano, T., Hiriashi, A. (1995). Transfer of *Acidiphilium facilis* and *Acidiphilium aminolytica* to the genus *Acidocella* gen. nov., and emendation of the genus *Acidiphilium*. *Systematic and Applied Microbiology*, **18**, 85-91.

Kocur, M. (1986). Genus *Micrococcus* Cohn 1872. In: Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holt, J. G. *Bergey's Manual of Systematic Bacteriology, Vol. 2.*, Williams and Wilkins, 1004-1008.

Koseoglu-Imer, D.Y., Keskinler, B. (2013). Immobilization of *Acidithiobacillus ferrooxidans* on sulfonated microporous poly(styrene–divinylbenzene) copolymer with granulated activated

carbon and its use in bio-oxidation of ferrous iron. *Materials Science and Engineering C*, **33**, 53–58.

Kozubal, M.A., Macur, R.E., Jay, Z.J., Beam, J.P., Malfatti, S.A., Tringe, S.G., Kocar, B.D., Borch, T., Inskeep, W.P. (2012). Microbial iron cycling in acidic geothermal springs of Yellowstone National Park: integrating molecular surveys, geochemical processes, and isolation of novel Fe-active microorganisms. *Frontiers in Microbiology*, **3**, 109.

Kristjánsson, J.K., Hreggvidsson, G.O. (1995). Ecology and habitats of extremophiles. *World Journal of Microbiology and Biotechnology*, **11**, 17-25.

Kunin,V., Engelbrektson, A., Ochman, H., Hugenholtz, P. (2010). Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environmental Biology*, **12**, 118-123.

Kusube, M., Sugihara, A., Moriwaki, Y., Ueoka, T., Shimane, Y., Minegishi, H. (2014). *Alicyclobacillus cellulosilyticus* sp. nov., a thermophilic, cellulolytic bacterium isolated from steamed Japanese cedar chips from a lumbermill. *International Journal of Systematic and Evolutionary Microbiology*, **64**, 2257-2263.

Lauber, C. L., Hamady, M., Knight, R., Fierer, N. (2009). Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. *Applied and Environmental Microbiology*, **75**, 5111–5120.

Lee, J. Y., Kim, S., H., Jeong, H. S., Oh, S. H., Kim, H. R., Kim, Y., H., Lee, J. N., Kook, J.-K., Kho, W-G., Bae, I. K., Shin, J. H. (2009). Two cases of peritonitis caused by *Kocuria marina* in patients undergoing continuous ambulatory peritoneal dialysis. *Journal of Clinical Microbiology*, **47**, 3376–3378.

Lewis, G.N. (1923). *Valence and the structure of atoms and molecules*. The Chemical Catalog Co.

Li, L., Mendis, N., Trigui, H., Oliver, J. D., Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Fronteers in Microbiology*, **5**, 258.

Liljeqvist, M., Valder, J., Holmes, D. H., Dopson, M. (2011). Draft genome of the psychrotolerant acidophile *Acidithiobacillus ferrivorans* SS3. Journal of Bacteriology, **193**, 4304-4305.

Liu, J., Hua, Z-S., Chen, L-X., Kuang, J-L., Li, S-J., Shu, W-S., Huang, L-S. (2014). Correlating microbial diversity patterns with geochemistry in an extreme and heterogeneous mine tailings environment. *Applied and Environmental Microbiology*, **80**, 3677-8366.

Lobos, J. H., Chisolm, T. E., Bopp, L. H., Holmes, D. S. 1986). *Acidiphilium organovorum* sp. nov., an acidophilic heterotroph isolated from *Thiobacillus ferrooxidans* culture. *International Journal of Systematic Bacteriology*, **36**, 139-144.

Lowry, T.M. (1923).THe electronic theory of valency. Part 1. Intramolecular ionisation. *Transactions of the Faraday Society*, **18**, 285-295.

Lovely, D. L. (1987). Organic mineralization with the reduction of ferric iron: A review. *Geomicrobiology*, **5**, 375-399.

Lu, S., Gischkat, S., Reiche, M., Akob, D.M., Hallberg, K.B., Küsel, K. (2010). Ecophysiology of Fe-cycling bacteria in acidic sediments. *Applied and Environmental Microbiology*, **76**, 8174–8183.

Ludwig, W., Euzéby, J., Whitman, W. B. (2012). Taxonomic outline of the phylum *Actinobacteria*. In: Kämpfer P., Busse H.-J., Trujillo M. E., Suzuki K.-i., Ludwig W., Whitman W. B. (Eds.). *Bergey's Manual of Systematic Bacteriology, Volume 5, Part A. Second edition*. Springer, 31.

Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., Stahl, D. A., Brock, T. (Eds.) (2014). *Brock Biology of Microorganisms, 14th Edition*. Pearson,

Malki, M., De Lacey, A., Rodríguez, N., Amils, R., Fernandez, V. M. (2008). Preferential use of an anode as an electron acceptor by an acidophilic bacterium in the presence of oxygen. *Applied and Environmental Microbiology*, **74**, 4472–4476.

Martiny, J.B.H., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., Horner-Devine, M.C., Kane, M., Krumins, J.A., Kuske, C.R., Porin, P.J., Naeem, S., Øverås, L., Reysenbach, A-L., Smith, V.H., Staley, J.T. (2006). Microbial biogeography: Putting microorganisms on the map. *Nature Reviews Microbiology*, **4**, 102-112.

Mather, J. D. (2013). Britain's spa heritage; a hydrological appraisal. In: Duffin, C. J., Moody, R. T. J., Gardner-Thorpe, C. (Eds.). *A history of Geology and Medicine*. Geological Society Special Publication 375, 243-260.

Matin, A. (1999). pH homeostasis in acidophiles. *Novartis Found Symposium* *221*,152-63.

Matsubara, H., Goto, K., Matsumara, T., Mochida, K., Iwaki, M., Niwa, M., Yamasato, K. (2002). *Alicyclobacillus acidiphilus* sp. nov., a novel thermo-acidophilic, ω-alicyclic fatty acid-containing bacterium isolated from acidic beverages. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 1681-1685.

Matsuzawa, Y., Kanbe, T., Suzuki, J., Hiriashi, A. (2000). Ultrastructure of the acidophilic aerobic photosynthetic bacterium *Acidiphilium rubrum*. *Current Microbiology*, **40**, 398–401.

Mazuelos, A., Moreno, J. M., Carranza, F., Palomino, C., Torres, A., Villalobo, E. (2012). Biotic factor does not limit operational pH in packed-bed bioreactor for ferrous iron biooxidation. *Journal of Industrial Microbiology and Biotechnology*, **39**, 1851-1858.

McFarlane, H., Döring, A., Persson, S. (2014). The cell biology of cellulose synthesis. *Annual Reviews of Plant Biology*, **65**, 69-94.

McGinness, S., Johnson, D. B. (1992). Selective solid media for isolating and enumerating acidophilic bacteria. *Journal of Microbiological Methods*, **23**, 205-218.

McKenna, D., Spence, D., Haggan, S.E., McCrum, E., Dornan, J.C., Lappin, T. R. (2003). A randomized trial investigating an iron-rich natural mineral water as a prophylaxis against iron deficiency in pregnancy. *Clinical and Laboratory Haematology*, **25**, 99-103.

Melamud, V. S., Pivovarova, T. A., Tourova, T. P., Kolganova, T. V., Osipov, G. A., Lysenko, A. M., Kondrateva, T. F., Karvaiko, G. I. (2003). *Sulfobacillus sibiricus* sp. nov., a new moderately thermophilic bacterium. *Microbiology*, **72**, 605-612.

Mendez, M. O., Neilson, J. W., Maier, R. M. (2008). Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site. *Applied and Environmental Microbiology*, **74**, 3899–3907.

Merriman, R. J., Highley, D. E., Cameron, D. G. (2003). Definition and characteristics of very fine-grained sedimentary rocks: clay, mudstone, shale and slate. Commissioned report CR/03/281N, British Geological Society.

Mitchell, D., Harneit, K., Meyer, G., Sand, W., Stackebrandt, E. (2004). Systematic analysis of our culture collection for "genospecies" of *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans and Leptospirillum ferrooxidans*. In: Ciminelli, V. S. T., Garcia, O. Jr. (Eds.). *Biohydrometallurgy: fundamentals, technology and sustainable development*. Elsevier, Amsterdam, 1369–1378.

Moya-Beltrán, A., Cárdenas, J. P., Covarrubias, P. C., Issotta, F., Ossandon, F. J., Grail, B. M., Holmes, D. S., Quatrini, R., Johnson, D. B. (2014). Draft genome sequence of the nominated type strain of "*Ferrovum myxofaciens*," an acidophilic, iron-oxidizing *Betaproteobacterium*. *Genome Announcements*, **2**, 4.

Ñanchucheo, I., Johnson, D.B. (2010). Production of glycolic acid by chemolithotrophic iron- and sulfur-oxidizing bacteria and its role in delineating and sustaining acidophilic sulfide mineral-oxidizing consortia. *Applied and Environmental Microbiology*, **76**, 461–467.

