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Moderately thermophilic acidophiles and their use in mineral processing

Okibe, Naoko

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MODERATELY THERMOPHILIC ACIDOPHILES AND THEIR USE IN MINERAL PROCESSING

A Thesis submitted to the University of Wales
in candidature for the degree of Philosophiae Doctor

by

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Naoko Okibe

Master of Engineering
Bachelor of Engineering

School of Biological Sciences,
Memorial Building
University of Wales, Bangor,
Gwynedd LL57 2UW,
United Kingdom

2002

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Abstract

This research project focused on moderately thermophilic acidophilic microorganisms and their role in the oxidation of pyrite. A major objective of the work was to assess the relative efficiencies of defined combinations of moderate thermophiles in oxidising pyrite under defined conditions. In addition, various aspects of the physiology and phylogeny of moderately thermophilic acidophiles were investigated. Moderately thermophilic acidophiles, including novel acidophiles (a thermotolerant *Leptospirillum* and a *Ferroplasma* sp.), were isolated from a commercial stirred-tank pilot plant. Pyrite oxidation by mixed cultures of different combinations of moderate thermophiles, including the novel isolates, was assessed in preliminary shake flask experiments. Data from these experiments were used to select microbial consortia in later experiments in temperature- and pH-controlled bioreactors. These involved monitoring rates of mineral oxidation, and relative numbers of the different microorganisms included in the original inoculum, using a plating technique in conjunction with a molecular approach (FISH). The results from the pyrite oxidation studies indicated that mixed populations of acidophiles may accentuate or diminish the rates and extent of pyrite oxidation, relative to pure cultures. The thermotolerant *Leptospirillum* isolate was found to be unable to oxidise a pyrite concentrate when grown in pure culture, though this inhibition was overcome when the iron-oxidiser was grown in mixed cultures with various Gram-positive acidophiles. Investigation of the effects of fifteen individual and mixtures of flotation chemicals on moderately thermophilic acidophiles revealed different degrees of toxicities of the different reagents and sensitivities of the microorganisms, with the *Leptospirillum* isolate generally being the most sensitive of those tested. The phenomenon of pH-related ferric iron toxicity to moderately thermophilic and mesophilic Gram-positive bacteria was also investigated. ARDREA (Amplified Ribosomal DNA Restriction Enzyme Analysis) using the 16S rRNA gene sequences of known acidophilic bacteria, was refined and developed, and applied successfully to identify moderate thermophiles isolated from environmental samples.

Acknowledgements

I wish to express my sincere gratitude to my supervisor, Dr. Barrie Johnson, for his attentive supervision, support and encouragement. His consistent warm support always made me confident and comfortable during my time at the University of Wales, Bangor.

I would like to thank Dr. Kevin Hallberg for his valuable comments and criticism, especially for molecular side of my work.

My special thanks to Mr Stewart Rolf, for his technical assistance and sense of humour. There were always good laughs wherever he was.

Many thanks to my colleagues in G11 laboratory, Sakurako and Kris, for their assistance and being good company with me.

The writing of this thesis was made possible through grants from Glaxo International Scholarship Foundation, Institute of Mining and Metallurgy and Gen Foundation, and I would like to acknowledge here the generosity of these organisations.

My great respect and gratitude go to my mother, Minako Okibe and my father, Masao Okibe, for their endless love, support and encouragement.

Finally, but very importantly, a special thanks to my husband, Franz, for being there for me whenever I needed him.

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Abbreviations

%	percent	Fe^{3+}	ferric iron
µl	microlitre	Fe(III)	ferric iron
µg	microgram	Feo	ferrous iron overlay medium
µm	micrometre		
µmoles	micromoles	FeSo	ferrous iron/tetrathionate overlay medium
A	adenine		
AAS	Atomic Absorbance Spectroscopy	FISH	Fluorescent *In Situ* Hybridisation
ADP	adenosine diphosphate	g	gram
AMD	Acid Mine Drainage	G	guanine
ANOVA	analysis of variance	GTP	guanosine triphosphate
ARDREA	Amplified Ribosomal DNA Restriction Enzyme Analysis	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
ATCC	American Type Culture Collection	HOQNO	*n*-heptyl-4-hydroxyquinolone *N*-oxide
ATP	adenosine triphosphate	L	litre
A_x	absorbance at x nm	M	molar
BLAST	Basic Local Alignment Search Tool	mg	milligram
		MIC	Minimum Inhibitory Concentration
bp	base pair		
BSA	Bovine Serum Albumin	min	minute
C	cytosine	ml	millilitre
°C	degrees Celsius	mm	millimetre
CFUs	Colony Forming Units	mM	millimolar
CTP	cytidine triphosphate	mmols	millimoles
DAPI	4',6-diamidino-2-phenylindole	$mS\ cm^{-1}$	millisiemens per centimetre
		MS	metal sulfide
DGGE	Denaturing Gradient Gel Electrophoresis	mV	millivolts
		NAD^+	nicotinamide adenine dinucleotide, oxidised form
dH_2O	distilled water		
DMSO	dimethylsulfoxide	NADH	nicotinamide adenine dinucleotide, reduced form
DNA	deoxyribonucleic acid		
DOC	Dissolved Organic Carbon	ng	nanogram
DSMZ	Deutsche Sammlung von Mikroorganismen und Zelkulturen	nm	nanometre
		nmols	nanomoles
		OD_x	optical density at x nm
EDTA	Na_2 ethylenediaminetetra-acetic acid	PCR	Polymerase Chain Reaction
		PFA	paraformaldehyde
Eh	redox potential	PFGE	Pulse Field Gel Electrophoresis
EPS	Extracellular Polymeric Substances	PHYLIP	Phylogeny Inference Package
Fe/YE	ferrous iron/yeast extract solid medium	ppm	parts per million
Fe^{2+}	ferrous iron	RAPD	Random Amplification of Polymorphic DNA
Fe(II)	ferrous iron		

Rep-APD	Repetitive Primer Amplified DNA
RFLP	Restriction Fragment Length Polymorphism
RISC	Reduced Inorganic Sulfur Compound
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	Sodium Dodecyl Sulfate
S°	elemental sulfur
SX/EW	solvent extraction and electrowinning
T	thymine
t_d	culture doubling time
T_m	melting temperature
Tris	tris(hydroxymethyl)methylamine
TSB	Tryptone Soya Broth
TTP	thymidine triphosphate
UK	United Kingdom
UV	ultraviolet light
UWB	University of Wales, Bangor
v	volume
w	weight
YE	Yeast Extract

-This thesis is dedicated to my parents.

Chapter 1

General Introduction

1.1 Bioleaching, Biooxidation and Depyritization

Bioleaching refers to the use of microorganisms to extract metals with economic value, such as copper, zinc, uranium, nickel and cobalt, from sulfide minerals. During the oxidation, the microorganisms release the metals of value into the solution phase, facilitating their recovery by, for example, solvent extraction and electrowinning (SX/EW; Brierley, 1997).

Biooxidation refers to a pre-treatment process of sulfide ores or concentrates, usually pyrite or arsenopyrite, which host gold, silver, or both. This process uses similar microorganisms as bioleaching to degrade minerals. Degradation of ores or concentrates by microorganisms in the biooxidation process facilitates the release of precious metals either by chemical extraction or by bioleaching from the solid residue. Therefore, biooxidation leaves the metal values in the solid phase and the solution is discarded (Brierley, 1997).

Depyritization refers to the removal of inorganic (pyritic) sulfur from coal, and is based on a complex combination of spontaneous (non-biological) and microbiologically-catalysed oxidation of inorganic sulfidic minerals present in coal. This combination of reactions leads to the dissolution of the sulfidic minerals present in the coal. By separating the coal from the process fluid, a fuel is obtained with a lower sulfur content (Bos *et al.*, 1992).

1.2 Commercial Biomining Operations

The use of microorganisms to solubilise sulfide minerals is a technology that has a long history. "Precipitation ponds" within which sulfide-rich rocks and boulders were subjected to leaching, were in place in the 18th and 19th centuries at Rio Tinto (Spain) and Parys Mountain (Wales), both of which are copper mining sites which date back to pre-Roman times (Hallberg and Johnson, 2001a). In 1947, the occurrence of iron-oxidising bacteria in acid rock drainage was discovered and in 1951, the bacterium was named as *Thiobacillus ferrooxidans* (subsequently reclassified as *Acidithiobacillus ferrooxidans* by Kelly and Wood (2000)). This organism is able to oxidise elemental sulfur, and reduced inorganic sulfur compounds (RISCs) and ferrous ions at much higher rates than can be achieved abiotically under similar conditions. Since then, research on microorganisms involved in mineral oxidation has been ongoing, and mineral bioprocessing has developed as an expanding area of biotechnology. Between 1986 and 1996, 11 commercial biooxidation/bioleaching plants were commissioned (Brierley, 1997), and bioleaching/biooxidation has developed into one of the most successful and important areas of biotechnology. This success story is based upon many advantages to using biooxidation/bioleaching over conventional mineral processing methods (Brierley, 1997):

1. Cost-effectiveness; capital costs are significantly lower (by as much as 50%) than those of the traditional smelting and refining processes.
2. Operational simplicity; simple stepwise expandability by a single reactor or in modules of reactors.
3. Environmental friendliness; the use of natural components, dust, SO_2 free, the ability to handle and dispose of arsenic in a stable form.
4. High performance (relative to smelting).

5. Qualities-robust nature.

6. Health and safety benefits.

Overall, therefore, biooxidation/bioleaching satisfies the mining industry requirements regarding ease of construction, use, expandability and maintenance, environmentally friendliness, as well as competitive economics.

Current biooxidation/bioleaching processes can be categorised as dump bioleaching, bioheaps, *in situ* bioleaching, and stirred-tanks.

1.2.1 Copper Dump Leaching

Dump leaching, the earliest engineering technology used, is very basic in concept. Copper dump leaching is used as a scavenger technology by copper industries to extract copper from run-of-mine, sub marginal ores (which are otherwise waste materials). The submarginal ore is piled to depths of up to 350 meters. The ore piles are acidified and the leaching bacteria facilitate the extraction of copper, which may be recovered using SX/EW (Brierley, 1997).

1.2.2 Bioheap Biooxidation/Bioleaching

Later developments in engineering and hydrometallurgical aspects of biomining have involved the use of bioheap oxidation/leaching operations. Bioheap reactors are commonly used to pre-treat low-grade, refractory-sulfidic gold ores and to leach copper from chalcocite ores. Thin layer heaps of ores are acidified with sulfuric acid and stacked on lined pads with the height of the ore being varied from 2-10 meters depending on heat generation and dissipation, acid balance throughout the heap, and air/water permeability. Heaps are generally actively aerated during bioleaching (Brierley, 1997).

In chalcocite heap leaching, the solution percolates through the heap where bacteria catalyse the release of copper. Soluble copper is usually recovered by SX/EW. Approximately 20% of western copper production arises from the processing of leachable copper ores by heap leaching (Schnell, 1997).

For refractory-sulfidic gold ores, the crushed ore is irrigated with acid and/or an acidic ferric sulfate solution containing mineral-oxidising bacteria. The biooxidised ore is water-washed and restacked on lined pads and leached with a dilute cyanide solution to extract the gold.

Due to larger ore particle sizes used in bioheap leaching, overall metal recovery tends to be lower than is achieved by alternative process methods such as stirred tank bioreactors (Brierley, 1997).

1.2.3 *In Situ* Bioleaching

In situ bioleaching has been commercially used for nearly 30 years to scavenge uranium and copper from depleted underground mines. The underground mining site is first blasted to fragment the ore. The deep mine is aerated *via* shafts and acidified solutions are percolated through the fragmented ores. Indigenous leaching bacteria become established and facilitate metal extraction. Metal-bearing solutions are ultimately pumped to the surface for metal recovery (Brierley, 1997).

1.2.4 Stirred-Tank Biooxidation

Most recently, stirred-tanks have been used in biomining. Aerated stirred-tanks require the preliminary fine grinding of ores to produce a flotation feed and concentrate. This biooxidation process involves three or more stages in series. The first stage has several tanks placed in parallel to allow longer retention of the feed, followed by single tanks in

series in subsequent stages. Due to the considerable heat generated by the process, tanks are typically equipped with cooling systems to maintain optimum temperatures for the bacteria of 35-45°C for mesophilic and thermotolerant *Acidithiobacillus/Leptospirillum* species, and of 45-55°C for moderately thermophilic bacteria. The microbes that oxidise the mineral release ferric iron and sulfate, and the leach solutions are typically maintained at pH 1.5-1.6. For refractory sulfidic gold concentrates, the solid residue is separated from liquid and then is water-washed, neutralised and leached in a cyanide circuit to recover gold. Most of the commissioned, commercial stirred-tank plants are technically biooxidation facilities as they operate with refractory sulfidic gold; however, a bioleaching operation for extraction of cobalt has been in place in Kasese, Uganda since 1999 (d'Hugues *et al.*, 1999).

1.3 Mechanisms of Biooxidation

A variety of metals including those of economic value (copper, lead, zinc etc.) occur as sulfides. Other metals, such as uranium and gold, may be found associated with sulfides in ore bodies. Of all sulfides, the most abundant in the lithosphere is pyrite (FeS_2). Pyrite is formed under reducing conditions (e.g. in sediments) and is unstable in aerobic, moist environments. Current consensus is that, in acidic liquors, ferric iron is responsible for pyrite oxidation, whereas at neutral or alkaline pH, oxygen is the important oxidant because of low solubility of ferric iron (Lowson 1982; Luther 1987; Moses *et al.*, 1987).

At least three mechanisms have been described to account for the bacterial oxidation of sulfide minerals. (i) The direct mechanism, in which bacteria attach to the mineral surface directly and enhance the mineral dissolution *via* a simultaneous enzymatic oxidation of the minerals. Iron ions, either ferric or ferrous are not involved in the

reaction. The reduced iron and sulfur compounds are utilised as electron donors and energy sources for bacterial growth. Highly oxidising, acidic liquors are produced by these reactions, which may promote chemical or indirect leaching. (ii) The indirect mechanism, in which ferric iron generated by iron-oxidising acidophiles in the bulk solution chemically oxidises sulfide minerals; the ferrous ions produced by the indirect attack can be rapidly reoxidised aerobically by the bacteria to complete the leaching cycle. (iii) The indirect contact mechanism, in which attached bacteria oxidise ferrous ions to ferric ions within layers of bacteria and expolymeric material, and the ferric ions within this layer leach the mineral (Crundwell, 2001). At low pH (pH<3.5), indirect solubilisation of the mineral by ferric iron hexahydrate is the major mechanism of mineral attack producing ferrous iron and thiosulfate (Sand *et al.*, 1995). It is generally agreed that bioprocessing of minerals is optimum under high redox potentials and well-aerated conditions, and high ratios of Fe^{3+}/Fe^{2+} in mine effluents and leach liquors generally indicate biological activity. Other natural mechanisms of iron oxidation in acid (pH 1-2) leach liquors are kinetically so slow that their contribution to the overall balance of Fe^{3+}/Fe^{2+} is insignificant when contrasted with bacterial oxidation.

Though evidence in favour of direct leaching came from experiments with synthetic metal sulfides, with repeatedly-washed iron-free cells in an iron-free solution (Rickard and Vanselow, 1978), it still remains to be proven whether or not the direct attack mechanism really occurs with natural sulfide minerals. Sand *et al.* (1995) reported a complete loss of any measurable substrate degradation ability of *At. ferrooxidans*, which had been subcultured in an iron-free solution. Two variations of the "indirect mechanism", the thiosulfate mechanism (Figure 1.1) and the polysulfide mechanism (Figure 1.2), have been described by Schippers *et al.* (1996) and Schippers and Sand (1999).

1.3.1 The Thiosulfate Mechanism

In this mechanism (Figure 1.1), acid-insoluble metal sulfides, such as pyrite (FeS_2), molybdenite (MoS_2), and tungstenite (WS_2) are chemically attacked by ferric hexahydrate ions, generating thiosulfate. The mechanism is exclusively based on the oxidative attack of ferric ions (equation [1.1]).

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow S_2O_3^{2-} + 7\,Fe^{2+} + 6\,H^+ \quad [1.1]$$

Thiosulfate is not stable at acidic pH, and is oxidised chemically or enzymatically to tetrathionate. A series of reactions follows, resulting in the formation of disulfane-monosulfonic acid, trithionate and sulfate (equation [1.2]):

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 2\,SO_4^{2-} + 8\,Fe^{2+} + 10\,H^+ \quad [1.2]$$

Elemental sulfur also occurs as a by-product (Schippers and Sand, 1999). Schippers *et al.* (1996) reported that considerable amounts of elemental sulfur accumulated, and that tetrathionate and pentathionate were produced, during the oxidative dissolution of pyrite by the iron-oxidising acidophile *Leptospirillum ferrooxidans.* A similar result was obtained for chemical oxidation assays with sterile acidic ferric ion-containing solutions. On the other hand, in the case of *At. ferrooxidans,* only small amounts of elemental sulfur were detectable because of the organism's capacity to oxidise sulfur compounds. In this mechanism, the function of iron-oxidising bacteria is to regenerate ferric iron. The oxidation products in the case of FeS_2 and MoS_2 consisted of up to 90% sulfate and about 1 to 2% polythionates (Schippers and Sand, 1999).

In the case of pyrite, the oxidation state of iron is +2 and that of sulfur is –1; therefore, for each mole of FeS_2 to be fully oxidised, one mole electron is released from the iron moiety, and 14 moles electron are released from the sulfur moiety. As a result, it is likely that, despite the iron-oxidisers being often the key microbes in pyrite oxidation in that they supply the ferric iron that oxidises the mineral, sulfur-oxidisers are often

abundant and even numerically dominant due to their ability to oxidise the more energy-rich RISCs (Reduced Inorganic Sulfur Compounds) that are produced during mineral dissolution. For example, the moderately thermophilic sulfur-oxidiser *Acidithiobacillus caldus* has been reported to be the most numerous microorganism in some stirred tank ore-leaching bioreactors, despite its inability to oxidise acid-insoluble sulfide minerals in pure cultures (Norris *et al.*, 2000; Rawlings *et al.*, 1999).

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Figure 1.1: The thiosulfate mechanism (Schippers and Sand, 1999; Schippers *et al.*, 1996).

1.3.2 The Polysulfide Mechanism

In this mechanism (Figure 1.2), acid-soluble metal sulfides, such as sphalerite (ZnS), galena (PbS), hauerite (MnS_2) orpiment (As_2S_3) and realgar (As_4S_4) are attacked by protons as well as by ferric iron, generating intermediary polysulfides, such as disulfide, trisulfide and tetrasulfide (equation [1.3]).

$$MS + Fe^{3+} + H^{+} \rightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+}\ (n \geq 2) \quad [1.3]$$

Polysulfides are oxidised chemically or biologically to sulfur (equation [1.4]).

$$0.5\ H_2S_n + Fe^{3+} \rightarrow 0.125\ S_8 + Fe^{2+} + H^{+} \quad [1.4]$$

Due to its stability, degradation of sulfur occurs only in the presence of sulfur-oxidising bacteria, such as *At. thiooxidans*, which results in the ongoing supply of protons needed for further dissolution of the minerals (equation [1.5]).

$$0.125\ S_8 + 1.5\ O_2 + H_2O^{-} \rightarrow SO_4^{2-} + 2\ H^{+} \quad [1.5]$$

The bacterial function in this mechanism is to supply protons (by generating sulfuric acid) for hydrolytic attack, and/or to replenish ferric iron for an oxidative attack. Though ferric iron is not required in this mechanism, dissolution of the sulfide mineral proceeds more rapidly in the presence of ferric iron (Tributsch and Bennett, 1981a and b). Over 90% of the sulfur products formed *via* chemical attack (by ferric chloride) on acid-soluble sulfides were found by Schippers and Sand (1999) to be elemental sulfur.

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Figure 1.2: The polysulfide mechanism (* indicates radicals) (Schippers and Sand, 1999).

1.4 Cell Attachment to Sulfide Minerals

The attachment of bacteria to mineral surfaces, such as sulfides, is a common phenomenon. It was suggested that a thin film exists between the attached bacterial cell outer membrane and the sulfide mineral, and that the corrosion process occurs within this interfacial film (Rodriguezleiva and Tributsch, 1988). Leaching bacteria such as *At. ferrooxidans* and *L. ferrooxidans* attach strongly to the surface of sulfidic ores by means of extracellular polymeric substances (EPS) (e.g., lipopolysaccharides), which provides a "reaction compartment" (Arredondo *et al.* 1994; Gehrke *et al.*, 1995). The exact nature of EPS and their interaction with the mineral sulfide surface is unknown. Sand *et al.* (1995) reported that extracellular polymers produced by *At. ferrooxidans* and *L. ferrooxidans* were associated with considerable amounts of ferric iron (between 0.5% and 5%), which were not removable by any washing procedure. It was indicated that strains of *At. ferrooxidans* that had a relatively large concentration of Fe^{3+} associated with their extracellular polymers, possessed a higher mineral oxidation activity than those with lesser amount of iron (Kinzler *et al.*, 2001; Gehrke *et al.*, 2001). Natural sulfide ores are usually associated with iron compounds (only synthetic sulfides are free from iron compounds). Therefore, iron compounds in any given natural ore are not limiting the microbial oxidising activity due to their easy accessibility for iron-oxidisers. Blake *et al.* (1994) reported that metal cations in the exopolymers of the bacteria caused a shift of the net charge on the surface from negative to slightly positive, which overcame the repulsion between negatively charged sulfide minerals or sulfur, and microbial cells. The mechanism for site recognition for attachment is still unclear. It was demonstrated that a chemotactic mechanism is involved in site recognition, with Ni^{2+}, Fe^{2+} and Cu^{2+} ions being positive attractants for *L. ferrooxidans* (Acuna *et al.* 1992). Also, the

constitutive nature of chemotaxis by *At. ferrooxidans* toward thiosulfate has been reported (Chakraborty and Roy, 1992).

1.5 Microbial Consortia Involved in Mineral Processing

Since the discovery from acid mine drainage in the late 1940's and isolation of the iron-oxidising acidophile *At. ferrooxidans* in 1951, this chemolithotroph was assumed, for many years, to be the sole or dominant bacterium in sulfide mineral dissolution. This was refuted, however, by reports indicating that *L. ferrooxidans* was actually more abundant than *At. ferrooxidans* in some environments (Schrenk *et al.*, 1998; Rawlings, 1995; Walton and Johnson, 1992).

Microorganisms used in commercial mineral leaching operations are *At. ferrooxidans, L. ferrooxidans* and thermophilic species of *Sulfobacillus, Acidianus* and *Sulfolobus*; however, in the majority of cases, defined microbial inocula are not used in industrial mineral processing. The microorganisms involved in mineral processing in industrial operations are generally those that are associated with the original ore body. An exception is the moderately thermophilic culture used in the 'BacTech' process (Miller, 1997).

In both the natural and the industrial mineral oxidation environments, there are many physico-chemical changes that can provide selective pressures on microbial populations, such as pH, temperature, redox potential (Eh), conductivity, concentrations of soluble metals and organic matter, and oxygen concentration. Changes in one or more of these factors over time may lead to major shifts in the indigenous microflora in leaching sites.

There is now a great amount of information on the biodiversity of acidophilic microorganisms (section 1.7), and by understanding the behaviour of these microorganisms, how they respond to physico-chemical changes, and how they interact

with each other, it is possible, in theory at least, to put together and manipulate bioleaching microflora to enhance both rates and efficiencies of mineral oxidation.

1.5.1 Use of Mixed Cultures

It has become increasingly apparent over recent years that a considerable diversity of microbial life exists in extremely acidic environments. The microorganisms that are involved in ore processing include various acidophilic bacteria and archaea. In recent years, researchers have become increasingly aware of the importance of mixed populations of acidophiles rather than pure cultures in promoting rapid and efficient mineral processing.

In the absence of sulfur-oxidising prokaryotes, a part of the sulfur moiety may accumulate as elemental sulfur during pyrite oxidation, while in mixed cultures with *Acidithiobacillus* spp., pyrite oxidation by *L. ferrooxidans* proceeds to the level of sulfate (Merretting *et al.*, 1989). Mineral dissolution of pyrite was shown to be more extensive by mixed cultures dominated by *L. ferrooxidans* (Norris and Kelly, 1982; Norris, 1983) compared to pure *At. ferrooxidans* cultures. Similarly, Lizama and Suzuki (1989) reported enhanced rates of chalcopyrite and sphalerite leaching by a mixed culture of *At. ferrooxidans* and *At. thiooxidans*, compared with the respective rates by pure cultures.

In contrast to the extensive research undertaken on the activities of the acidophilic chemolithotrophs, the contribution of heterotrophic acidophiles to mineral dissolution is poorly understood. Acidophilic heterotrophic bacteria may affect mineral bioleaching indirectly by interacting with iron-oxidising chemolithotrophs. The indirect theory of sulfide mineral oxidation suggests that any biological system that is capable of regenerating ferric iron should promote mineral dissolution. In fact, several strains of

heterotrophic iron-oxidising acidophilic bacteria have been shown to oxidise pyrite in the presence of yeast extract and ferrous iron (Bacelar-Nicolau and Johnson, 1999). On the other hand, the reduction of ferric iron by some heterotrophic acidophiles (Johnson *et al.*, 1993; Bridge and Johnson, 2000) could have negative effects on mineral dissolution. Contrasting results have been reported from laboratory studies on mixed cultures of acidophilic heterotrophs and chemolithotrophs. Negative effects may result from the production of extracellular polymeric materials on mineral surfaces by heterotrophic bacteria that may shield minerals from oxidative attack (Johnson, 1991b; Kishimoto *et al.*, 1991). On the other hand, production of vitamins, cofactors, chelating agents and surfactants by acidophilic heterotrophs may enhance sulfide mineral leaching by chemolithotrophic acidophiles (Tuovinen *et al.*, 1991). No enhancement of depyritization of coal was found in mixed cultures of iron-oxidising moderate thermophiles and *Alicyclobacillus*-like heterotrophs compared with pure cultures of iron-oxidising moderate thermophiles (Johnson, 1991a). Similarly, pyrite leaching by *At. ferrooxidans* was found not to be influenced by *A. acidophilum* (Norris and Kelly, 1982). In contrast, metal leaching of sludge by *At. ferrooxidans* was found to be stimulated by heterotrophic organisms, including *Rhodotorula rubra* and indigenous sludge microflora (Fournier *et al.*, 1998). Increased solubilisation of cobalt sulfide ores by *At. ferrooxidans* in the presence of a number of acidophilic heterotrophic bacteria in cultures supplemented with ferrous iron or glucose was reported by Wichlacz and Thompson (1988). Pyrite leaching by a mixed culture of *L. ferrooxidans* and *Acidiphilium* SJH was found to be more rapid than that brought about either by pure cultures of *L. ferrooxidans* or by pure or mixed (with *Acidiphilium* SJH) cultures of *At. ferrooxidans* (Johnson *et al.*, 1990).

Mixed cultures that include microorganisms with different physiological characteristics would expand the range of microbial adaptability to variations in physico-chemical parameters. Based on known acidophilic microbial interactions, it would be possible to design microbial consortia to be more robust and more efficient in processing minerals.

1.5.2 Use of Thermophilic Microorganisms in Mineral Leaching

Interest in using thermophilic bacteria in mineral processing lies in the potential for improving the leach rates of sulfide minerals, such as pyrite and arsenopyrite, and overcoming the problems encountered in bioleaching of refractory copper sulfides, such as chalcopyrite, that exist at lower temperatures.

Sulfide mineral oxidation is an exothermic reaction and may involve a considerable amount of heat evolution during the active phase of the process. Moderately elevated temperatures within the range of 50-60°C have been recorded in waste rock dumps (Harries and Ritchie, 1981; Murr and Brierley, 1978). Though such temperatures are well in excess of the upper growth limits of mesophilic bacteria, moderate thermophiles have been readily isolated from a variety of environments such as copper leach dumps (Brierley, 1978), a large-scale experimental copper waste leaching facility (Murr and Brierley, 1978), and coal spoil heaps (Marsh and Norris, 1983a; 1983b). The potential advantages of operating at higher temperature with moderate thermophiles in continuous leaching of a refractory, gold-bearing pyrite/arsenopyrite concentrate has been demonstrated (Liu *et al.*, 1993). A commercial bioreactor has been developed to utilise such organisms at 45-50°C for extraction of gold from a pyrite/arsenopyrite concentrate (Brierley and Brans, 1994).

Temperatures in excess of 60°C have been reported in a variety of mining operations. The first extremely thermophilic acidophile isolated from a mine site (a coal spoil heap)

was a *Sulfolobus* sp. (Marsh and Norris, 1983b). Extreme thermophiles have also been found in drainage from a copper mine (Gomez *et al.*, 1993). *Metallosphaera prunae*, which grows between 55°C and 80°C by oxidising pyrite, sphalerite, chalcopyrite or molecular hydrogen was isolated in a uranium mine (Fuchs *et al.*, 1996b).

Sulfolobus spp. are effective in leaching sulfide minerals, including molybdenite and chalcopyrite-containing ore materials (Brierley and Brierley, 1986) as well as pyrite and arsenopyrite concentrates (Lawrence and Marchant, 1988; Lindström and Gunneriusson, 1990). Mixed cultures of mesophiles, moderate thermophiles and *Sulfolobus*-like extreme thermophiles have been used for leaching a gold-bearing pyrite/arsenopyrite concentrate at 5% w/v solids, and displayed greater rates of metal extraction as the temperature increased (Hutching *et al.*, 1987). Maximum rates of mineral leaching were found with chalcopyrite concentrates (~15%, w/v) in air-lift reactors containing *Sulfolobus metallicus* (Le Roux and Wakerley, 1988) and with pyrite (6-8%, w/v) in stirred reactors containing "*Sulfolobus acidocaldarius*" (Lindström *et al.*, 1993).

It is known that the efficient extraction of copper from chalcopyrite concentrates cannot readily be achieved at low temperatures. Chalcopyrite is the major copper sulfide of commercial interest. However, chalcopyrite typically exhibits slow leach kinetics and low copper extractions at mesophilic temperatures and ambient pressure in acid ferric sulfate leach solutions, due to passivation of the mineral surface (Munoz *et al.*, 1995). Thermophilic microorganisms, operating at a temperature of between 60 and 78°C, can solubilise chalcopyrite, which is perhaps the most important potential application of bioleaching at high temperatures.

It was pointed out that progress in leaching at high mineral concentrations is required before the potential of these organisms can be commercially realised (Clark and Norris, 1996b). To overcome the problem of high sensitivity of thermophiles to high solids

concentration, selection or isolation of more robust bacteria or the development of improved reactor designs may be required. Ultimately, the use of moderately and extremely thermophilic microorganisms could represent a major breakthrough in the bioleaching of sulfide minerals.

1.6 Bioenergetics of Acidophilic Microorganisms

1.6.1 Iron

1.6.1.1 Ferrous Iron Oxidation

Iron is the fourth most abundant element in the earth's crust, accounting for approximately 5% of its weight. Iron plays essential roles for living organisms not only as enzyme-associated iron, which is responsible for a large variety of biological redox reactions, but also as a substrates, for microbial energy metabolism. In neutral and alkaline environments, chemical oxidation rates of ferrous iron are so fast that both ferrous iron and ferric iron are unstable in solution. On the other hand, in acidic environments (<pH 2.3), chemical oxidation rates of ferrous iron are very low and both ferrous and ferric irons are soluble.

Some acidophilic microorganisms gain energy for growth and cell maintenance by oxidation of ferrous irons and/or reduced sulfur compounds, using oxygen as the electron acceptor. The Fe^{3+}/Fe^{2+} redox couple has a very positive standard electrode potential (+770 mV) at pH 2.0 and, as a result, only oxygen is able to act as a natural electron acceptor for ferrous iron oxidation (equation [1.6]):

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

Because of the comparatively small amount of energy available from ferrous iron oxidation (-30 kJ/mol at pH 2.0), iron-oxidising bacteria require to oxidise large amounts of iron to grow. *At. ferrooxidans* has been the major focus of study in

understanding the microbial respiratory mechanism. Several models have been proposed for the iron respiratory chain, which differ with regard to the electron carriers and the side of the cytoplasmic membrane on which oxygen reduction takes place (Ingledew *et al.*, 1977; Ehrlich *et al.*, 1991; Yamanaka, *et al.*, 1991; Blake *et al.*, 1992) (Figure 1.3).

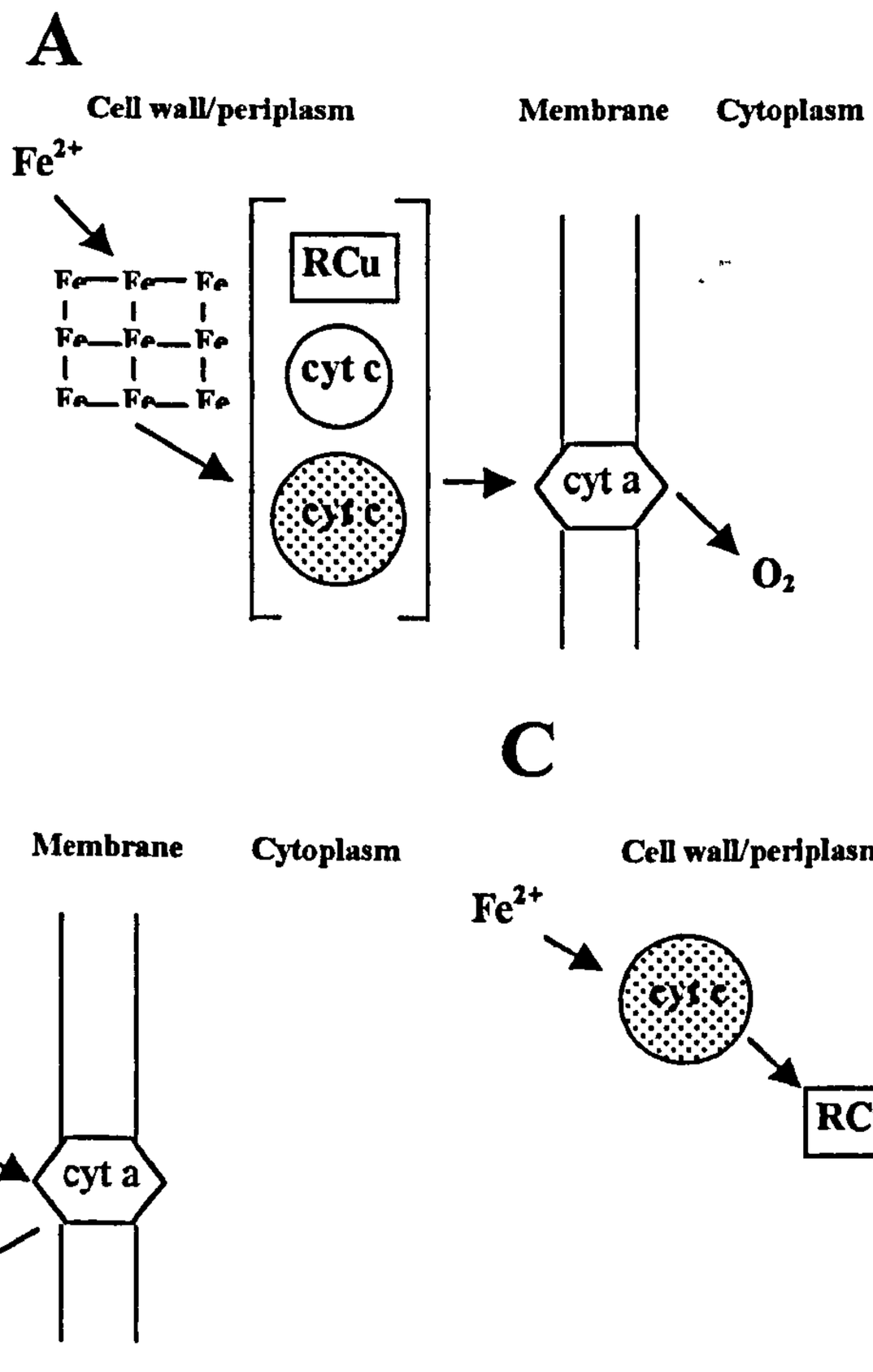

Figure 1.3: Various hypotheses suggested for the pathway for electron transfer from Fe^{2+} to molecular oxygen in *At. ferrooxidans*. (A) Working model adapted from Ingledew (Ingledew *et al.*, 1997; Ehrlich *et al.*, 1991). (B) Working model adapted from Yamanaka *et al.* (1991). (C) Working model adapted from Blake *et al.* (1992). Components: Fe-S, iron-sulfur protein; RCu, rusticyanin; cyt c, cytochrome *c* (shaded and unshaded symbols denote different polypeptide chains); cyt a, cytochrome *a*; and [...-Fe-...], polynuclear iron complex. The arrows in A, B, and C indicate the direction of electron flow.