Ňancucheo, N., Johnson, D. B. (2013). Acidophilic algae isolated from mine-impacted environments and their roles in sustaining heterotrophic acidophiles. *Frontiers in Microbiology*, **3**, 325.

Ni, Y., Wan, D., He, K. (2008). 16S rDNA and 16S–23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Acidithiobacillus* phylogeny. *Microbiology*, **154**, 2397–2407.

Nicolaus, B., Improta, R., Manaca, M. C., Lama, L., Esposito, E., Gambacorta, A. (1998). *Alicyclobacilli* from an unexplored geothermal soil in Antarctica: Mount Rittmann. *Polar Biology*, **19**, 133-141.

Nordstrom, D. K., Alpers, C. N., Ptacek, C. J., Blowes, D. W. (2000). Negative pH and extremely acidic mine waters from Iron Mountain, California. *Environmental Science and Technology*, **34**, 254-258.

Nordstrom, D. K. (2011). Mine Waters: acidic to circum-neutral. *Elements*, **7**, 393-398.

Norris, P. R., Barr, D. W., Hinson, D. (1988). Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite. In: Norris, P. R., Kelly, D. P. (Eds.) *Biohydrometallurgy*, *Proceedings of the International Symposium*, Kew: Science and Technology Letters. 43-59.

Norris, P. R., Clark, D. A., Owen, J. P., Waterhouse, S. (1996). Characteristics of *Sulfobacillus acidophilus* sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing bacteria. *Microbiology*, **142**, 775-783.

Norris, P. R., Davis-Belmar, C. S., Brown, C. F., Calvo-Bado, L. A. (2011). Autotrophic, sulfur-oxidizing actinobacteria in acidic environments. *Extremophiles*, **15**, 155-163.

Norris, P. R., Ingledew, W. J. (1992). Acidophilic bacteria: adaptations and applications. In: Herbert, R. A. & Sharp R. J. (Eds), *Molecular biology and biotechnology of extremophiles*. Blackie, Glasgow, 115–142.

Offre, P., Spang, A., Schleper, C. (2013). Archaea in biogeochemical cycles. *Annual Reviews of Microbiology*, **67**, 437–457.

Ohmura, N., Sasaki, K., Matsumoto, N., Saiki, H. (2002). Anaerobic respiration using Fe^{3+}, S^0, and H^2 in the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans. Journal of Bacteriology*, **184**, 2081–2087.

Okamura, K., Kawai, A., Wakao, N., Yamada, T., Hiriashi, A. (2015). *Acidiphilium iwatense* sp. nov., isolated from an acid mine drainage treatment plant, and emendation of the genus *Acidiphilium*. *International Journal of Systematic and Evolutionary Microbiology*, **65**, 42-48.

Olem, H., Unz, R. F. (1977). Acid mine drainage treatment with rotating biological contactors. *Biotechnology and Bioengineering*, **19**, 1475-1491.

Oliver, J.D. (2005). The viable but nonculturable state in bacteria. *The Journal of Bacteria*, **43**, 93-100.

Osorio, H., Mangold, S., Denis, Y., Ñancucheo, I., Esparza, M., Johnson, D. B., Bonnefoy, V., Dobson, M., Holmes, D. F. (2013). Anaerobic sulfur metabolism coupled to dissimilatory iron reduction in the extremophile *Acidithiobacillus ferrooxidans. Applied and Environmental Microbiology,* **79**, 2172–2181.

O'Sullivan, A. S. (1997). Cellulose: the structure slowly unravels. *Cellulose*, **4**, 173-207.

Pankratov, T.A. (2012). *Acidobacteria* in microbial communities of the bog and tundra lichens. *Microbiology*, **81**, 56-63.

Park, D., Lee, D.S., Joung, J.Y., Park, J.M. (2005). Comparison of different bioreactor systems for indirect H_2S removal using iron-oxidizing bacteria. *Process Biochemistry*, **40**, 1461–1467.

Pearce, J. B., Kemp, S. J. (1994). A geochemical study of acid mine drainage from a pyrite mine, North Wales. *International Mine Water Association Proceedings*, 1994.

Peccia, J., Marchand, E.A., Silverstein, J., Hernandez, M. (2000). Development and application of small-subunit rRNA probes for assessment of selected ***Thiobacillus*** species and members of the genus ***Acidiphilium***. *Applied and Environmental Microbiology,* **66**, 3065–3040.

Pikuta, E. V., Hoover, R. B. (2007). Microbial extremophiles at the limits of life. *Critical Reviews in Microbiology*, **33**, 183–209.

Pronk, J.T., Meesters, P.J.W., van Dijken, J.P., Bos, P., Kuenen, J.G. (1990). Heterotrophic growth of *Thiobaciilus acidophilus* in batch and chemostat cultures. *Archives of Microbiology*, **153**, 392-398.