The bioenergetics of iron oxidation by *At. ferrooxidans* is of biochemical interest because of the much more positive reduction potential of the Fe^{3+}/Fe^{2+} couple (+770 mV at pH 2.0) than that of the NAD(P)-NAD(P)H couple (305 mV at pH 6.5, the cytoplasmic pH of *At. ferrooxidans*) (Cox *et al.*, 1979). Therefore, the reduction of NAD(P) using ferrous iron as sole energy source, is not thermodynamically feasible. To overcome this, an uphill electron transfer model in *At. ferrooxidans* was proposed (Elbehti *et al.*, 1999; Elbehti *et al.*, 2000) (Figure 1.4). Electrons arising from Fe^{2+} (coupled to oxygen reduction *via* cytochrome oxidase) establish a proton motive force which, when the ATP/ADP ratio is low, is used by ATP synthase to synthesise ATP. As long as ATP is used in protein synthesis, this ratio is low and the ATP synthase synthesises ATP. But when no carbon is available, ATP is no longer used and the ATP/ADP ratio increases. When ATP accumulates, the proton motive force established *via* cytochrome oxidase therefore decreases, and ATP synthase functions like an ATPase, generating a proton motive force; this proton electrochemical gradient will then be used for the reverse electron transfer through the bc_1 and NDH-1 complexes, leading to the formation of NAD(P)H required for CO_2 fixation. The ATP/ADP ratio will then decrease, cytochrome oxidase will be activated, and ATP synthase again will synthesise ATP. The ATP/ADP ratio controls the balance of the reducing equivalents from Fe^{2+} in favour of either cytochrome oxidase or the uphill electron transfer (Elbehti *et al.*, 2000) Acidic environments facilitate the passage of H^+ into the cell *via* the reversible membrane bound ATPase, coupled with ATP synthesis (Figure 1.4).

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Figure 1.4: Model for the balance of reducing equivalents from ferrocytochrome *c* between the exergonic cytochrome oxidase and the endergonic bc_1 and NDH-1 pathways (Elbehti *et al.*, 2000).

Acidophilic bacteria take advantage of the pre-existing transmembrane proton gradients for energy genesis (Norris and Ingledew, 1992). Acidophilic bacteria are capable of maintaining a near-neutral cytoplasmic pH (Michels and Bakker 1985) by removal of H^+ from within the cytosol to ensure that the H^+ concentration does not increase to levels where cytosolic acidification would eventually result in cell death. In the case of *At. ferrooxidans*, the H^+ concentration is controlled by internal proton consumption in the reduction of oxygen at the inner surface of the cell membrane, linked to ferrous iron oxidation (Figure 1.5).

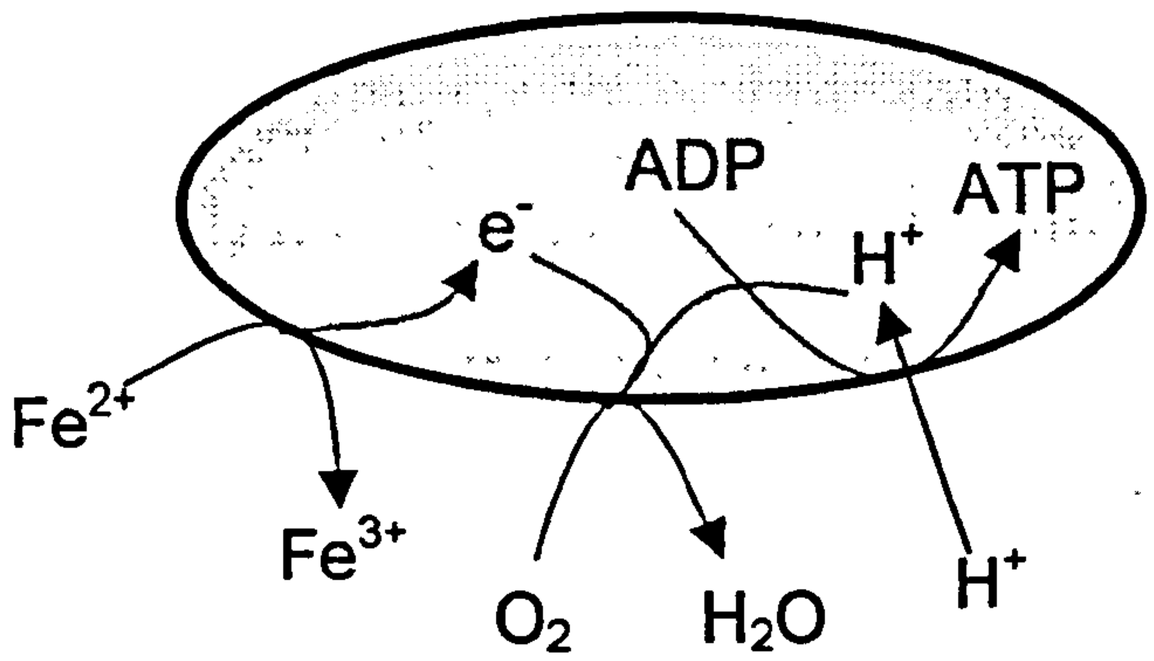

Figure 1.5: Energy gain from ferrous iron oxidation by acidophilic bacteria.

On the other hand, relatively little is known about the enzymology of other ferrous iron oxidising acidophiles. In *L. ferrooxidans*, an acid stable cytochrome that is slowly oxidised by ferrous iron has been described (Barr *et al.*, 1990). Phylogenetically distinct organisms express copious quantities of spectrally distinct redox-active biomolecules during autotrophic growth on soluble iron (Blake *et al.*, 1993). *At. ferrooxidans*, *L. ferrooxidans*, *Sb. thermosulfidooxidans*, and *Metallosphaera sedula* possess iron respiratory chains dominated by a blue copper protein, a novel red cytochrome, a novel yellow protein, and a novel yellow cytochrome, respectively (Blake *et al.*, 1993).

1.6.1.2 Ferric Iron Reduction

Dissimilatory reduction of ferric iron has also been reported to occur in highly acidic, metal-rich environments (e.g., Johnson *et al.*, 1996; Fortin *et al.*, 1996). Ferric iron, generated from ferrous iron oxidation (coupled to CO_2 reduction) by anoxygenic phototrophic bacteria (Widdel *et al.*, 1993) may have been the principle electron acceptor during the early phases of the evolution of life, when the earth's atmosphere was anoxic (Walker, 1987).

As the redox potential of the Fe^{3+}/Fe^{2+} couple (+770 mV, pH 2.0) is almost as positive as that of O_2/H_2O, ferric iron is a thermodynamically highly attractive alternative electron acceptor to oxygen. In very acidic environments, ferric iron is stable in solution and if oxygen depletion occurs, ferric iron can serve as an alternate electron acceptor coupled to the oxidation of RISCs or organic electron donors (Johnson, 1998a).

The reduction of ferric iron coupled to the oxidation of hydrogen or organic compounds may have been the first globally significant mechanism for the oxidation of organic matter to carbon dioxide (Walker, 1987). The process plays an important role in the oxidation of natural and contaminant organic compounds in a variety of environments and contributes to other phenomena of widespread significance such as the release of metals and nutrients to water supplies, the magnetisation of sediments, and the corrosion of metal (Lovley, 1991). Brock and Gustafson (1976) first demonstrated ferric iron reduction by acidophiles, showing that *Thiobacillus* and *Sulfolobus* were able to reduce ferric iron when growing on elemental sulfur as an energy source (equation [1.7]).

$$S + 6Fe^{3+} + 4H_2O \rightarrow SO_4^{2-} + 6Fe^{2+} + 8H^+ \quad [1.7]$$

Pronk *et al.* (1991) later demonstrated growth of *At. ferrooxidans* by the oxidation of sulfur coupled to the reduction of ferric iron. Ohmura *et al.* (1999) reported H_2-coupled ferric iron reduction by *At. ferrooxidans*. *At. ferrooxidans* was also shown to be able to grow by reducing ferric iron with tetrathionate as substrate (Hallberg *et al.*, 2001). In the same study, *At. thiooxidans* and *At. caldus* were found not to grow anaerobically using ferric iron as electron acceptor.

The contribution of heterotrophic microorganisms to iron cycling in extremely acidic environments was prompted by the observation that ferric iron reduction can be coupled to the oxidation of a variety of organic compounds (Johnson and McGinness, 1991b).

Mesophilic and moderately thermophilic heterotrophs, *Acidiphilium* spp. and *Alicyclobacillus-like* isolates, have been shown to reduce ferric iron at low pH (Pronk and Johnson, 1992). Also, the iron-oxidising moderate thermophiles, *Sb. thermosulfidooxidans*, *Sb. acidophilus*, and *Am. ferrooxidans*, were shown to be capable of reducing ferric iron to ferrous iron when grown under oxygen limitation conditions; iron reduction was most readily observed when these bacteria were grown heterotrophically using glycerol as an electron donor (Bridge and Johnson, 1998). Cycling of ferrous and ferric iron by mixed populations of iron-oxidising and iron-reducing mesophilic bacteria, and by pure cultures of moderate thermophiles, has been demonstrated (Johnson *et al.*, 1996). Recently, strains of *Acidiphilium cryptum* were found to be able to grow anaerobically by coupling the oxidation of many organic compounds to the reduction of ferric iron (Kusel *et al.*, 1999).

A few components of the ferric iron-reducing system have been identified in acidophilic autotrophs. Sasaki *et al.* (2001) reported that *At. ferrooxidans* synthesised a significant amount of a red coloured protein with a typical spectrum of *c*-type cytochrome when grown anaerobically. Sugio *et al.* (1992a; 1992b) have claimed that a hydrogen sulfide: ferric ion oxidoreductase that catalyses the oxidation of elemental sulfur with ferric ions as an electron acceptor to produce ferrous and sulfite ions occurs in iron-oxidising bacteria, such as *At. ferrooxidans*, *L ferrooxidans*, and some moderately thermophilic iron-oxidising bacteria including *Sb. thermosulfidooxidans* BC1, *Sb. acidophilus* ALV and *Acidimicrobium ferrooxidans* TH3. Pronk *et al.* (1992) demonstrated that a respiratory-chain poison inhibited the anaerobic sulfur-dependent ferric iron reduction and ferrous iron dependent oxygen reduction, which led to the conclusion that a single oxidoreductase was involved in the oxidation and reduction of iron. The single oxidoreductase theory is also supported by the observation that rusticyanin is present in

anaerobically grown *At. ferrooxidans* with hydrogen as electron donor (Ohmura *et al.*, 1999).

Corbett and Ingledew (1987) suggested that *bcl* complex is involved in anaerobic ferric iron dependent sulfur oxidation in *At. ferrooxidans*, by demonstrating that the oxidation can be inhibited by HOQNO (*n*-heptyl-4-hydroxyquinoline *N*-oxide), a specific inhibitor of the *bcl* complex of the respiratory chain.

This microbial reduction of ferric iron would have, in terms of its impact on redox potential, a negative impact on mineral oxidation, especially where the aeration is limited. On the other hand, there may be considerable potential in using microorganisms with an ability of ferric iron reduction. During the leaching of iron-containing sulfide minerals, ferric iron may precipitate in a variety of mineralogical forms to produce passivation layers of secondary sulfides including covellite or Fe^{3+}-complexes such as jarosite (Stott *et al.*, 2000; Carlson *et al.*, 1992; Tuovinen *et al.*, 1994). Such secondary minerals may seriously reduce the efficiency of ore processing (Stott *et al.*, 2001). Modifications to the nutrient medium (by limiting sulfate and monovalent cation concentrations) to limit jarosite deposition was found to have little effect on the copper leaching rate with *Sulfolobus metallicus* (Stott *et al.*, 2001). The ability of heterotrophic acidophiles to reduce not only soluble but also solid-phase ferric iron compounds, such as amorphous and crystalline forms, could be advantageous to leaching processing of sulfide minerals. "*Sulfobacillus yellowstonensis*" YTF1 was shown to bring about the reductive dissolution of three ferric iron-containing minerals (ferric hydroxide, jarosite, and goethite) when grown under restricted aeration conditions with glycerol as a carbon and energy source (Bridge and Johnson, 1998). The acidophilic heterotroph *Acidiphilium* SJH was shown to catalyse the reductive dissolution of a wide range of ferric iron-containing minerals (akageneite, goethite, jarosite, natrojarosite, and

amorphous ferric hydroxide) and of the mixed ferrous/ferric mineral, magnetite; rates of dissolution varied with the structural stabilities of the minerals. Among those minerals tested, amorphous ferric hydroxide ($Fe(OH)_3$) was the most readily solubilised, and jarosite and akageneite were the least (Bridge and Johnson, 2000). As indicated by the following equation (equation [1.8]):

$$Fe^{3+}\text{solid phase} \leftrightarrow Fe^{3+}\text{soluble} \rightarrow Fe^{2+}\text{ (biological reduction)} \quad [1.8],$$

the reduction of soluble ferric iron should result in the equilibrium between solid-phase Fe^{3+} and soluble-phase Fe^{3+} being shifted somewhat, accelerating the dissolution of the mineral (Bridge and Johnson, 1998).

Improved mineral oxidation rates may be achieved by control of microbial populations specifically to remove ferric iron precipitates (e.g. by promoting occasional anoxic conditions), which might result in more efficient commercial mineral processing.

1.6.2 Reduced Inorganic Sulfur Compounds (RISCs)

In environments where acidophilic microorganisms are found, sulfuric acid is generated by the oxidation of RISCs and mineral sulfides. RISCs play important roles as electron donors for acidophiles as they offer more electrons per mole of substrate than do ferrous iron.

Attempts to investigate sulfur oxidation pathways have proved difficult, due to the chemical reactivity and hence lack of stability of many sulfur intermediates. Also, disproportionation, where electrons removed from sulfur compounds are used to reduce other molecules of the same type, has been frequently observed, making the oxidation pathway difficult to elucidate. The oxidation pathway where tetrathionate and thiosulfate are intermediates during the oxidation of each was proposed for *At. ferrooxidans* (Pronk *et al.*, 1990), *A. acidophilum* (Meulenberg *et al.*, 1992), *At.*

thiooxidans (Chan and Suzuki, 1994) and *At. caldus* (Hallberg *et al.*, 1996). Also, the conversion of thiosulfate to tetrathionate, and the subsequent oxidation of tetrathionate *via* thiosulfate, was also found to occur in *Sulfolobus* strain LM (Nixon and Norris, 1992). Although the actual mechanism of many of these reactions has been doubted, the ultimate oxidation product of RISCs is sulfate, which causes decrease in solution pH.

1.7 Diversity of Acidophilic Microorganisms

1.7.1 Mesophilic Acidophilic Bacteria

Mesophilic bacteria have optimum temperatures for growth of 25-37°C and are generally incapable of growth above 45°C. Bacteria most commonly isolated from inorganic mining environments are *At. ferrooxidans*, *At. thiooxidans* and *L. ferrooxidans*, and these are the most widely studied of acidophilic organisms. Heterotrophic acidophiles (e.g., *Acidiphilium* spp.) are also readily isolated from low temperature acidic environments. Acidophilic chemolithotrophic bacteria generally derive their energy for growth by oxidising various inorganic compounds, particularly iron and reduced sulfur compounds, whereas acidophilic heterotrophs oxidise organic substrates.

1.7.1.1 Autotrophic Acidophilic Bacteria

1.7.1.1.1 Genus *Acidithiobacillus*

The genus *Acidithiobacillus* was recently proposed for some species (*T. thiooxidans*, *T. ferrooxidans*, *T. caldus* and *T. albertensis*) previously classified as *Thiobacillus* spp. (Kelly and Wood, 2000). The genus *Acidithiobacillus* includes obligately acidophilic (optimum pH < 4.0) and aerobic, Gram-negative rods that can use reduced sulfur compounds to support autotrophic growth. Some species oxidise ferrous iron or use

natural and synthetic metal sulfides for energy generation; some species can also oxidise hydrogen (Kelly and Wood, 2000).

Since the first isolation of *At. ferrooxidans* from acidic mine drainage (Temple and Colmer, 1951), it has been the most studied and well known acidophilic organism in biological leaching systems. *At. ferrooxidans* is a Gram-negative, autotrophic rod-shaped bacterium that can derive energy for growth by oxidising ferrous ion in addition to RISCs. Consequently it has the capacity to degrade sulfide minerals such as pyrite and chalcopyrite. In contrast to other *Acidithiobacillus* spp., *At. ferrooxidans* is a facultative anaerobe and is capable of growth by coupling the oxidation of elemental sulfur and RISCs to the reduction of ferric iron. Also, *At. ferrooxidans* can use hydrogen as electron donor coupled to the reduction of oxygen (under aerobic conditions) or ferric iron (anaerobic conditions). The bacterium was reported to capable of growth at a pH of 1.5 after selection in continuous culture (Vian *et al.*, 1986).

Acidithiobacillus thiooxidans was the first acidophilic prokaryote to be isolated (Waksman and Joffe, 1921). It was recently reported that *At. thiooxidans* is more closely clustered with *At. ferrooxidans* than other species of the genus *Acidithiobacillus* (Kelly and Wood, 2000). *At. thiooxidans* is phylogenetically closely related to *At. ferrooxidans* and it shares morphological characteristics with the iron-oxidiser. However, *At. thiooxidans* tends to be more motile than *At. ferrooxidans,* and is unable to oxidise ferrous iron to degrade pyrite or chalcopyrite. *At. thiooxidans* is an obligate aerobe and derives energy for growth by oxidation of S° and variety of RISCs to sulfate. Ferric iron may be reduced by *At. thiooxidans*, but this does not support its growth in the absence of oxygen (Brock and Gustafson, 1976). Although *At. thiooxidans* is not capable of iron or pyrite oxidation, it can grow on pyrite in co-culture with *L. ferrooxidans*, where it utilises RISCs produces as intermediate products (Sand *et al.*,

1992). *At. thiooxidans* is considerably more resistant to low pH than *At. ferrooxidans,* and it is capable of growth at pH of less than 0.8. *At. thiooxidans* falls into a monophyletic group within the γ-*Proteobacteria* together with other Acidithiobacilli, which is close to the cusp between the β and γ subgroups (Hallberg and Johnson, 2001a).