Purity, S., Saranathan, R., Prashanth, K., Narayanan, K., Asir, J., Devi, S. C., Amaranth, S. K. (2013). The expanding spectrum of human infections caused by *Kocuria species*: a case report and literature review. *Emerging Microbes and Infections*, **2**, doi:10.1038/emi.2013.71.

Quinteros, A., Farré, R., Lagarda, M. J. (2001). Optimization of iron speciation (soluble, ferrous and ferric) in beans, chickpeas and lentils. *Food Chemistry*, **75**, 365–370.

Rappé, M.S., Giovannoni, S.J. (2003). The uncultured microbial majority. *Annual Reviews of Microbiology*, **57**, 369-394.

Rawlings, D.E. (2005). Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. *Microbial Cell Factories*, **4**, 13.

Rawlings, D. E., Kusano, T. (1994). Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiological Reviews*, **58**, 39-55.

Rawlings, D.E., Tributsch H., Hansford, G.S. (1999). Reasons why '*Leptospirillum*'-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. *Microbiology*, **145**, 5–13.

Reasoner, D. J., Geldrich, E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*, **49**, 1-7.

Richard, H. T., Foster, J.W. (2003). Acid resistance in *Escherichia coli. Advances in Applied Microbiology*, **52**,167-186.

Richard, H., Foster, J.W. (2004). *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. *Journal of Bacteriology*, **186**, 6032-6041.

Rittenberg, S.C. (1969). The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. *Advances in microbial Physiology*, **3,** 159-196.

Rittenberg, S.C. (1971). The obligate autotroph – the demise of a concept. *Antonie van Leeuwenhoek*, **38**, 457-478.

Rohwerder, T.,Gehrke. T., Kinzler, K., Sand, W. (2003). Bioleaching review part A: Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Applied and Microbiology and Biotechnology*, **63**, 239–248.

Rohwerder, T., Sand, W. (2007). Oxidation of inorganic sulfur compounds in acidophilic prokaryotes. *Engineering in Life Sciences*, **7**, 301-309.

Rossello-Mora, R., Lucio, M., Pena, A., Brito-Echeverria, J., Lopez-Lopez, A., Valens-Vadell, M., Frommberger, M., Anton, J., Schmitt-Kopplin, P. (2008). Metabolic evidence for biogeographic isolation of the extremophilic bacterium *Salinibacter ruber*. *International Society of Microbial Ecology Journal*, **2**, 242–253.

Rowe, O.R. (2007). *A study of the geochemical and microbial interactions in acidic environments and their potential application for the bioremediation of acid mine drainage*. Ph.D. thesis, University of Wales.

Rowe, O.R., Johnson, D.B. (2007). Comparison of ferric iron generation by different species of acidophilic bacteria immobilized in packed-bed reactors. *Systematic and Applied Microbiology*, **31**, 68-77.

Rowe, O.F., Sánchez-España, J., Hallberg, K.B. and Johnson, D.B. (2007). Microbial communities and geochemical dynamics in an extremely acidic, metal-rich stream at an abandoned sulfide mine (Huelva, Spain) underpinned by two functional primary production systems. *Environmental Microbiology,* **9**, 1761-1771.

Rusconi, R., Lecuer, S., Guglielmini, L., Stone, H. A. (2010). Laminar flow around corners triggers the formation of biofilm streamers. *Interface*, **6**, 1293-9.

Sánchez-Andrea, I., Rodríguez, N., Amils, R., Sanz, J. L. (2011). Microbial diversity in anaerobic sediments at Rio Tinto, a naturally acidic environment with a high heavy metal content. *Applied and Environmental Microbiology*, **77**, 6085–6093.

Sánchez-Andrea, I., Stams, A.J., Hedrich, S., Ňancucheo, I., Johnson, D.B. (2015). *Desulfosporosinus acididurans* sp. nov.: an acidophilic sulfate-reducing bacterium isolated from acidic sediments. *Extremophiles*, **19**, 39-47.

Sand, W., Gehrke, T. (2006). Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron (III) ions and acidophilic bacteria. *Microbiology*, **157**, 49–56.

Sand, W., K. Rohde, B. Sobotke, Zenneck, C. (1992). Evaluation of *Leptospirillum ferrooxidans* for leaching. *Applied and Environmental Microbiology*, **58**, 85-92.

Santofimia, E., González-Toril, E., López-Pamo, E., Gomariz, M., Amils, R., Aguilera, A. (2013). Microbial diversity and its relationship to physicochemical characteristics of the water in two extreme acidic pit lakes from the Iberian Pyrite Belt (SW Spain). *PLoS One*, **8**, e66746.

Schippers, A., Jozsa, P., Sand, W. (1996). Sulfur chemistry in bacterial leaching of pyrite. *Applied and Environmental Microbiology*, **62**, 3424–3431.

Schloss, P. D., Gevers, D., Westcott, S. L. (2011). Reducing the Effects of PCR Amplification and sequencing artifacts on 16S rRNA-based studies. *PLOS One*, **6**, e27310. doi:10.1371/journal.pone.0027310.

Schwertmann, U., Taylor, R. M. (1977). Iron oxides. In: Dixon, J. B., Weed, S. B. (Eds.). *Minerals in Soil Environments, Soil Science Society of America*, 145-180.

Shade, A., Hogan, C. S., Kimowicz, A. K., Linske, M., McManus, P. S., Handelsman, J. (2012). Culturing captures members of the soil rare biosphere. *Environmental Microbiology,* **14**, 2247-2252.

Sieber, J. R., McInterney, M.J., Gunslaus, R.P. (2012). Genomic insights into syntrophy: The paradigm for anaerobic metabolic cooperation. *Annual Reviews of Microbiology*, **66**, 429-452.

Sirinivasan, V., Morowitz, H.J., Huber, H. (2012). What is an autotroph? *Archives of Microbiology*, **194**, 135-140.

Simbahan, J., Drijber, R., Blum, P. (2004). *Alicyclobacillus vulcanalis* sp. nov., a thermophilic, acidophilic bacterium isolated from Coso Hot Springs, California, USA. *International Journal of Systematic Bacteriology*, **54**, 1703-1707.

Smeulders, M. J., Pol, A., Venselaar, H., Barends, T. R. M., Hermans, J., Jetten, M. S. M., Op den Camp, H. J. M. (2013). Bacterial C_S2 hydrolases from *Acidithiobacillus thiooxidans* strains are homologous to the archaeal catenane C_S2 hydrolase. *Journal of Bacteriology*, **195**, 4046–4056.

Smith, N. A., Kelly, D. P. (1988). Isolation and physiological characterization of autotrophic sulfur bacteria oxidizing dimethyl disulfide as sole source of energy. *Journal of General Microbiology*, **134**, 1407–1417.

Smitt, Y., Cameron, M., Venter, P., Witthuhn, R., C. (2011). *Alicyclobacillus* spoilage and isolation - A review. *Food Microbiology*, **28**, 331-349.

Stackebrandt, E. (2012). *Dermacoccus.* In: Whitman, W. B., Parte, A., Goodfellow, M., Kämpfer, P., Busse, H., Trujillo, M. E., Ludwig, W., Suzuki, K. (Eds.). (2012). *Bergey's Manual of Systematic Bacteriology: Volume 5.* Springer Science & Business Media, 737-741.

Stackenbrandt, E., Goebel, B. M. (1994). Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, **44**, 846-849.

Stackebrandt, E., Rainey, F. A., Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis. nov.. *International Journal of Systematic Bacteriology*, **47**, 479-491.

Staley, J.T., Konopka, A. (1985). Measurement of in situ activities of non-photosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Reviews in Microbiology*, **39**, 321-346.

Steven, J.R., Chang, Y-J., Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Flemming, C., A., White, D.C. (1999). Effect of toxic metals on indigenous soil b-subgroup *Proteobacterium* ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. *Applied and Environmental Microbiology*, **65**, 95-101.

Stookey, L. L. (1970). Ferrozine-A new spectrophotometric reagent for iron. *Analytical Chemistry*, **42**, 778-781.

Streten-Joyce, C., Manning, J., Gibb, K. S., Neilan, B. A., Parry, D. L. (2013). The chemical composition and bacteria communities in acid and metalliferous drainage from the wet–dry tropics are dependent on season. *Science of the Total Environment*, **443**, 65–79.

Stumm, W., Lee, G. F. (1961). Oxygenation of ferrous iron. *Industrial and Engineering Chemistry*, **53**,143–146.

Stumm, W., Morgan, J. J. (1981). *Aquatic Chemistry*. Wiley-Interscience, New York, 780.

Sulonen, M. L. K., Kokko, M. E., Lakaniemi, A-M., Puhakka, J. A. (2015). Electricity generation from tetrathionate in microbial fuel cells by acidophiles. *Journal of Hazardous Materials*, **284**, 182–189.

Suter, D., Branwart, S., Stumm, W. (1991). Dissolution of hydrous Iron (III) oxides by reductive mechanism. *Langmuir*, **7**, 809-813.