Acidithiobacillus albertensis (originally *Thiobacillus albertis*) was isolated from acidic soil adjacent to a sulfur stockpile in Alberta, Canada (Bryant *et al.*, 1983). *At. albertensis* differs from the other three species by its relatively high G+C content of its DNA, together with its possession of a tuft of flagella and a glycocalyx (Kelly and Wood, 2000).

1.7.1.1.2 Other "*Thiobacillus*" -like isolates

Strain m-1, isolated from coal mine drainage water in Missouri, USA, was considered to be a strain of *T. ferrooxidans*. However, strain m-1 was shown to have little DNA homology with strains of *At. ferrooxidans* and also have significantly higher G+C content of its chromosomal DNA (Harrison, 1982).

"*Thiobacillus prosperus*" is a halotolerant bacterium that can grow in 6% sodium chloride (Huber and Setter, 1989). This bacterium grows by oxidation of a variety of sulfide minerals, but somewhat poorly on elemental sulfur or ferrous iron. According to the 16S rRNA gene analysis, this bacterium is not related to other *Thiobacillus* (or *Acidithiobacillus*) species, and the name of this species requires revision.

1.7.1.1.3 Genus *Leptospirillum*

Another ferrous iron-oxidiser, *Leptospirillum ferrooxidans* was first isolated from copper deposits in Armenia (Markosyan, 1972). Unlike *At. ferrooxidans, L. ferrooxidans* is not capable of direct oxidation of sulfur, however, it has been shown that this organism is able to degrade pyrite (Sand *et al.*, 1992) and pyritic coal (Merretting *et al.*, 1989) in pure culture. The genus *Leptospirillum* currently includes three species; *L. ferrooxidans*, *L. thermoferrooxidans*, and *L. ferriphilum* (Hippe, 2000; Coram and Rawlings, 2002). A putative group/species has been identified in clone libraries obtained from an abandoned pyrite mine at Iron Mountain, California (Bond *et al.*, 2000a). *L. ferrooxidans* is readily distinguished from *At. ferrooxidans* by its cell shape, which varies from curved rods to spirals. The bacterium possess a polar flagellum and more motile than *At. ferrooxidans*. *Leptospirillum* is more resistant to low pH than *At. ferrooxidans* and will grow at pH as low as 1.2 (Norris, 1983). Many *L. ferrooxidans* strains form aggregates of cells, which appear as floc-like structures in liquid media and cause the flocculation of fine grain minerals, such as pyrite. Growth on iron usually proceeds with macroscopic aggregation of cells embedded in slime. Exopolymer production is most evident with growth at low temperatures (e.g., 15 to 20°C; Norris, 1990).

Due to its slower growth rate than *At. ferrooxidans* in enrichment cultures, the importance of *L. ferrooxidans* has been overlooked for a long time. However, there have been a number of reports supporting the wide distribution of *L. ferrooxidans* as an important leaching organism in leaching environments (Schrenk *et al.*, 1998; Rawlings, 1995). *L. ferrooxidans* was found to gradually dominate and to support more extensive leaching than *At. ferrooxidans* in mixed cultures with *At. ferrooxidans* growing on

pyrite (Norris and Kelly, 1982), particularly at low pH values (Norris *et al.*, 1988) or at higher temperatures (Sand *et al.*, 1992).

L. thermoferrooxidans, a thermophilic *Leptospirillum*, is described elsewhere (section 1.7.2.1.2.).

The name *L. ferriphilum* was proposed for a group of *Leptospirillum* isolates (group II) that have G+C moles percent ratios of 55 to 58% and have two copies of *rrn* genes, whereas the other major group of isolates (group I) had G+C mole percent ratios between 49 and 52% and had three copies of *rrn* genes. Based on 16S rRNA gene sequence data, group I isolates clustered together with the *L. ferrooxidans* type strain (DSM2705; Coram and Rawlings, 2002). The two groups could also be distinguished from the sizes of their 16S-23S rRNA gene spacer regions. Members of the two species can be rapidly distinguished from each other by amplification of their 16S rRNA genes and by carrying out restriction enzyme digests of the products. Several, but not all, isolates of the group II leptospirilla, but none from group I (*L. ferrooxidans*), were capable of growth at 45°C (Coram and Rawlings, 2002).

1.7.1.1.4 Genus *Thiomonas*

Thiomonas cuprina (originally classified as *Thiobacillus cuprinus*) was isolated from solfatara fields in Iceland and a uranium mine in Federal Republic Germany (Huber and Stetter, 1990). *Tm. cuprina* has its optimum pH of 3.0-4.0. *Tm. cuprina* occurs as Gram-negative motile rods, and is capable of heterotrophic growth on complex organic substrates and pyruvate, and autotrophic growth on elemental sulfur, RISCs and various sulfidic ores. Autotrophic growth is less efficient on elemental sulfur and single sulfidic ore than in ore mixtures and arsenopyrite, and *Tm. cuprina* does not grow on pyrite (Huber and Stetter, 1990). Recently, novel moderate acidophilic *Thiomonas*-like

isolates have been isolated from ferruginous water draining a coal mine in south Wales (Dennison *et al.*, 2001). Like other *Thiomonas* spp., these isolates oxidise RISCs but, in addition, they appear to oxidise ferrous iron.

1.7.1.2 Heterotrophic Acidophilic Bacteria

Mesophilic acidophilic heterotrophs have been isolated directly from acid mine drainage, acidic coal refuse and supposedly pure cultures of *At. ferrooxidans* as contaminants (Harrison *et al.*, 1980; Wichlacz and Unz, 1981; Johnson and Kelso, 1983).

1.7.1.2.1 Genus *Acidiphilium*

The genus *Acidiphilium* was first proposed for aerobic, mesophilic rod-shaped bacteria that grow in "lean" organic media (Harrison, 1981) and currently comprises six *bona fide* species, *A. cryptum* (Harrison, 1981), *A. organovorum* (Lobos *et al.*, 1986), *A. rubrum*, *A. angustum* (Wichlacz *et al.*, 1986), *A. multivorum* (Wakao *et al.*, 1994) and *A. acidophilum* (Guay and Silver, 1975; Hiraishi *et al.*, 1998). *Acidiphilium* spp. appear to be the most widely distributed mesophilic, obligately heterotrophic bacteria found in acidic environments.

A. acidophilum (originally classified as *Thiobacillus acidophilus*) was isolated from a supposedly pure culture of *At. ferrooxidans* (Guay and Silver, 1975). This organism has a highly versatile metabolism, as it can grow autotrophically on a variety of RISCs, mixotrophically using both inorganic and organic carbon, and heterotrophically on a variety of single organic compounds (Hiraishi *et al.*, 1998).

The type species, *A. cryptum*, is an adept scavenger, capable of surviving multiple, serial subcultures in acidified basal salts to which organic substrates have not been

added. *A. cryptum* has been shown to live on the trace amounts of organic compounds leaked by *At. ferrooxidans* and *L. ferrooxidans* (Harrison, 1984). Although *A. cryptum* was thought to grow only in lean organic media, it can grow also in organic-rich media, which are supplemented with yeast extract (typically 0.02% w/v) to high cell densities (<10^9/ml). *Acidiphilium* SJH was shown to catalyse the reductive dissolution of a wide range of ferric iron-containing minerals (Bridge and Johnson, 2000).

The 16S rRNA gene sequence analysis lead to transfer two previous *Acidiphilium* isolates including *A. facilis* (Wichlacz *et al.*, 1986) and *A. aminolytica* (Kishimoto *et al.*, 1993) to the genus *Acidocella* (Kishimoto *et al.*, 1995).

1.7.1.2.2 Genus *Acidocella*

Acidocella spp. tend to be less acidophilic and less tolerant of some metals than *Acidiphilium* spp., though some *Acidocella* strains have been shown to exhibit high metal resistance (Ghosh *et al.*, 1997). "*Ac. aromatica*" (proposed name for strains WJB-3 and LGS-3) is unique in being able to catabolise a wide range of aromatic compounds, including benzoic acid, phenol, and naphthalene (Hallberg *et al.*, 1999). Although this bacterium appears unable to utilise organic substrates that are used by all the other acidophilic heterotrophs (such as glucose and glycerol), it can use fructose as the sole carbon and energy source, as well as various aliphatic acids (Gemmell and Knowles, 2000).

1.7.1.2.3 Other Acidophilic Heterotrophs

Acidomonas methanolica is a phylogenetically distinct, methylotrophic acidophilic bacterium (Urakami *et al.*, 1989).

Acidisphaera rubrifaciens was recently isolated from hot springs and AMD in Japan (Hiraishi *et al.*, 2000). This bacterium is an obligate aerobe, and cells are coccoid or rodococcus, and it grows between pH 3.5 and 6.0.

Acidobacterium capsulatum is a saccharolytic capsulated bacterium originally isolated from acid mine drainage (AMD) in Japan (Kishimoto *et al.*, 1991). This bacterium is relatively less acidophilic with a pH range of 3-6 to grow. Bacteria sharing 94% 16S rRNA gene sequence homology with the original Japanese isolate have recently been isolated from the site of the former Wheal Jane tin mine, Cornwall, UK (Hallberg and Johnson, 2001b).

Bacelar-Nicolau and Johnson (1999) reported some novel strains, the 'T-series' bacteria, including "*Ferrimicrobium acidophilum*". "*Fm. acidophilum*" is able to conserve energy for growth from the oxidation and reduction of ferrous and ferric iron, respectively. Mixed cultures of "*Fm. acidophilum*" and *At. thiooxidans* or *A. acidophilum* were shown to accelerate the oxidative dissolution of pyrite, presumably due to the latter providing the former with organic carbon compounds to support its growth and the iron oxidation.

Table 1.1: Characteristics of mesophilic, acidophilic autotrophic bacteria.

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Table 1.2: Characteristics of mesophilic, acidophilic heterotrophic bacteria.

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1.7.2 Moderately Thermophilic Acidophilic Prokaryotes

Moderately thermophilic, acidophilic prokaryotes display optimum growth temperatures of about 45-55°C and have been isolated from various geothermal, self-heating coal spoils, leach dumps and soils. Moderately thermophilic prokaryotes include four main groups: (i) Gram-negative, sulfur-oxidising autotrophs; (ii) Gram-negative, iron-oxidising autotrophs; (iii) Gram-positive, *Bacillus*-like facultative chemolithotrophs and heterotrophs; and (iv) archaeal isolates. At elevated temperatures, the solubility of oxygen, carbon dioxide, and other gases is lowered. Many moderately thermophilic iron-oxidising bacteria, unlike their mesophilic and extremely thermophilic counterparts, are relatively inefficient in assimilating carbon dioxide.

1.7.2.1 Moderately Thermophilic Bacteria

1.7.2.1.1 *Acidithiobacillus caldus*

At. caldus grows as Gram- negative, motile rods, having a pH optimum for growth of 2-2.5 and an optimum growth temperature of 45°C. *At. caldus* is capable of chemolithotrophic growth on reduced sulfur substrates and molecular hydrogen and can also grow mixotrophically with sulfur or tetrathionate and yeast extract or glucose (Hallberg and Lindström, 1994). Mixed culture studies have shown that *At. caldus* is able to out-compete other sulfur oxidising acidophiles (including *At. thiooxidans*) even at relatively low (30°C and above) temperatures (Hallberg *et al.*, 2001). *At. caldus* has been reported to be the dominant prokaryote in stirred tank cultures leaching mineral ores (Norris *et al.*, 2000).

1.7.2.1.2 *L. thermoferrooxidans*

Leptospirillum thermoferrooxidans was first described by Golovacheva *et al.* (1992) as an aerobic thermoacidophilic, chemolithoautotrophic, iron-oxidising bacterium with many phenotypic similarities to *Leptospirillum ferrooxidans*. It was isolated from acid iron-containing hydrothermal springs on Kuril Islands, Japan. *L. thermoferrooxidans* differs from other known *L. ferrooxidans* by its optimal temperature for growth of 45-50°C (maximum temperature for growth of 55-60°C), its greater resistance to high Fe^{2+} concentrations, and its greater G+C content in the chromosomal DNA (56.2 mol% G + C; Golovacheva *et al.*, 1992). This isolate, however, has unfortunately been lost.

1.7.2.1.3 Genus *Sulfobacillus*

Moderately thermophilic *Bacillus*-like acidophiles have been isolated from hot springs, coal spoil heaps, and acidic water at mine sites from various parts of the world. Sulfobacilli are generally non-motile spore-forming iron-oxidising rods, and the genus currently contains five species, not all of which have been officially classified: *Sb. thermosulfidooxidans* (Golovacheva, 1979), *Sb. acidophilus* (Norris *et al.*, 1996), "*Sb. yellowstonensis*" (Ghauri and Johnson, 1991), "*Sb. montserratensis*" (Yahya *et al.*, 1999) and *Sb. disulfidooxidans* (Dufresne *et al.*, 1996). *Sulfobacillus* spp. display considerable metabolic versatility. Both *Sb. thermosulfidooxidans* and *Sb. acidophilus* were reported to grow autotrophically on ferrous iron and on pyrite, mixotrophically on ferrous iron, on elemental sulfur in the presence of yeast extract, and heterotrophically on yeast extract. Autotrophic growth on elemental sulfur was observed only with *Sb. acidophilus*, and *Sb. thermosulfidooxidans* was capable of sulfur oxidation in the presence of yeast extract (Norris *et al.*, 1996). *Sulfobacillus* spp. are facultative anaerobes, and may grow in the absence of oxygen by anaerobic respiration using ferric

iron as terminal electron acceptor and either an organic (e.g. glycerol) or inorganic (e.g. tetrathionate) as electron donor (Bridge and Johnson, 1998).

Sb. disulfidooxidans was described as a disulfide-oxidising bacterium that can grow autotrophically on elemental sulfur and pyrite as sole energy sources and can grow heterotrophically on organic substrates such as glutamate and glucose. (Dufresne *et al.*, 1996). Unlike other classified *Sulfobacillus* spp., *Sb. disulfidooxidans* does not oxidise ferrous iron, and it is phylogenetically more closely related to *Alicyclobacillus* spp.. The presence of the diagnostic ω–alicyclic fatty acid in *Sb. disulfidooxidans*, in addition to 16S rRNA phylogeny, justifies the reclassification of this organism as "*Alicyclobacillus disulfidooxidans*" (Hallberg and Johnson, 2001a).

1.7.2.1.4 Genus *Alicyclobacillus*

The genus *Alicyclobacillus* was proposed by Wisotzkey (Wisotzkey *et al.*, 1992) and currently comprises four species: *Alb. acidocaldarius* (Darland and Brock, 1971), *Alb. acidoterrestris* (Deinhard *et al.*, 1987a), *Alb. cycloheptanicus* (Deinhard *et al.*, 1987b) and *Alb. hesperidum* (Albuquerque *et al.*, 2000). *Alicyclobacillus* spp. are Gram-positive (or Gram variable), rod-shaped spore-formers, and are phylogenetically closely related to *Sulfobacillus* spp. with which they share a *Bacillus*-like morphology and capacity for endospore formation, though *Alicyclobacillus* spp. are differentiated from *Sulfobacillus* spp. by being obligately heterotrophic. Also, *Alicyclobacillus* spp. possess ω-alicyclic fatty acids as the major natural membranous lipid component, which is not found in any other *Bacillus* species (Wisotzkey *et al.*, 1992). *Alb. cycloheptanicus* differs from other *Alicyclobacillus* spp. in possessing ω-cycloheptane fatty acids in the cell membrane rather than ω-cyclohexane fatty acids that are commonly found in other species (Deinhard *et al.*, 1987b).

Several *Alicyclobacillus*-like isolates, including strains YTH1 and YTH2 isolated from hot springs in Yellowstone National Park, USA have also been described (Johnson *et al.*, 2001b).

1.7.2.1.5 Genus *Acidimicrobium*

The genus *Acidimicrobium* currently comprises a single species, *Am. ferrooxidans*, which includes strain TH3 isolated from a copper leach dump in New Mexico (Brierley, 1978: Norris and Barr, 1985) and strain ICP (the type strain) isolated from an Icelandic geothermal site (Clark and Norris, 1996a). These are iron-oxidising, Gram-positive, rod-shaped cells, occasionally forming elongated chains and filaments.

Am. ferrooxidans is distinguished from *Sulfobacillus* spp. by its lack of spore formation, its lower tolerance of ferric iron, and by its chromosomal DNA base composition. Anaerobic growth has been reported for strain TH3, using glycerol as electron donor and ferric iron as electron acceptor (Bridge and Johnson, 1998).

1.7.2.1.6 Strain GSM

Mine spoil materials from the Golden Sunlight mine, Montana, were analysed for indigenous acidophilic microflora by plating dispersed samples onto selective solid media (Johnson *et al.*, 2001a). One of these, originally isolated on plates incubated at 30°C, was later shown to be a moderate thermophile with a temperature optimum of about 46°C. This isolate (GSM) was capable of oxido-reduction of iron. GSM is far more "heterotrophically inclined" than *Sulfobacillus* spp. and grows readily on a range of organic substrates, producing high cell yields (>10^9/ml). It can also grow mixotrophically and autotrophically (in yeast extract-containing and yeast extract-free

media, respectively), and oxidises elemental sulfur. The iron-oxidising system in GSM appears to be inducible and can be suppressed by subculturing in heterotrophic media (Johnson *et al.*, 2001a). Phylogenetically, this isolate is positioned more closely to *Alicyclobacillus* spp. (obligate heterotrophs) than to the iron-oxidising *Sulfobacillus* spp., though its low sequence similarity (88% to *S. thermosulfidooxidans* and 93% to *Alicyclobacillus cycloheptanicus*) is again sufficient to warrant a novel genus label.

1.7.2.2 Moderately Thermophilic Acidophilic Archaea

There are currently three genera of moderately thermophilic acidophilic archaea recognised, *Thermoplasma*, *Picrophilus* and *Ferroplasma*, all of which are members of the order *Thermoplasmales*.

1.7.2.2.1 Genus *Thermoplasma*

Thermoplasma spp. have been found in solfatara fields and currently contains two species; *Tp. acidophilum* (Darland *et al.*, 1970) and *Tp. volcanium* (Segerer *et al.*, 1988). They are irregular cocci, varying from spherical to filamentous structures, and are facultative anaerobic heterotrophs that can grow anaerobically by sulfur respiration, producing H_2S (Segerer *et al.*, 1988).

1.7.2.2.2 Genus *Picrophilus*

Two species (*P. oshimae* and *P. torridus*) belonging to the genus *Picrophilus* were isolated from solfataras fields in northern Japan (Schleper *et al.*, 1995; Schleper *et al.*, 1996). In contrast to other acidophilic *Euryarchaeota*, *Picrophilus* cells possess a wall-like outer structure, though the cells are irregular cocci, displaying duplex or triplex

forms. They are aerobic hyperacidophilic heterotrophs, which grow on yeast extract and poorly on tryptone under aerobic conditions at temperatures between 45-65°C with optimal temperature at 60°C, and at pH between 0.0-3.5 with optimal pH of 0.7 (Schleper *et al.*, 1995).

1.7.2.2.3 Genus *Ferroplasma*

Recently, the genus *Ferroplasma*, within the order '*Thermoplasmales*', was proposed by Golyshina (Golyshina *et al.*, 2000).

Ferroplasma acidiphilum was isolated from a bioleaching pilot plant, and described as a strictly aerobic, ferrous-iron-oxidising, cell-wall-lacking archaeon. Cells are irregular cocci, varying from spherical to filamentous, forming duplex and triplex forms. Addition of yeast extract is essential for growth of *Fp. acidiphilum*. Growth of strain Y-T was observed in the range of pH 1.3-2.2 with the optimal pH of 1.7, and at temperatures between 15 and 45°C with an optimal temperature of 35°C (Golyshina *et al.*, 2000).

A new species name, "*Ferroplasma acidarmanus*", was suggested for an iron-oxidising archaeon, isolate fer1, isolated from a sulfide ore body at Iron Mountain, California. The isolate is capable of heterotrophic growth using yeast extract as the sole energy source, and is able to grow between pH 0-2.5 with the optimal pH 1.2. This species was dominant in the environment studied (slimes and sediments) and constituted up to 85% of the microbial community when solute concentrations were high (conductivity of 100 to 160 mS cm^{-1}; Edwards *et al.*, 2000b).

Table 1.3: Characteristics of moderately thermophilic acidophilic bacteria.

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Table 1.4: Characteristics of moderately thermophilic acidophilic archaea.

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1.7.3 Extremely Thermophilic, Acidophilic Prokaryotes

This group currently comprises mostly archaea and there has been only one bacterium reported so far that grows optimally above 60°C in extremely acidic liquors. These prokaryotes have often been isolated from hot springs and thermal acidic soils or sediments.

1.7.3.1 Extremely Thermophilic, Acidophilic Bacteria

1.7.3.1.1 *Hydrogenobacter acidophilus*

Hydrogenobacter acidophilus was originally isolated from a solfatara in Japan and grows aerobically using hydrogen with elemental sulfur as electron donor (Shima and Suzuki, 1993). This organism is the most thermophilic of all known acidophilic bacteria (temperature optimum and maximum 65°C and ~70°C, respectively).

1.7.3.2 Extremely Thermophilic, Acidophilic Archaea

1.7.3.2.1 Genus *Sulfolobus*

The genus *Sulfolobus*, the most diverse of the acidophilic archaeon genera, was first described by Brock *et al.* (1972), and species are characterised by aerobic growth at high temperatures and low pH in the presence of elemental sulfur. Known species of *Sulfolobus* include *S. acidocaldarius* (Brock *et al.*, 1972), *S. solfataricus* (Zillig *et al.*, 1980), *S. shibitae* (Grogan *et al.*, 1990), *S. metallicus* (Huber and Stetter, 1991), *S. hakonensis* (Takayanagi *et al.*, 1996) and *S. yangmingensis* (Jan *et al.*, 1999). In contrast to earlier reports, neither *S. acidocaldarius* nor *S. solfataricus* are now considered to oxidise sulfur, and both are obligate heterotrophs (Norris and Johnson, 1998). *S. metallicus* is an obligate autotroph that grows by oxidising elemental sulfur, RISCs,

ferrous iron, and sulfide ores (Huber and Stetter, 1991). *S. metallicus*, together with *Metallosphaera* spp., are probably the most significant mineral-oxidising microorganisms at >60°C (Norris *et al.*, 2000). In contrast, *S. hakonensis* and *S. yangmingensis* are facultative autotrophs.

1.7.3.2.2 Genus *Acidianus*

Acidianus spp. grow as facultative anaerobes by lithotrophic growth, aerobically by means of oxidation of elemental sulfur or anaerobically by means of reduction of elemental sulfur with molecular hydrogen as electron donor (Segerer *et al.*, 1986). *Acidianus* currently comprises three species, two of which, *Ac. infernus and Ac. ambivalens* (formerly *Desulfurolobus ambivalens*; Zillig *et al.*, 1986) are obligate chemolithotrophs while *Ac. brierleyi* grows autotrophically on reduced sulfur (or ferrous iron) or heterotrophically in organic media. *Ac. brierleyi* was also reported to be able to oxidise and grow autotrophically on pyrite (Larsson *et al.*, 1990).

1.7.3.2.3 Genus *Metallosphaera*

Metallosphaera spp. are aerobic facultative chemolithotrophs. Currently two species are recognised: *Metallosphaera sedula* (Huber *et al.*, 1989) and *Metallosphaera prunae* (Fuchs *et al.*, 1996b). *Metallosphaera* spp. are able to oxidise sulfur, sulfide minerals, and hydrogen, or grow on complex organic substrates, such as yeast extract.

1.7.3.2.4 Genus *Sulfurococcus*

The genus *Sulfurococcus* has been proposed for facultatively autotrophic sulfur-oxidising species, that are able to grow heterotrophically and mixotrophically on

organic compounds with pH range 1.0-5.6 (Golovacheva *et al.*, 1987). The genus *Sulfurococcus* currently comprises two species, *Sc. mirabilis* (Golovacheva *et al.*, 1987) and *Sc. yellowstonii* (Karavaiko *et al.*, 1994). *Sc. yellowstonii* is able to oxidise ferrous iron and sulfide minerals, as well as reduced sulfur.

1.7.3.2.5 *Stygiolobus azoricus*

The first strictly anaerobic members of the order *Sulfolobales*, *Stygiolobus azoricus*, was described by Segerer *et al.* (1991). This archaeon grows at pH range 1.0-5.5 and grows obligately chemolithotrophically by reduction of elemental sulfur with hydrogen, forming hydrogen sulfide.

1.7.3.2.6 *Sulfurisphaera ohwakuensis*

Sulfurisphaera ohwakuensis is a facultatively anaerobic archaeon, capable of growth at up to 92°C with pH range 1.0-5.0. Elemental sulfur reduction-dependent anaerobic growth of this organism was reported by Kurosawa *et al.* (1998).

1.7.3.2.7 *Acidilobus aceticus*

Acidilobus aceticus is a second obligate anaerobic thermoacidophilic archaeon reported (Prokofeva *et al.*, 2000). *Al. aceticus* grows at pH 2.0-6.0 by fermenting starch to acetate. *Al. aceticus* can use elemental sulfur as an electron sink.

Table 1.5 (a): Characteristics of extremely thermophilic acidophiles.

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Table 1.5 (b): Characteristics of extremely thermophilic acidophiles.

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1.8 Microbial Interactions in Extremely Acidic Environments

Acidophilic microorganisms exist as mixed populations in natural environments, in industrial mineral leaching heaps, and in mineral processing bioreactors. In such environments, a variety of interactions occur between acidophilic organisms, including competition, predation, mutualism, synergism, and ammensalism (Johnson, 1998a; Hallberg and Johnson, 2001a).

Competition occurs between acidophiles for substrates, such as inorganic electron donors, as well as organic electron donors. Competition between *At. ferrooxidans* and *L. ferrooxidans* has been well demonstrated. Because of its higher affinity for ferrous iron, greater tolerance of ferric iron, and greater tolerance of very low pH, *L. ferrooxidans* tends to be dominant over *At. ferrooxidans* in ferrous iron-limited mixed cultures of the organisms, and when pyrite is the substrate and excess acid is produced (Norris *et al*, 1988; Norris and Kelly, 1982; Rawlings *et al.*, 1999). In contrast, the faster growth rate of *At. ferrooxidans* generally results in it emerging as the dominant iron-oxidiser where ferrous iron concentrations are relatively high. Slightly elevated (35-40°C) temperatures also favour *L. ferrooxidans*, because of its greater thermo-tolerance; *At. ferrooxidans* is more effective at lower temperatures (<25°C). Heterotrophic acidophiles compete with autotrophic organisms for inorganic nutrients and, most importantly, for dissolved oxygen.

Predation of acidophilic bacteria by acidophilic protozoa has been observed. A biflagellated protozoan, isolated from an acidic drainage stream located inside a disused pyrite mine, was found to graze a range of acidophilic bacteria, including the chemolithotrophs *At. ferrooxidans*, *L. ferrooxidans*, and the heterotroph *A. cryptum*. In mixed cultures of *At. ferrooxidans* and *L. ferrooxidans*, the protozoan isolate displayed preferential grazing of the former (McGinness and Johnson, 1992). Acidophilic

protozoa including flagellates, a ciliate and an amoeba, were isolated from acid mine water and a coal biotreatment plant, and grown in mixed cultures with acidophilic bacteria. In cultures of pyritic coal, protozoa grazed iron-oxidising and heterotrophic bacteria, but to varying extents. The presence of protozoa was noted to effect changes in acidophilic populations, in particular often causing *L. ferrooxidans* to become the dominant iron- oxidiser at an earlier stage than in corresponding protozoa- free controls (Johnson and Rang, 1993).

Mutualistic interactions result in both microbial partners gaining benefit, e.g. interactions between chemolithotrophic and heterotrophic acidophiles. Chemolithotrophic acidophiles are generally very sensitive to organic acids and some other organic materials. In acidic environments, a large transmembrane pH gradient exists between the near-neutral internal pH and the external pH. Such a pH gradient may cause the accumulation of weak acids in the cell cytosol resulting in cytosolic acidification and inhibition of bacterial growth (Ingledew, 1982; Figure 1.6).

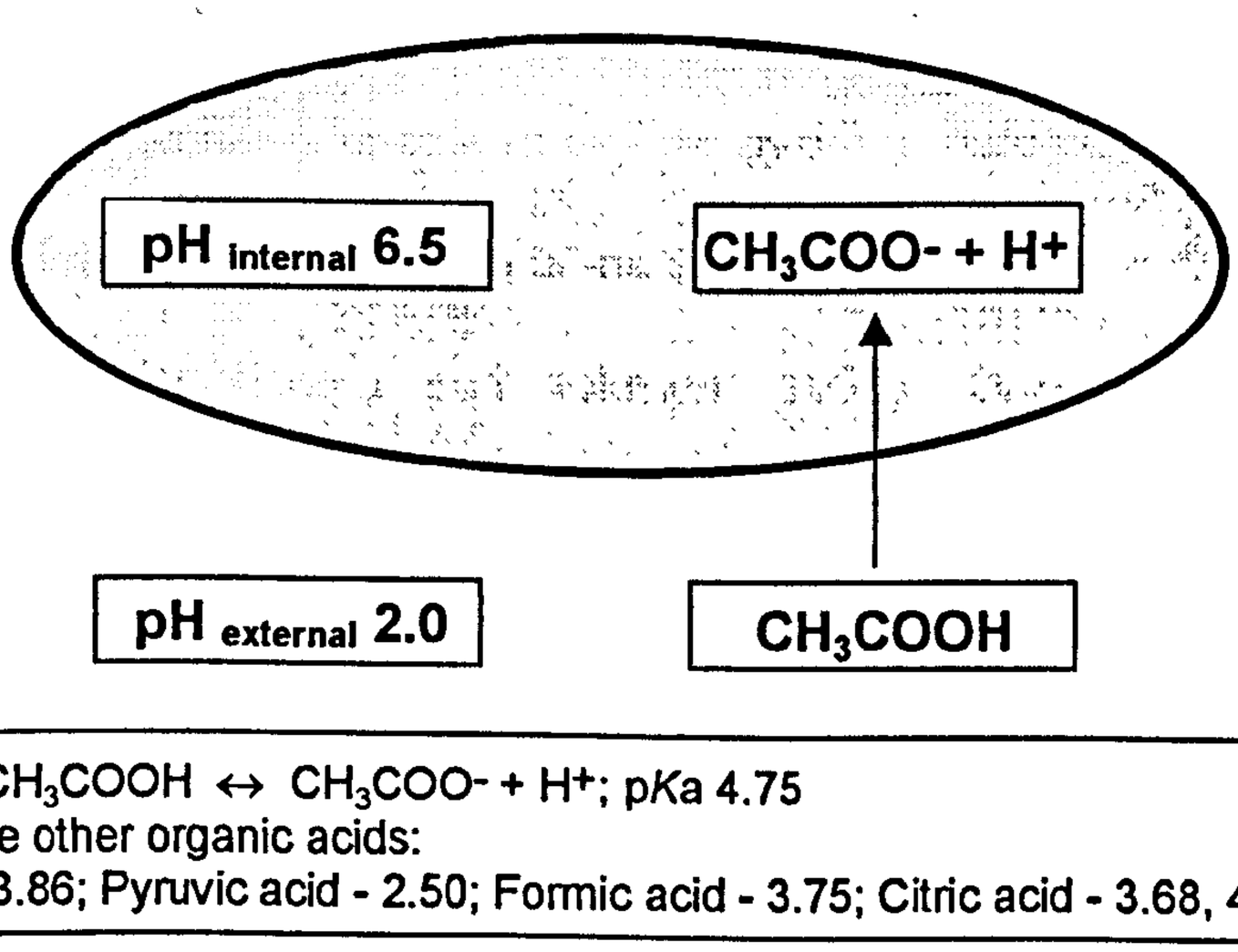

Figure 1.6: Acidification of acidophile cells resulting from exposure to small molecular weight organic acids.

This inhibition may be eliminated by acidophilic heterotrophs that maintain concentrations of organic compounds at non-inhibitory levels for the autotrophs (Johnson *et al.*, 1987). Growth of *At. ferrooxidans* has been found to be enhanced in the presence of *Acidiphilium* spp. (Harrison, 1984; Wichlacz and Thompson, 1988). The 'overlaid' solid media, in which an acidophilic heterotrophic bacterium is incorporated into the underlayer of a two-layer gel in order to allow the growth of chemolithotrophs, was developed on the basis of this observation (Johnson and McGinness, 1991a).

Mutualism between acidophilic heterotrophs and autotrophs is observed also in the cycling of iron, involving ferrous iron-oxidising chemolithotrophs and ferric iron-reducing heterotrophs, in situations where dissolved oxygen concentrations vary spatially or temporally. Reduction of ferric iron to ferrous by *Acidiphilium* spp. 'regenerates' the substrate used by *L. ferrooxidans* and *At. ferrooxidans*, and cycling between the two ionic forms has been observed in mixed cultures (Johnson, 1998b).

Carbon flow from active, senescent and dead chemolithotrophs to acidophilic heterotrophs has also been demonstrated. The iron-oxidising heterotroph "*Fm. acidophilum*" T23, which is unable to oxidise pyrite in pure culture in the absence of added organic carbon, is able to do so in mixed cultures with either *At. thiooxidans* or *A. acidophilum* (Bacelar-Nicolau and Johnson, 1999). Organic carbon to sustain heterotrophic growth was considered to originate from the CO_2-fixing acidophiles, which oxidise reduced sulfur compounds produced *via* ferric iron attack on pyrite (Bacelar-Nicolau and Johnson, 1999; Figure 1.7).

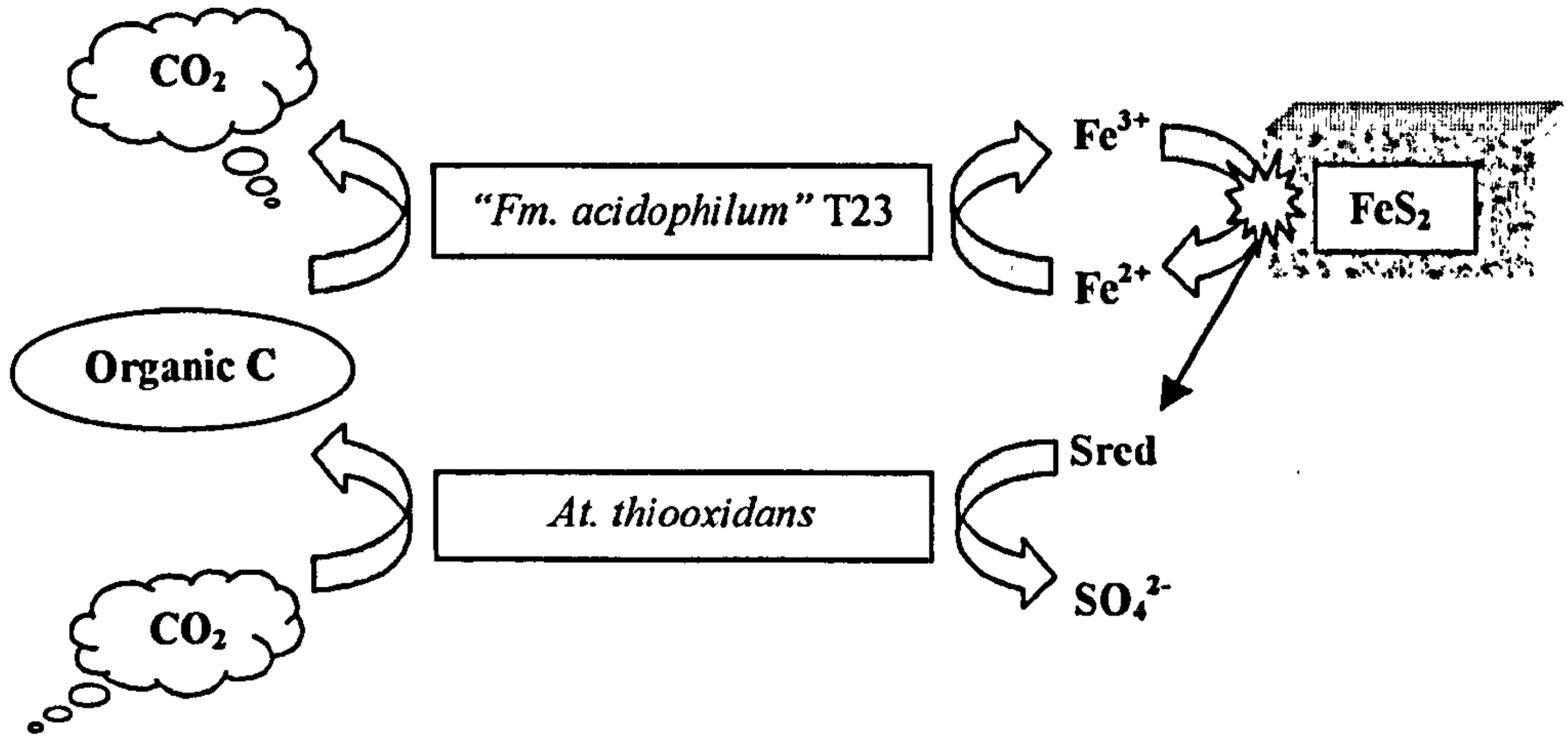

Figure 1.7: Hypothetical scheme for the oxidation of pyrite by mixed cultures containing the sulfur-oxidising organism *At. thiooxidans* and heterotrophic iron-oxidising bacteria.