Suzuki, K., Wakao, N., Sakurai, Y., Kimura, T., Sakka, K., Ohmiya, K. (1997). Transformation of *Escherichia coli* with a large plasmid of *Acidiphilium multivorum* AIU 301 encoding arsenic resistance. *Applied and Environmental Microbiology*, **63**, 2089-2091.

Suzuki, K., Wakao, N., Kimura, T., Sakka, K., Ohmiya, K. (1998). Expression and regulation of the arsenic resistance operon of *Acidiphilium multivorum* AIU 301 plasmid pKW301 in *Escherichia coli*. *Applied and Environmental Microbiology*, **64**, 411-418.

Tindall, B., J., Roselló-Móra, R., Busse, H-J., Ludwig, W., Kämpfers, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology,* **60**, 249-266.

Tischler, J.S., Jwair, R. J., Gelhaar, N., Drechsel, A., Skirl, A.M., Wiacek, C., Janneck, E., Schlömann, M. (2013). New cultivation medium for "*Ferrovum*" and *Gallionella*-related strains. *Journal of Microbial methods*, **95**, 138-144.

Tsuroka, N., Isono, Y., Shida, O., Hemmi, H., Nakayama, T., Nishino, T. (2003). *Alicyclobacillus sendaiensis* sp. nov., a novel acidophilic, slightly thermophilic species isolated from soil in Sendai, Japan. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1081-1084.

Tuttle, J. H., Randles, C. I., Dugan, P. R. (1968). Activity of Microorganisms in acid mine water 1. Influence of acid water on aerobic heterotrophs of a normal stream. *Journal of Bacteriology*, **95**, 1495-1503.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., Soloyev, V. V., Rubin, E. M., Rokhsar, D. S., Banfield, J. F. (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature*, **428**, 37-43.

Ueda, T., Suga, Y., Yahiro, N., Matsuguchi, T. (1997). Remarkable N2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *Journal of Bacteriology*, **177**, 1414-1417.

Valdés, J., Ossandon, F., Quatrini, R., Dopson, M., Holmes, D. S. (2011). Draft genome sequence of the extremely acidophilic biomining bacterium *Acidithiobacillus thiooxidans* ATCC 19377 provides insights into the evolution of the *Acidithiobacillus* genus. *American Society for Microbiology*, **193**, 7003–7004.

Valdés, J., Pedroso, I., Quatrini, R., Holmes, D. S. (2008). Comparative genome analysis of *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *A. caldus*: Insights into their metabolism and ecophysiology. *Hydrometallurgy*, **94**, 180–184.

Vartoukian, S. R., Palmer R. M., Wade, W. G. (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiology Letters*, **309**, 1-7.

Wakao, N., Mashina, M., Sakurai, Y., Shiota, H. (1984). Bacterial pyrite oxidation III. Adsorption of *Thiobacillus ferrooxidans* cells to solid surfaces and its effect on iron release from pyrite. *Journal of General and Applied Microbiology*, **30**, 63–77.

Wakao, N., Nagasawa, N., Matsuura, T., Matsukura, H., Matsumoto, T., Hirashi, A., Sakurai, Y., Shiota, H. (1994). *Acidiphilium multivorum* sp. nov., an acidophilic chemoorganotrophic bacterium from pyritic acid mine drainage. *Journal of General and Applied and Microbiology*, **40**, 143-159.

Wakao, N., Yokoi, N., Isoyama, N., Hiraishi, A., Shimada, K., Kobyashi, M., Kise, H., Iwaki, M., Itoh, S., Takatchi, S., Sakurai, Y. (1996). Discovery of natural photosynthesis using Zn-containing bacteriochlorophyll in an aerobic bacterium *Acidphilium rubrum*. *Plant Cell Physiology*, **37**, 889-893.

Watling, H.R., Perrot, F.A., Shiers, D.W. (2008). Comparison of selected characteristics of *Sulfobacillus* species and review of their occurrence in acidic and bioleaching environments. *Hydrometallurgy*, **93**, 57–65.

Watling, H. R., Watkin, E. L. J., Ralph, D. E. (2010).The resilience and versatility of acidophiles that contribute to the bio-assisted extraction of metals from mineral sulphides. *Environmental Technology*, **31**, 915–933.

Watkin, E. L. J., Keeling, S. E., Perrot, F. A., Shiers, D. W., Palmer, M-L., Watling, H. R. (2009). Metals tolerance in moderately thermophilic isolates from a spent copper sulfide heap, closely related to *Acidithiobacillus caldus*, *Acidimicrobium ferrooxidans* and *Sulfobacillus thermosulfidooxidans*. *Journal of Industrial Microbiology & Biotechnology*, **36**, 461-465.