Mixed cultures of *Am. ferrooxidans* with either *Sb. thermosulfidooxidans* or *Sb. acidophilus* displayed more rapid oxidation of ferrous iron than pure cultures of these bacteria (Clark and Norris, 1996). The greater part of ferrous iron oxidation in mixed cultures probably resulted from activity of the *Sulfobacillus* spp., which possess a greater tolerance of ferric iron, and which presumably grew mixotrophically utilising organic compounds from *Am. ferrooxidans* (Clark and Norris, 1996). It is also possible that the oxidation of organic compounds by heterotrophs may increase the level of CO_2 available for fixation by *At. ferrooxidans* (Wichlacz and Thompson, 1988).

The presence of mixed populations is, in many ways, beneficial to the leaching process, as it may greatly extend the range of microbial metabolic capabilities required for effective oxidation of sulfide minerals.

Ammensalism refers to the repression of one or more species by toxins produced by another. Heterotrophic acidophiles vary in their sensitivities to hydrogen ions and some heavy metals. *Acidocella* spp. are, in general, more sensitive to both than are *Acidiphilium* spp.. Therefore, end metabolic products of acidophilic iron- or sulfur-

oxidisers can suppress populations of more rapidly growing *Acidocella* spp. in favour of slower growing *Acidiphilium* spp. (Hallberg and Johnson, 2001a).

1.9 Enumeration and Identification of Acidophilic Prokaryotes

A number of methods have been reported to investigate microbial diversity in environmental or industrial samples and microbial population dynamics during mineral processing operations.

1.9.1 Enrichment Techniques and Solid Media

Since the initial isolation of *At. ferrooxidans* about 50 years ago, enrichment cultures have been widely used to isolate acidophilic microorganisms. However, enrichment cultures tend to select acidophiles that suit the imposed conditions of the growth medium, and often result in selecting a particular bacterium with faster growth, rather than the most important *in situ*. This is illustrated in the case of *At. ferrooxidans*, which tends to be selected for when ferrous sulfate enrichment cultures are used, even though *L. ferrooxidans* may be more numerous in original sample (Harrison, 1984). Solid media have been used to isolate and enumerate acidophilic microorganisms from environmental and industrial samples. (Johnson and McGinness, 1991a; Johnson, 1995b; Lopez-Archilla and Amils, 1999) Until about a decade ago, most of the solid media developed were not effective and reproducible, and in general, it was difficult to grow obligatory autotrophs that tend to be sensitive to organic materials. Therefore, the use of solid media has been much criticised for underestimating the number and diversity of microbes on gelled media. In 1991, Johnson and McGinness (1991a) developed a selective solid medium in which an acidophilic heterotrophic bacterium is

incorporated into the underlay of a two-layer gel in order to degrade the organic materials present in the gelling agent (principally agar and agarose hydrolysis products), thereby allowing isolation of mesophilic and thermophilic microorganisms from environmental and industrial samples (Johnson, 1995b). Environmental samples can be plated directly on to solid media to evaluate microbial diversity *in situ*, whereas plating from enrichment cultures tends to lead to the isolation of specific organisms.

1.9.2 Immunological Techniques

Immunological assays using antibodies are among the other 'classical' approaches which have been used to detect acidophilic microorganisms. These methods have the advantage over culturing techniques in that they produce more rapid results. In an immunological assay, a primary antibody generated against whole cells of a given bacterial species is reacted specifically with the antigens of the microorganisms to be identified, followed by application of a secondary antibody specific to the primary antibody. A secondary antibody can be conjugated with an enzyme that reacts with a coloured substance to reveal the interaction. Specific antibodies that differentiate various species of acidophiles include those specific for *At. ferrooxidans* (Apel *et al.*, 1976; Arredondo and Jerez, 1989; Muyzer *et al.*, 1987), *L. ferrooxidans* (Jerez and Arredondo, 1991), *At. caldus* and *Sulfolobus* (probably *S. metallicus*) strain BC65 (Amaro *et al.*, 1994). The immunological assays provide much more immediate results than either enrichment or plate cultures. However, these assays have disadvantages such as much lower sensitivity (minimum of ~ 10^3-10^4 bacteria/ml) (Jerez and Arredondo, 1991) and the phenomenon of multiple serotypes displayed by different isolates of a single species (Hallberg and Lindström, 1996).

1.9.3 Molecular Techniques

1.9.3.1 Determination of the 16S rRNA Genes

The biodiversity of acidophilic microorganisms in a particular ecological niche can be assessed by ribosomal gene sequence analysis. Ribosomal RNA genes are essential for all organisms to survive and are highly conserved in the bacterial and other evolutionary domains. The characterisation of the 16S rRNA gene is now a reliable, standardised technique that enables a phylogenetic classification of the microorganisms in acidic as well as in other environments (Woese, 1987).

Chromosomal DNA extracted from the environmental sample or enrichment culture is used as template DNA for Polymerase Chain Reaction (PCR) by using, for example, 'universal' primers to amplify the 16S ribosomal RNA genes. The 16S ribosomal RNA gene sequences are determined either from the cloned gene or from the PCR product itself. By comparing the determined 16S rRNA gene sequence with other known sequences in database, it is possible to identify and classify the target organism.

16S rRNA gene libraries were prepared from DNA samples obtained at an abandoned pyrite mine at the Iron Mountain site (Bond *et al.*, 2000a) and at acidic geothermal sites on the volcanic island Montserrat (Burton and Norris, 2000). Probably due to the less acidity at the site, a greater diversity of microorganisms was found in the latter studies.

1.9.3.2 PCR-Based Techniques

De Wulf-Durand *et al.* (1997) developed designed PCR primers based on published 16S rRNA gene sequences to amplify 16S rRNA genes from the DNA of six bacterial phylotypes associated with acidic mining environments (*Acidiphilium* spp., *At. thiooxidans*, *At. ferrooxidans*, *At. caldus*, *L. ferrooxidans*, and *Sb. thermosulfidooxidans*). They found that *L. ferrooxidans*, *At. caldus* and a *Sulfobacillus*

spp. were detected in the leachate-liquor but *At. ferrooxidans* and *At. thiooxidans* were not. Moreira and Amils (1996) developed a PCR-mediated method for the detection of *Tm. cuprina* using specific oligonucleotide primers that target variable regions of the 23S rRNA coding gene and of the 16S/23S intergenic spacer region. However, high mutuality in some parts of the 23S rRNA gene and in the ISR could preclude the detection of these microorganisms in environmental samples. The random amplification of polymorphic DNA (RAPD) is a PCR-based technique where a variety of oligonucleotides are synthesised and used for the amplification of short segments of DNA. Since the oligonucleotides are designed to bind to DNA randomly during the annealing step of PCR, no prior DNA sequence data are needed. Novo *et al.* (1996) used the RAPD method to assess genomic variability among eight *At. ferrooxidans* strains. RAPD fingerprints showed variation for the thirty primers used, giving a total of 269 polymorphic bands. Most primers divided *At. ferrooxidans* strains into two distinct groups. Selenska-Pobell *et al.* (1998) also used RAPD to discriminate thiobacilli accompanied by two other PCR-based techniques, ARDREA (amplified ribosomal DNA restriction enzyme analysis; section 1.9.3.3) and Rep-APD. In Rep-APD, primers specific to short, repetitive DNA segments, which are found in most microorganisms, are used to amplify the DNA between these repeats. The amplified DNAs vary from one strain to another and are thus a useful method for quick comparative genome analysis. In this study, it was found that both RAPD and Rep-APD were much more discriminatory than ARDREA, where limited genetic material is available for comparison.

1.9.3.3 Amplified Ribosomal DNA Restriction Enzyme Analysis (ARDREA)

Rawlings (1995) amplified 16S rRNA genes from strains of *At. ferrooxidans, At. thiooxidans* and *L. ferrooxidans* by PCR to obtain their restriction enzyme sites maps. Populations of these acidophilic bacteria in a biooxidation tank were estimated by comparing the restriction site patterns: Results showed that *At. ferrooxidans* were absent, whereas *At. thiooxidans* and *L. ferrooxidans* were present. Kamimura *et al.* (2001) amplified the 16S rRNA gene from ten strains of *At. ferrooxidans* and the amplified products were compared by performing restriction enzyme digestion. The results showed that iron-oxidising bacteria isolated from natural environments were rapidly identified as *At. ferrooxidans* by the method combining ARDREA with physiological analysis.

1.9.3.4 Spacer Region Analysis

To obtain preliminary data on microbial diversity in copper bioleaching system, Pizarro *et al.* (1996) investigated the composition of bacterial populations in copper bioleaching systems by amplifying the spacer regions between 16S and 23S rRNA genes from DNA obtained either directly from ores or leaching solutions, or from laboratory enrichment cultures. Products were compared using gel electrophoresis and compared with those of cultures of the known acidophiles. Identification of the bacteria was achieved by partial sequencing of the 16S rRNA genes adjacent to the spacer regions. It was shown that the relative abundance of *At. ferrooxidans, L. ferrooxidans* and *At. thiooxidans* was highly dependent on ferrous iron concentrations (*At. ferrooxidans* at high ferrous iron concentration and *At. thiooxidans* and *L. ferrooxidans* at low ferrous iron concentration). Bacterial populations developed in copper sulfide ores leached with 0.3 M sulfuric acid were examined by characterisation of the spacer regions between 16S

and 23S rRNA genes obtained after PCR amplification of the DNA extracted from the leached ore. The spacers observed had sizes found in strains of *L. ferrooxidans* and *At. thiooxidans* (Vasquez and Espejo, 1997).

1.9.3.5 Denaturing Gradient Gel Electrophoresis (DGGE)

Separation of DNA fragments in DGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). In denaturing gradient gel electrophoresis (DGGE), DNA fragments of the same length migrate through the gel until they reach a point at which the double strand opens and migration stops. DNA fragments stop at the different point depending on their melting behaviour, therefore on their G+C content. Stoner *et al.* (1996) demonstrated the efficacy of a direct 5S rRNA assay for the characterisation of mixed microbial populations by analysing the migration patterns of 5S rRNA extracted from some important bioleaching bacteria using DGGE. The migration patterns of different acidophilic bacteria were readily distinguishable from each other, and this allowed the species composition of a mixed microbial community to be readily assessed. DGGE is a very discriminating technique for the study of nucleic acids from microbial populations (Muyzer, 1999), though this approach has its disadvantage in requiring sufficient biomass to be able to detect the genetic material studied.

1.9.3.6 Pulse Field Gel Electrophoresis (PFGE)

Genomic DNA fragments digested by a certain restriction enzyme can be separated by pulse field gel electrophoresis (PFGE) and the restriction patterns analysed. The genomic DNA extracted from various thiobacilli give different restriction patterns,

which facilitates preliminary differentiation and possibly identification by comparison with reference patterns (Irazabal *et al.*, 1995).

1.9.3.7 Fluorescent *In Situ* Hybridisation (FISH)

Fluorescent *in situ* hybridisation allows the detection of specific nucleic acid sequences in cells by binding oligonucleotide probes labelled with a fluorescent dye to their complementary target sequences. The methodology using oligonucleotide probes has progressed rapidly from simple assessing of presence or absence measurements, to fluorescent *in situ* hybridisation (FISH) methods targeting specific sequences within the 16S rRNA of individual cells. Relative numbers of particular microbes can be assessed by comparing the numbers that hybridise to a particular probe to the total number of cells, obtained by using a general fluorescent stain such as the DNA-binding stain 4',6-diamidino-2-phenylindole (DAPI). This method has opened up many new areas of environmental microbiological research, as studies move towards analysing cells *in situ* and avoiding biases introduced by culturing techniques. Initial problems regarding cell wall permeability and access into the fixed cells by the oligonucleotide probe have been largely overcome for many types of bacteria, and the use of oligonucleotide probes with enzyme labels for signal amplification has also been demonstrated (Zarda *et al.*, 1991; MacNaughton *et al.*, 1994). To attempt to boost signal intensities, multiple probes have been used (Lee *et al.*, 1993), as have probes with multiple fluorochrome labels (Trebessius *et al.*, 1994). The brightness of the signal obtained after FISH has been correlated to rRNA content and thus protein synthesis and cell activity (Ruimy *et al.*, 1994).

Fluorescently-labelled oligonucleotide probes with various levels of specificity (domain-, genus- species- or strain-specific) may be used for enumeration of different

microorganisms, including those which have not actually been isolated and cultured (Amann *et al.*, 1990). The diversity of microbes present in slimes and water in an abandoned mine at Iron Mountain, California, was assessed using rRNA probes designed on the basis of previously reported sequences (Bond and Banfield, 2001; Bond *et al.*, 2000a; Edwards *et al.*, 1999; Schrenk *et al.*, 1998). Results confirmed that *At. ferrooxidans* accounted for low proportions of the microbial communities associated with the ore body. However, *At. ferrooxidans* was readily detectable in less acidic liquors (Edwards *et al.*, 1999; Schrenk *et al.*, 1998). Oligonucleotide probes can be used also to evaluate changes in microbial populations. At Iron Mountain, relative proportions and absolute numbers of microorganisms varied spatially and seasonally, and correlated with geothermical and physical conditions (pH, temperature, conductivity, and rainfall (Edwards *et al.*, 1999). *At. ferrooxidans* was most abundant at moderate pHs and temperatures, *L. ferrooxidans* was more abundant at higher temperatures and lower pHs, and archaea dominated microbial populations over the summer months when ionic strength of the pools and streams was greatest (Edwards *et al.*, 1999). Seven oligonucleotide probes for the detection of the *Thermoplasmales* group, a new group of *Leptospirillum*, the genus *Sulfobacillus*, the *Acidiphilium* genus, *Acidimicrobium* and relatives, and of organisms within the δ-*Proteobacteria* were designed and used to examine the abundance and distribution of these organisms at Iron Mountain (Bond and Banfield, 2001). *Thermoplasmales* were abundant and "*Fp. acidarmanus*" was a stable and dominating member of these samples (Bond and Banfield, 2001). The FISH technique was also used by Peccia *et al.* (2000) to identify members of the genus *Acidiphilium*, *At. thiooxidans* and *At. ferrooxidans* in laboratory reactors and environmental samples. For rapid counting of numbers of microbes in a mixed population, fluorescent probes can be combined with flow cytometry (Porter and

Pickup, 2000), a technique that may be more suited to industrial processes such as biomining.

1.10 Scope of The Current Project

This project focused on mineral processing by moderately thermophilic acidophiles. A major objective was to investigate which combinations of moderate thermophiles were most effective at oxidising pyrite under defined conditions. In other parts of the work, various aspects of the physiology and phylogeny of moderately thermophilic acidophiles were investigated.

The study is subdivided as follows:

(i) Characterisation of novel moderate thermophiles.

(ii) Pyrite oxidation experiments using various combinations of moderate thermophiles (preliminary experiments in flasks and further experiments in bioreactors).

(iii) Investigating the effects of flotation chemicals on growth of acidophiles.

(iv) Development of ARDREA (Amplified Ribosomal DNA Restriction Enzyme Analysis) method for "rapid" identification of acidophilic bacteria.

(v) Investigation of ferric iron sensitivity in some moderately thermophilic, iron-oxidising bacteria

The study started by characterising some moderately thermophilic acidophiles isolated from a commercial pilot plant. Following this, mixed cultures of different combinations of moderate thermophiles, including the novel isolates, were tested for pyrite oxidation as preliminary experiments in shake flasks. Data from preliminary experiments were used to 'fine-tune' microbial communities in further experiments using bioreactors.

Bioreactor experiments included monitoring rates of mineral breakdown and fates of the different microorganisms included in the original inoculum, using a plating technique in conjunction with a molecular approach (FISH).

The project also involved the application of ARDREA to identify acidophilic bacteria from environmental and industrial samples, and examination of the toxicity of ferric iron species and flotation chemicals to the bacteria used in leaching experiments.

Chapter 2

Materials and Methods

Materials and methods described in this chapter are those used routinely throughout the present study. Those used for specific experiments, and details of any modifications applied to the basic procedures, are described in their respective chapters. All chemicals used were supplied by either Merck-BDH Laboratory Supplies, or Sigma Chemicals Ltd., unless stated otherwise, and were, where possible, of AnalaR grade.

2.1 Microorganisms

The microorganisms used in this study are listed in Tables 2.1-2.3.