Welander, P., Hunter, R., Zhang, L., Sessions, A., Summons, R., Newman, D. (2009). Hopanoids play a role in membrane integrity and pH homeostasis in *Rhodopseudomonas palustris* TIE-1. *Journal of Bacteriology*, **191**, 6145–6156.

Whortman, A. T., Voelz, H., Lantz, R. C., Bissonnette, G. K. (1986). Effect of acid mine water on *Escherichia coli*: Structural damage. *Current Microbiology*, **14**, 1-5.

Wichlacz, P.L., Unz, R.F. (1981). Acidophilic, heterotrophic bacteria of acidic mine waters. *Applied and Environmental Microbiology*, **41**, 1254-1261.

Wichlacz, P., Unz, P., Langworthy, T. (1986). *Acidiphilium angustum* sp. nov. *Acidiphilium facilis* sp. nov. And *Acidiphilium rubrum* sp. nov. : Acidophilic heterotrophic bacteria isolated from acidic coal mine drainage. *International Journal of Systematic Bacteriology*, **36**, 197-201.

Williams, K. P., Kelly, D. P. (2013). Proposal for a new class within the phylum *Proteobacteria*, *Acidithiobacillia* classis nov., with the type order *Acidithiobacillales*, and emended description of the class *Gammaproteobacteria*. *International Journal of Systematic and Evolutionary Microbiology*, **63**, 2901-2906.

Wisotzkey, J. D., Jurtshuk Jr., P., Fox, G. E., Deinhard, G., Poralla, K. (1992). Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacilluscycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *International Journal of Systematic and Evolutionary Bacteriology*, **42**, 263-269.

Xia, J. L., Peng, A. A., He, H., Yang, Y., Liu, X. D. & Qiu, G. Z. (2007). A new strain *Acidithiobacillus albertensis* BY-05 for bioleaching of metal sulfides ores. *Transactions of Nonferrous Metals Society of China*, **17**, 168-175.

Yin, H., Cao, L., Xie, M., Chen, Q., Qiu, G., Zhou, J., Wu, L., Wang, D., Liu, X. (2008). Bacterial diversity based on 16S rRNA and gyrB genes at Yinshan mine, China. *Systematic and Applied Microbiology*, **31**, 302–311.

You, X-Y., Guo, X., Zheng, H-J., Zhang, M-J., Liu, L-J., Zhu, Y-Q., Zhu, B., Wang, S-Y., Zhao, G-P., Poetsch, A., Jiang, C-Y., Liu, S-J. (2011). Unravelling the *Acidithiobacillus caldus* complete genome and its central metabolisms for carbon assimilation. *Journal of Genetics and Genomics*, **38**, 243-252.

Younger, P. L. (1997).The longevity of mine water pollution: a basis for decision-making. *The Science of Total Environment*, **194/195**, 457-466.

Younger, P. L. (2000). Predicting temporal changes in total iron concentrations in groundwaters flowing from abandoned deep mines: a first approximation. *Journal of Contaminant Hydrology*, **44**, 47–69.

Younger, P., Potter, H. A. B. (2012). *Parys in springtime: Hazard management and steps towards remediation of the UK's most polluted acidic mine discharge.* In: 9th International Conference on Acid Rock Drainage (ICARD), 20th-26th May, 2012, Ottowa, Canada

Zaitseva, S. V., Abidueva, E. Y., Namsaraeva, B. B., Wang, L., Wu, L. (2014). Microbial community of the bottom sediments of the brackish lake Beloe (Transbaikal Region). *Microbiology*, **83**, 861-868.

Zengler, K., Toledo, G., Rappé, M., Elkins, J., Mathur, E. J., Short, J. M., Keller, M. (2002). Cultivating the uncultured. *PNAS*, **99**, 15681-15686.

Zinger,L., Amaral-Zettler, L. A., Fuhrman, J. A., Horner-Devine, M. C., Huse, S. M., Welch, D. B. M., Martiny, J. B. H., Sogin, M., Boetius, A., Ramette, A. (2011). Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. *PLoS ONE*, **6**, e24570. doi:10.1371/journal.pone.0024570.

Appendix 1. List of publications and conference presentations

Journal papers

Jones, R. M., Hedrich, S., Johnson, D. B.. (2013). *Acidocella aromatica* sp. nov.: an acidophilic heterotrophic alphaproteobacterium with unusual phenotypic traits. *Extremophiles*, **17**, 841-850.

Jones, R. M., Johnson, D. B.. (2015). *Acidithrix ferrooxidans* gen. nov., sp. nov.; a filamentous and obligately heterotrophic, acidophilic member of the *Actinobacteria* that catalyzes dissimilatory oxido-reduction of iron. *Research in Microbiology*, **166**, 111-120.

Jones, R. M., Johnson, D. B.. (2015). Metabolism of small molecular weight aliphatic acids: a key physiological trait of the acidophilic alphaproteobacterium *Acidocella acidivorans*, sp. nov. *Extremophiles*, IN PRESS.

Conference paper and presentation

Jones, R. M., Johnson, D. B.. (2014). Characteristics of novel acidophilic bacteria with potential for remediating metal-rich mine waters. *Proceedings of Biohydrometallurgy 2014, Falmouth.*

Conference presentation

Jones, R. M.. (2014). Identification and isolation of acidophilic bacteria from an acidic chalybeate spring (Trefriw Wells Spa, North Wales*). Molecular Microbial Ecology Group (MMEG) meeting 2014, Bangor.*

Appendix 2. R 3 y-axis graph programme script

```
setwd("C:\\Users\\Dropbox\\Documents\\Rose\\Graph")
roseData <- read.csv("Coupling_curve.csv")
pdf("3YaxisGRAPH", width = 12, height = 8)

#Plot size defined to make room for additional axis
par(mar=c(4, 12, 5, 10) + 0.1)

#Graph with y values plotted
plot(x = roseData$Days, y = roseData$Fe2_mean, axes=F,
ylim=c(0,max(roseData$Fe2_mean)), xlab="",
ylab="",type="l",col="black", main="",xlim=c(0,12),lwd=2)
points(x = roseData$Days, y = roseData$Fe2_mean,,pch=19,col="black")

#y axis drawn and labelled
axis(2, cex=1.75, ylim=c(0,max(roseData$Fe2_mean)),col="black",lwd=2)
mtext(2,text="Iron oxidised (mM)",line=2.5, cex=1.75)

#Error bars added
feErrorUp <- roseData$Fe2_mean + roseData$Fe2_E
feErrorDown <- roseData$Fe2_mean - roseData$Fe2_E
arrows(x0 = roseData$Days, x1 = roseData$Days, y0 = roseData$Fe2_mean,
y1 = feErrorDown, angle = 90, length = 0.05)
arrows(x0 = roseData$Days, x1 = roseData$Days, y0 = roseData$Fe2_mean,
y1 = roseData$Fe2_mean + roseData$Fe2_E, angle = 90, length = 0.05)
```

```
#Next set of values overlaid on the previous graph plot
par(new=T)

#Next set of values plotted, and error bars added
plot(x = roseData$Days, y = roseData$Protein_mean, axes=F,
ylim=c(0,max(roseData$Protein_mean + 20)), xlab="", ylab="",
type="l",lty=1, main="",xlim=c(0,12),lwd=2)
axis(2, cex=2, ylim=c(0,140),lwd=2,line=5)
points(x = roseData$Days, roseData$Protein_mean,pch=17)
mtext(2,text="Protein (ug)",line=7, cex=1.75)
proErrorDown <- roseData$Protein_mean - roseData$Protein_E
proErrorUp <- roseData$Protein_mean + roseData$Protein_E
arrows(x0 = roseData$Days, x1 = roseData$Days, y0 = roseData$Protein_mean,
y1 = proErrorDown, angle = 90, length = 0.05)
arrows(x0 = roseData$Days, x1 = roseData$Days, y0 = roseData$Protein_mean,
y1 = proErrorUp, angle = 90, length = 0.05)

#Final Y values and error bars added
par(new=T)
plot(x = roseData$Days, y = roseData$Glucose_mean, axes=F, ylim=c(0,6), xlab="", ylab="",
type="l",lty=1, main="",xlim=c(0,12),lwd=2)
axis(4, ylim=c(0,max(roseData$Glucose_mean)),lwd=2, line = 1, cex=2)
points(x = roseData$Days, y = roseData$Glucose_mean,pch=15)
mtext(4,text="Glucose (mM)",line=3.5, cex=1.75)
proErrorDown <- roseData$Glucose_mean - roseData$Glucose_E
proErrorUp <- roseData$Glucose_mean + roseData$Glucose_E
```

```
arrows(x0 = roseData$Days, x1 = roseData$Days, y0 = roseData$Glucose_mean,
y1 = proErrorDown, angle = 90, length = 0.05)
arrows(x0 = roseData$Days, x1 = roseData$Days, y0 = roseData$Glucose_mean,
y1 = proErrorUp, angle = 90, length = 0.05)

#x axis drawn
axis(1,pretty(range(1:12,6)), cex = 2, lwd=2)
mtext("Time (Days)",side=1,col="black",line=2, cex=1.75)

dev.off()
```