Table 2.1: "Mintek" isolates (obtained from a commercial pilot plant in South Africa) used in this study (chapter 3).

Isolate code	Putative ID	Reference
MT1	*At. caldus*	This study
MT2	*At. caldus*	This study
MT6	*Leptospirillum* sp.	This study
MT16	*Ferroplasma* sp.	This study
MT17	*Ferroplasma* sp.	This study
NC	*Sulfobacillus* sp.	This study

Table 2.2: Mesophilic acidophiles used in this study.

Isolate code	Source	Reference
At. ferrooxidans[T] (ATCC 23270)	Acid mine drainage, eastern U.S.A.	Temple and Colmer, 1951
"*T. ferrooxidans*" m-1	Coal strip mine refuse, Missouri, U. S. A.	Harrison, 1982
L. ferrooxidans[T] (DSM 2705)	Copper mine, Armenia	Markosyan, 1972
"*Sb. montserratensis*" L15	Thermal pool, Montserrat, W. I.	Yahya *et al.*, 1999
Sulfobacillus Riv14	Thermal pool, Montserrat, W. I.	Yahya *et al.*, 1999
Isolates SLC1, SLC2 and SLC66	Weathering sulfidic regolith, Utah	Johnson *et al.*, 2001a
"*Fm. acidophilum*" T23 (DSM 11138)	Acid mine drainage, Wales	Johnson *et al.*, 2001a

Table 2.3: Moderately thermophilic acidophiles used in this study.

Isolate code	Source	Reference
At. caldus[T] (strain KU) (DSM 8584)	Kingsbury coal spoil enrichment culture, England	Marsh and Norris, 1983b
Sb. thermosulfidooxidans[T] (VKM 1269)	Sulfide mineral leach dumps	Karavaiko *et al.*, 1988
Sb. thermosulfidooxidans TH1	Thermal spring, Ireland	Brierley, 1978
Sb. acidophilus ALV	Self-heating coal spoil, England	Norris and Barr, 1985
"*Sb. yellowstonensis*" YTF1	Frying Pan hot spring, Yellowstone N. P.	Ghauri and Johnson, 1991
Sulfobacillus-like isolates YTF3, YTF5 and YTF17	Sylvan hot springs, Yellowstone National Park	Johnson *et al.*, 2001b
Sulfobacillus-like isolates Y002, Y006	Thermal spring, Gibbon river area, Yellowstone N. P.	This study
Sulfobacillus-like isolates Y0010, Y0015, Y0016, Y0017	Frying Pan hot spring, Yellowstone N. P.	This study
Sulfobacillus-like isolates G1, G2, Riv2	Galways, Montserrat	This study
Sulfobacillus-like isolates GG6/1, GG6/3, 8/30	Lower Gages, Montserrat	This study
Novel moderately thermophilic iron-oxidising isolate Y005	Thermal spring, Gibbon river area, Yellowstone N. P.	This study
Novel moderately thermophilic heterotrophs Y008, Y0012	Frying Pan hot spring, Yellowstone N. P.	This study
Novel moderately thermophilic heterotrophs Y0013, Y0014	Thermal spring, Gibbon river area, Yellowstone N. P.	This study
Am. ferrooxidans[T] (strain ICP)	Icelandic geothermal site	Clark and Norris, 1996a
Am. ferrooxidans TH3	Copper leach dump, New Mexico	Norris and Barr, 1985
Acidimicrobium-like isolate Y0018	Frying Pan hot spring, Yellowstone N. P.	This study
"*Alb. acidophilus*" YTH1	Frying Pan hot spring, Yellowstone N. P.	Johnson *et al.*, 2001a
Alicyclobacillus-like isolate Y004	Thermal spring, Gibbon river area, Yellowstone N. P.	This study
Isolate GSM	Golden Sunlight Mine, Montana	Johnson *et al.*, 2001a

Organisms were revived, when needed, from the Acidophile Culture Collection, maintained at the University of Wales, Bangor.

2.2 Microbiological Techniques

Acidophilic microorganisms were maintained in appropriate liquid media. Cultures were usually inoculated at 1-2% (v/v) in 100 ml Erlenmeyer flasks containing 50 ml medium, or in 250 ml Erlenmeyer flasks containing 100 ml medium. Autotrophic iron- and sulfur-oxidising bacteria were grown in ferrous iron liquid media (section 2.2.1.1.1) and tetrathionate liquid media (section 2.2.1.1.2), respectively. Mixotrophic and heterotrophic acidophiles were maintained in ferrous iron/yeast extract liquid medium (section 2.2.1.1.3). *Ferroplasma* isolates were grown in *"Ferroplasma"* medium (section 2.2.1.1.5). Culture purity was routinely checked by streaking onto overlay solid media (sections 2.2.1.2.1) or onto ferrous iron/yeast extract solid medium (section 2.2.1.2.2) to check for any heterotrophic contaminants. Flask cultures were shaken at 130 rpm unless otherwise stated.

2.2.1 Media and Culture Conditions

Liquid media were prepared using either autotrophic basal salts solution or heterotrophic basal salts solution (modified from autotrophic basal salts solution). The basal salts solutions were prepared as 50X concentrates:

Autotrophic Basal Salts Solution (50X)		**(g/l)**	**Heterotrophic Basal Salts Solution (50X)**		**(g/l)**
$(NH_4)_2SO_4$	;	7.5	$(NH_4)_2SO_4$	;	22.5
KCl	;	2.5	KCl	;	2.5
$MgSO_4 \cdot 7H_2O$	;	25	$MgSO_4 \cdot 7H_2O$	;	25
KH_2PO_4	;	2.5	KH_2PO_4	;	2.5
$Ca(NO_3)_2 \cdot 4H_2O$	;	0.7	$Ca(NO_3)_2 \cdot 4H_2O$	;	0.7
			$Na_2SO_4 \cdot 10H_2O$	;	16.1

For liquid media, that did not contain either yeast extract or tryptone soya broth, a trace elements solution was routinely added at 1 ml/l culture.

Trace Elements Stock Solution

		(g/l)
$ZnSO_4 \cdot 7H_2O$	;	10.0
$CuSO_4 \cdot 5H_2O$	;	1.0
$MnSO_4 \cdot 4H_2O$	;	1.0
$CoSO_4 \cdot 7H_2O$	;	1.0
$Cr_2(SO_4)_3 \cdot 15H_2O$	;	0.5
H_3BO_3	;	0.6
$Na_2MoO_4 \cdot 2H_2O$	;	0.5
$NaVO_3$	;	0.1
$NiSO_4 \cdot 6H_2O$	;	1.0
$Na_2SeO_4 \cdot 10H_2O$	;	1.0
$Na_2WO_4 \cdot 2H_2O$	;	0.1

The trace elements stock solution was acidified to pH 2.0 with H_2SO_4, filter sterilised through 0.22 µm nitrocellulose membranes (Millipore GVWP) and stored at 4°C.

2.2.1.1 Liquid Media

2.2.1.1.1 Ferrous Iron Medium

The medium comprised:

Autotrophic basal salts
20 mM Ferrous sulfate
Trace elements
Distilled water
(pH 2.0 with H_2SO_4)

A stock solution of 1 M ferrous sulfate (adjusted to pH 2.0 with H_2SO_4) was filter-sterilised through 0.22 µm nitrocellulose membranes. This was added to heat-sterilised (120°C, 20min) autotrophic basal salts/trace elements solution (also previously adjusted to pH 2.0 with H_2SO_4) to produce a medium containing 20 mM ferrous iron.

2.2.1.1.2 Tetrathionate Medium

The medium comprised:

Autotrophic basal salts
5 mM Potassium tetrathionate
0.1–1 mM Ferrous sulfate
Trace elements
Distilled water
(pH 2.5 with H_2SO_4)

Stock solutions of 100 mM potassium tetrathionate and 1 M ferrous sulfate (adjusted to pH 2.0 with H_2SO_4) were filter sterilised through 0.22 µm nitrocellulose membranes. These solutions were added to heat-sterilised (120°C, 20min) autotrophic basal salts/trace elements solution (also previously adjusted to pH 2.5 with H_2SO_4) to produce a medium containing 5 mM tetrathionate and 0.1–1 mM ferrous iron.

2.2.1.1.3 Heterotrophic Medium

The medium comprised:

Heterotrophic basal salts
0.02% (w/v) Yeast extract
10 mM Ferrous sulfate
Distilled water
(pH 2.0 with H_2SO_4)

A stock solution of 1 M ferrous sulfate (adjusted to pH 2.0 with H_2SO_4) was filter-sterilised through 0.22 µm nitrocellulose membranes. This was added to heat-sterilised (120°C, 20min) heterotrophic basal salts/yeast extract solution (also previously adjusted to pH 2.0 with H_2SO_4) to produce a medium containing 10 mM ferrous iron.

2.2.1.1.4 Pyrite Medium

The medium comprised:

Autotrophic basal salts (heterotrophic basal salts for bioreactor cultures)
Acid washed pyrite
Trace elements
Distilled water
(pH 1.5-2.0 with H_2SO_4)

Two samples of pyrite were used in leaching experiments. One of these was obtained by mill grinding of rock obtained from the abandoned Cae Coch pyrite mine, north Wales (McGinness and Johnson, 1993), and contained ~80% FeS_2, other minerals being mostly quartz. The second sample was supplied by Mintek Ltd., and was a concentrate containing *ca.* 60% pyrite and 40% quartz.

Prior to use, the pyrite was acid-washed with 100 mM hydrochloric acid with continuous stirring for about 30 minutes, to remove any ferric iron precipitates on the pyrite surface. The pyrite was then recovered by centrifugation and was rinsed with distilled water 2 to 3 times, until the yellow colour of the solution phase was not evident. After the final rinse, the pyrite was collected and dried at 100°C.

Acid-washed pyrite was added to the autotrophic basal salts/trace elements solution (adjusted to pH 1.5–2.0 with H_2SO_4), to the final concentration of 1–2% (w/v) for flask experiments and of 5% (w/v) for bioreactor experiments, and autoclaved (120°C, 20min).

For bioreactor experiments (chapter 5), heterotrophic basal salts (excluding $Na_2SO_4 \cdot 10H_2O$) were used instead of autotrophic basal salts to supply more nitrogen and also to avoid any precipitation (natrojarosite) from occurring due to the presence of sodium. The amount of water lost by evaporation during pyrite oxidising experiments was calculated by weighing the flasks before and after the each sampling and sterile water was added to supplement the evaporated water (except section 4.2).

2.2.1.1.5 "*Ferroplasma*" Medium

The medium comprised:

Heterotrophic basal salts
0.02% (w/v) Yeast extract
50 mM Ferrous sulfate
50 mM K_2SO_4
Trace elements
Distilled water
(pH 1.5 with H_2SO_4)

A stock solution of 1 M ferrous sulfate (adjusted to pH 1.5 with H_2SO_4) was filter-sterilised through 0.22 µm nitrocellulose membranes. This was added to heat-sterilised (120°C, 20min) heterotrophic basal salts/yeast extract/potassium sulfate/trace elements solution (also previously adjusted to pH 1.5 with H_2SO_4) to produce a medium containing 50 mM ferrous iron.

2.2.1.2 Solid Media

The media described here were developed by Johnson (1995b) and co-workers. This method used is based on the 'overlay' technique, in which an acidophilic heterotrophic bacterium (usually *Acidiphilium* SJH) is incorporated into the underlayer of a two-layer gel, in order to overcome the inhibitory effects of organic compounds (predominantly agarose hydrolysis products) on the growth of iron- and sulfur-oxidising acidophiles.

The "ferrous iron overlay" medium described by Johnson and McGinness (1991a) was reported to have a high plating efficiency for more than 50 iron-oxidising acidophilic isolates, including strains of *At. ferrooxidans*, *L. ferrooxidans* and moderately thermophilic bacteria, whereas the "ferrous iron/tetrathionate overlay" medium supports the growth of *At. thiooxidans*, *At. caldus* and other moderate thermophiles as well as iron-oxidising mesophiles and some heterotrophic acidophiles.

2.2.1.2.1 Overlay Solid Media

2.2.1.2.1.1 Ferrous Iron Overlay Medium (Feo)

Preparation of 400 ml medium was sufficient for 10–12 plates.

Solution A

40 ml Basal salts solution (10X concentrate (g/l): $(NH_4)_2SO_4$ (12.5); $MgSO_4 \cdot 7H_2O$ (5))
0.1 g TSB (Tryptone Soya Broth)
400 µl Trace elements (section 2.2.1)
250 ml Distilled water
(pH 2.5 with H_2SO_4)

Solution B

2 g	Agarose (e.g., Sigma Ltd. Type I)
100 ml	Distilled water

Solution C

1 M ferrous sulfate (adjusted to pH 2.0 with H_2SO_4): filter sterilised through 0.22 µm nitrocellulose membranes.

Procedure

Solutions A and B were prepared separately and heat sterilised (120°C, 20min). After cooling to ~50°C, the two solutions were mixed and 10 ml of solution C was added. The combined molten medium was split ~50:50 into two sterile containers. One solution was inoculated with 2 ml of an active culture of *Acidiphilium* SJH (pre-grown in a liquid medium containing basal salts solution, 10 mM galactose, 0.025% (w/v) TSB, and 25 mM ferrous sulfate (adjusted to pH 2.0-2.5 with H_2SO_4)), and was poured immediately as a thin underlayer in standard petri plates. The gelled underlayer was then covered with the same quantity of sterile medium.

2.2.1.2.1.2 Ferrous Iron/Tetrathionate Overlay Medium (FeSo)

The preparation of 400 ml medium was sufficient for 10–12 plates.

Solution A

40 ml Basal salts solution (10X concentrate (g/l): $(NH_4)_2SO_4$ (12.5); $MgSO_4 \cdot 7H_2O$ (5))
0.1 g TSB (Tryptone Soya Broth)
400 µl Trace elements (section 2.2.1)
250 ml Distilled water
(pH 2.5 with H_2SO_4)

Solution B

2 g Agarose (e.g., Sigma Ltd. Type I)
100 ml Distilled water

Solution C

1 M ferrous sulfate (adjusted to pH 2.0 with H_2SO_4), filter sterilised through 0.22µm nitrocellulose membranes.

Solution D

100 mM potassium tetrathionate, filter-sterilised through 0.22µm nitrocellulose membranes.

Procedure

Solutions A and B were prepared separately and heat sterilised (120°C, 20min). After cooling to ~50 °C, the two solutions were mixed and 10 ml of solution C and solution D were added. The combined molten medium was split ~50:50 into two sterile containers. One solution was inoculated with 2 ml of an active culture of *Acidiphilium* SJH (pre-grown in a liquid medium containing basal salts solution, 10 mM galactose, 0.025% (w/v) TSB, and 25 mM ferrous sulfate (adjusted to pH 2.0-2.5 with H_2SO_4)), and was poured immediately as a thin underlayer in standard petri plates. The gelled underlayer was then covered with the same quantity of sterile medium.

2.2.1.2.2 Ferrous Iron/Yeast Extract Solid Medium (Fe/YE)

A non-overlay, ferrous iron/yeast extract medium, (Fe/YE) was used during the course of this study to culture heterotrophic acidophilic bacteria (e.g. to check for culture purity). The preparation of 400 ml medium was sufficient for 10–12 plates.

Solution A

8 ml	Heterotrophic basal salts (section 2.2.1)
0.08 g	Yeast extract
292 ml	Distilled water

(pH 2.5–3.0 with H_2SO_4)

Solution B

2 g	Agarose (e.g., Sigma Ltd. Type I)
100 ml	Distilled water

Solution C

1 M ferrous sulfate (adjusted to pH 2.0 with H_2SO_4), filter sterilised through 0.22µm nitrocellulose membranes.

Procedure

Solutions A and B were heat sterilised (120°C, 20 min) and held at approximately 50°C prior to mixing. The solutions were combined and 0.2 ml of solution C was added, before pouring into sterile petri plates.

2.2.1.3 Bioreactor Cultures

Bench-scale bioreactors (2 L) (Electrolab P350) were used for batch culture growth of some isolates, and also for pyrite leaching experiments using mixed populations. Typically, 1.5 L of appropriate medium was prepared in the reactor and autoclaved (120°C, 40 min) before inoculation.

2.2.2 Determination of Microbial Biomass

2.2.2.1 Optical Densities

Bacterial biomass was measured spectrophotometrically by determining culture optical densities (OD) at 600 nm against a blank of distilled water. When yellowish-orange colours were evident due to the presence of ferric iron, 100 µl of 1M H_2SO_4 was added to 900 µl of culture; the resulting lower pH resulted in a greatly diminished colour of the ferric ion.

2.2.2.2 Total Cell Counts

2.2.2.2.1 Thoma Bacteria Counting Chamber

Operation

Bacteria in liquid samples were dispersed as thoroughly as possible, and a small drop of sample was placed on a glass slide and covered with a cover slip. The sample was viewed using a phase-contrast microscope (section 2.3.2) with an x40 objective lens. At least 50–100 bacteria were counted.

Calculation

This chamber consists of a glass slide, a section of which is precisely ground to a depth of 20 µm below the surface and this is surrounded by a moat. The distance between each of the parallel lines on the chamber is 50 µm. The area of each square was therefore 50 x 50 = 2500 μm^2, making the volume of each square to be 50,000 μm^3 (5 x 10^4 μm^3). The average number of bacteria per square was calculated and it was then multiplied by 2 x 10^7 (i. e. 1 ml/5 x 10^4 μm^3) to calculate the number of bacteria/ml.

2.2.2.2.2 DAPI (4 ',6-diamidino-2-phenylindole) Staining

DAPI is a DNA stain that allows the visualisation of microbes in samples, using a fluorescent microscope (section 2.3.3), to obtain total counts of microbes present in the sample. Microbes are first captured on a black polycarbonate filter, which is then mounted on a microscope slide to facilitate counting. As this is a very sensitive technique, it is essential that all reagents were prepared by filtering through a 0.22 µm membranes to remove all microbes from the reagents followed by autoclaving.

Reagents

Distilled water (dH_2O)
Distilled water (adjusted to pH 1.8 with H_2SO_4)

Stock DAPI solution: 1 mg/ml dH_2O, put into an Eppendorf tube wrapped with aluminium foil and stored at -20°C.

Diluted DAPI solution: 1 µg/ ml dH_2O

3X PBS: 22.79 g NaCl, 7.52 g $Na_2HPO_4 \cdot 12H_2O$ and 1.24 g $NaH_2PO_4 \cdot 1H_2O$ in 1 litre dH_2O (pH 7.2).
(1X PBS contains 130 mM NaCl, 7 mM $Na_2HPO_4 \cdot 12H_2O$ and 3 mM $NaH_2PO_4 \cdot 1H_2O$ (pH 7.2))

4% Paraformaldehyde fix:

33 ml dH_2O was heated gently and was added with 2 g paraformaldehyde. The solution was then added with one drop of 2 M NaOH and was stirred for about 2 minutes. When paraformaldehyde was dissolved, 16.5 ml of 3X PBS was added. The solution was adjust to pH 7.2 with NaOH or HCl and was filter sterilised through 0.2 µm membranes into a sterile container. The solution was stored at –20°C in small aliquots (750 µl).

Procedure

Microbes were fixed with paraformaldehyde, as described in section 2.5.12. A tower filtration apparatus (Millipore Inc.) was fitted with a 25 mm black polycarbonate filter (0.2 µm pore-size) per sample and the filters were pre-wetted by drawing through some filtered dH_2O. The filtration towers were filled with 10 ml of dH_2O (acidified if using iron-rich samples) the fixed sample added, and the liquid drawn through by applying a

vacuum. The filters were then washed with 10 ml dH_2O (acidified if using iron-rich samples followed by two further washings with 10 ml dH_2O (not acidified). DAPI solution (2-10 ml) was added and held for 10 minutes before applying a vacuum. The filters were then washed twice with 10 ml dH_2O. Excess water on the filters was removed on a piece of absorbent paper. A drop of immersion oil (non-fluorescing) was placed on a glass slide, the membrane put on top, followed by another drop of immersion oil. The number of microbes on the membrane was counted using a fluorescent microscope, ECLIPSE E600 (Nikon, Japan) (section 2.3.3). A minimum of 10 fields of view was counted.

Calculation

Each area of view with the x100 objective was 0.038 mm^2. The stained area of membrane using the Millipore tower was 185 mm^2, and that using the Millipore Manifold apparatus was 269 mm^2. The correction factor (per field of view) was, therefore, x4,868 for the tower set up and x7,079 for the Manifold apparatus.

2.2.2.3 Plate Counts on Solid Media

Samples of bacterial cells were diluted in acidified basal salts (pH 1.8) following a serial dilution procedure (10-fold), and 100 µl aliquots were spread onto selective solid media.

In the pyrite oxidation experiments (chapters 4 and 5), the microbial cultures were well mixed by vortexing to disperse pyrite equally.

Plates were incubated at 30°C (mesophiles) or 45°C (moderate thermophiles) for 1-2 weeks, and colonies were counted using a stereo-scan microscope (section 2.3.1).

Microorganisms in mixed cultures were identified and enumerated using methods described elsewhere (Johnson and Roberto, 1997).

2.3 Microscopy

2.3.1 Stereo-Scan Microscopy

A stereo-scan microscope (Leitz-Wild M32, Switzerland) was used to examine and characterise bacterial colonies grown on various types of solid media, using magnifications of x50 to x400. An Olympus OM-10 camera was fitted to the microscope to photograph colonies.

2.3.2 Phase-Contrast Microscopy

Phase contrast microscopy allows the visualisation of colourless, small specimens that do not absorb enough light to be seen by bright-field microscopy. A Leitz Labolux (Switzerland) phase contrast microscope, fitted with a Zenike condenser and objective (magnification x400), was used to record morphological and behavioural characteristics of bacterial cells.

2.3.3 Fluorescence Microscopy

Fluorescence microscopy required staining of the specimens with fluorescent dyes prior to viewing. A fluorescence microscope, ECLIPSE E600 (Nikon, Japan) attached to a super high pressure mercury lamp power supply, HB-10104AF (Nikon, Japan) and a digital camera, COOLPIX (Nikon, Japan) was used throughout these studies for bacterial counts using the DAPI method (section 2.2.2.2.2) and FISH method (section 2.5.12), with a magnification x1000. HQ FITC-LP filter, HQCy3 filter and UV-1A filter

were used for the Fluorescein (MWG Biotech, Germany)-, Cy3 (MWG Biotech, Germany)- and DAPI -treated specimens.

2.4 Analytical Techniques

2.4.1 Determination of pH and Redox Potential (E_h)

Culture pH and redox potentials (relative to a standard hydrogen electrode) were determined using an Accumet® 50 pH meter coupled to a pHase combination glass electrode (Merck) and a combination ORP electrode (Russell pH Ltd. UK).

2.4.2 Determination of Ferrous Iron

2.4.2.1 Titrimetric Method: Potassium Permanganate Assay

Reagents

1 mM Potassium permanganate ($KMnO_4$)
25% (v/v) Sulfuric acid (H_2SO_4)

Procedure

An aliquot of 1-5 ml sample was acidified with two or three drops of 25% (v/v) sulfuric acid prior to titration with permanganate and was titrated until the point where a faint pink colour appeared (indicating the end point).

Calculation

The correlation between permanganate and ferrous iron concentration is given by the reaction stoichiometry (equation [2.1]),

$$Fe^{2+} \rightarrow Fe^{3+} + e^-$$
$$MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_2O \qquad [2.1],$$

showing that 1 mole of permanganate reacts with 5 moles of ferrous iron. From this, concentrations of ferrous iron in sample aliquots could be determined. This method was used to determine ferrous iron when present at > 2.5 mM.

2.4.2.2 Colorimetric Method: Ferrozine Assay

This assay is based on the chelating of ferrous iron by the (-N=C-C-N) group of the ferrozine molecule (3-(2-pyridyl)5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine; Sigma Ltd.) to form a stable magenta-coloured ferrous iron-ferrozine complex, which exhibits a single sharp peak with a maximum absorbance at 562 nm (Stookey, 1970). The method used throughout these studies was a modification of the method described by Lovley and Phillips (1987) to determine concentrations of 0 to 1 mM ferrous iron. The complex formed was found to be stable for at least 1 hour in the dark.

Ferrozine Reagent

50 mM	Hepes buffer
1 g	Ferrozine
	(pH 7.0 with KOH; stored in the dark at 4 °C)

Standards

Standards were prepared with 10 mM ammonium ferrous sulfate ($(NH_4)_2SO_4.FeSO_4$ $6H_2O$, pH2.0) over the range of 0-1 mM Fe^{2+} to prepare a standard curve (Figure 2.1).

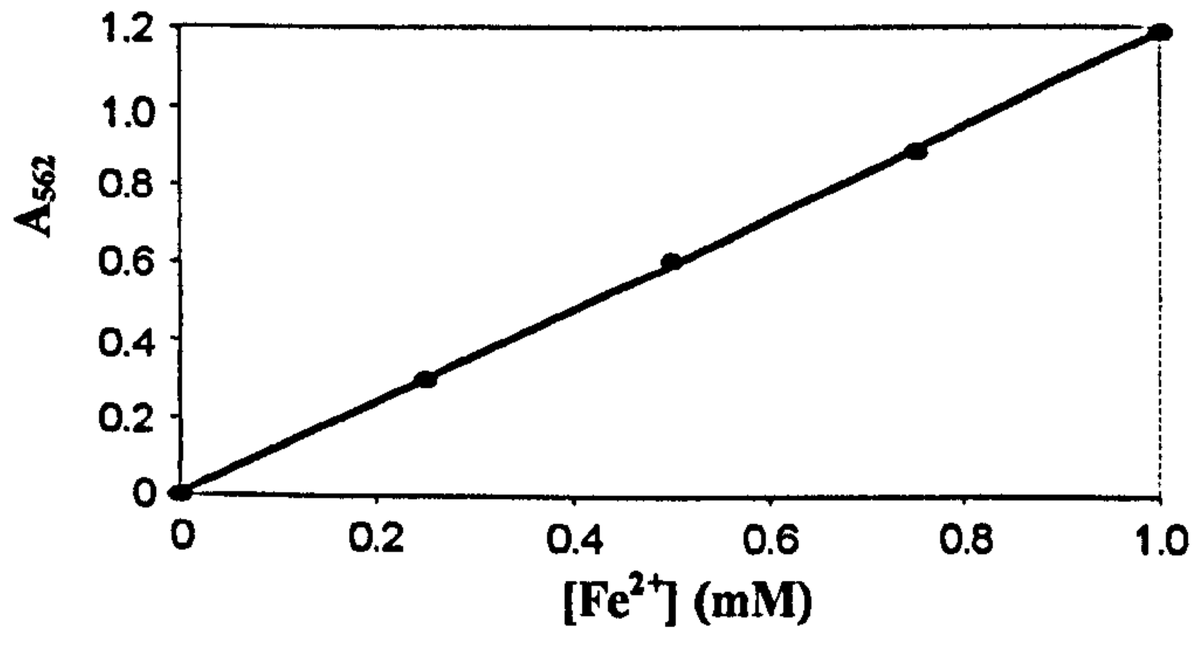

Figure 2.1: A typical standard curve for the assay of ferrous iron using ferrozine. The equation of the fitted line is y=1.188x + 0.004. Rval=0.9998.

Procedure

A 50 µl aliquot of sample was removed and added to 950 µl of ferrozine reagent. This was mixed thoroughly prior to measuring absorbance at 562 nm against a full reagent

blank. Samples were centrifuged prior to undertaking this assay, to remove any particulate matter (particularly ferric compounds).

2.4.3 Atomic Absorption Spectrophotometry

Reagents

6 M Hydrochloric acid (HCl)
0.5 M Hydrochloric acid (HCl)

Standards

Calibration curves were made using iron standard solution (BDH SpectrosoL®) over the range of 0 to 50 ppm (Figure 2.2a) or using copper standard solution (BDH SpectrosoL®) over the range of 0 to 10 ppm (Figure 2.2b).

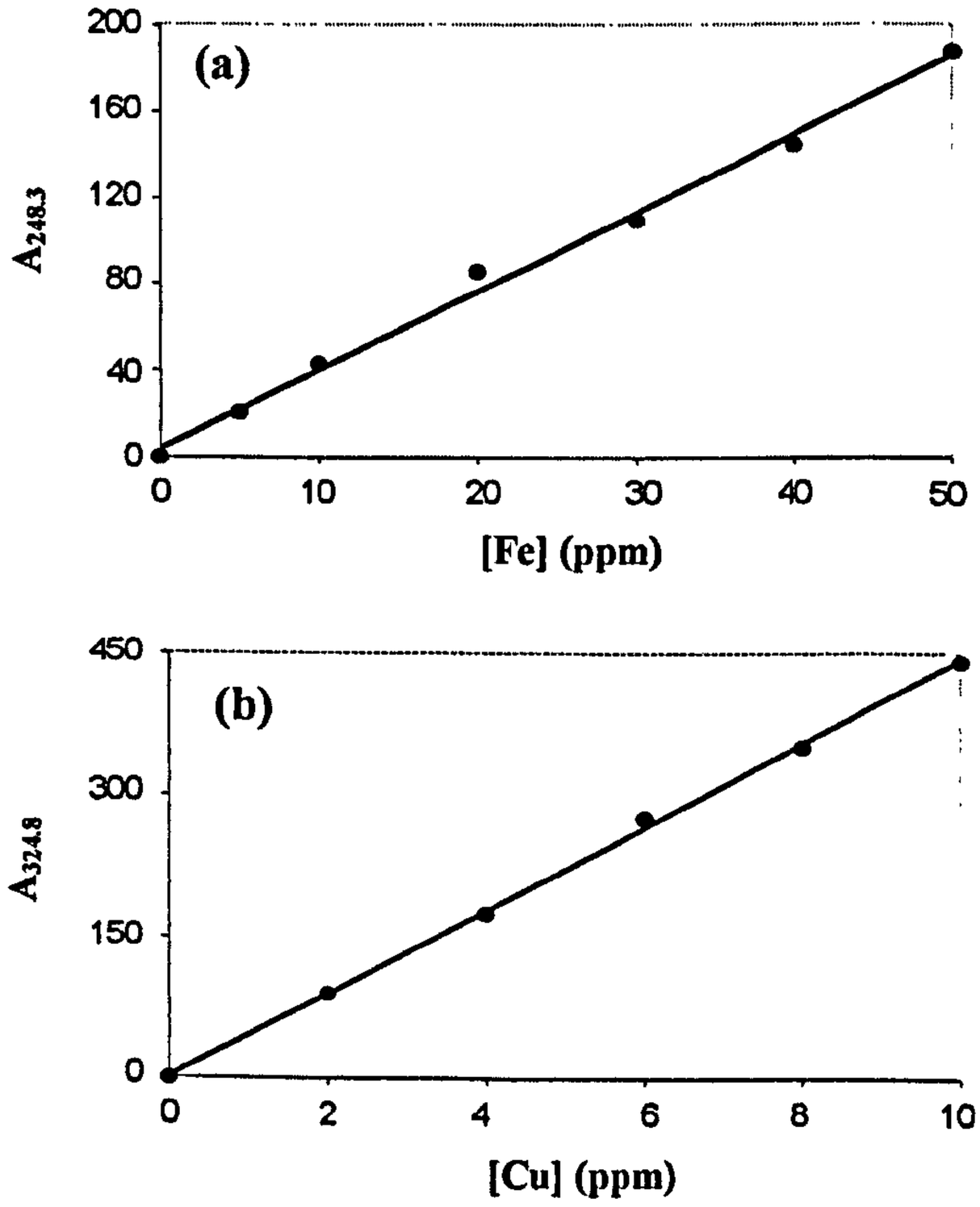

Figure 2.2: (a) A typical standard curve for the measurement of Fe ions using AAS. The equation of the fitted line is y=3.643x + 4.0478. Rval=0.9948. (b) A typical standard curve for the measurement of Cu ions using AAS. The equation of the fitted line is y=44x + 1.6667. Rval=0.9992.

Procedure

Determination of total iron/copper in solution phase:

1 ml of sample was centrifuged (13,000 rpm, 1 min) to remove cells and minerals. A 0.5 ml aliquot of the supernatant was combined with 0.5 ml of 6 M hydrochloric acid, and stored at room temperature until needed.

Determination of total soluble iron in solution and solid phase:

The minerals in the sample were dispersed as thoroughly as possible before taking 0.1 ml of sample. The sample was combined with 0.9 ml of 6 M HCl and incubated at 65°C for 30 minutes, followed by centrifugation at 15,000 rpm for 1 minute. Supernatant (0.5 ml) was removed and added to 0.5 ml of 0.5 M HCl, and stored at room temperature until needed.

The concentration of total iron was determined using a Pye Unicam SP2900 double beam atomic absorption spectrophotometer (AAS) fitted with a Pye Unicam SP9-10 gas controller unit, at wavelength 248.3 nm (iron) or 324.8 nm (copper), using a fuel-lean air/acetylene flame.

2.4.4 Determination of Tetrathionate

Reagents

Phosphate buffer: 50 ml 0.2 M NaH_2PO_4 plus 39 ml 0.2 M NaOH.
Ferric nitrate solution: 1.5 M $Fe(NO_3)_3$ in 4M $HClO_4$.
Potassium cyanide solution: 0.1 M KCN in dH_2O.

Standards

A standard curve was prepared using potassium tetrathionate over the range of 0–0.5 mM (Figure 2.3).

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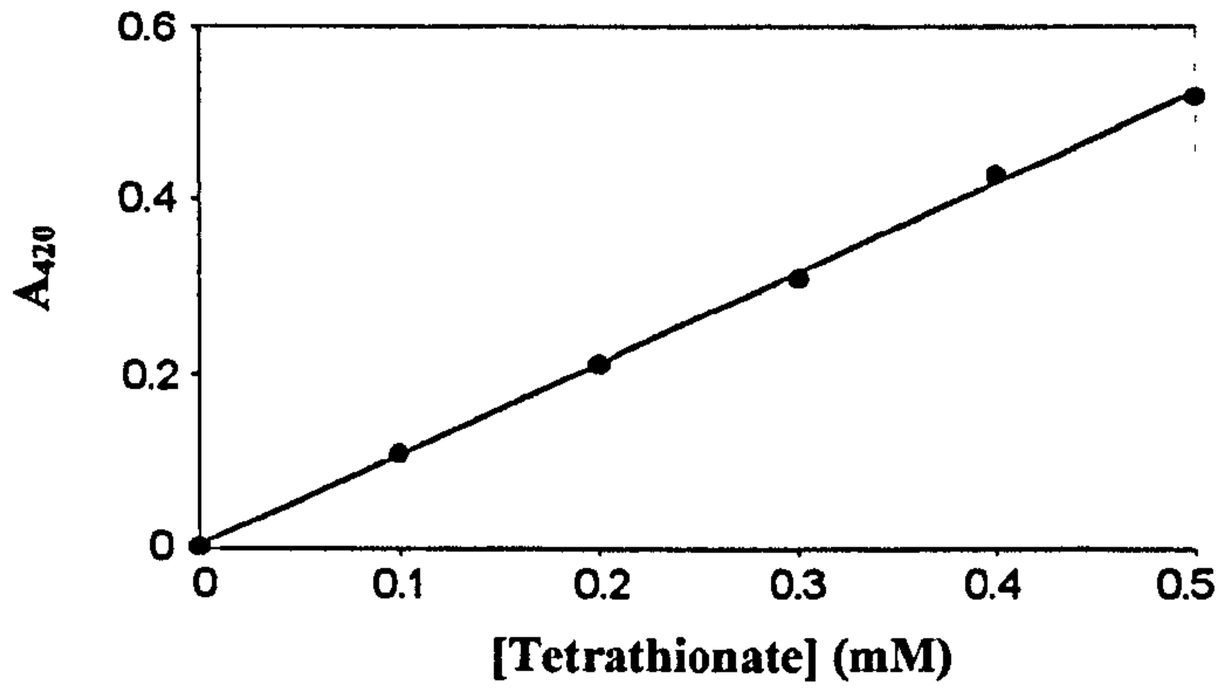

Figure 2.3: A typical standard curve for the determination of tetrathionate. The equation of the fitted line is y=1.043x + 0.0032. Rval=0.9992.

Procedure

A sample aliquot (0.24 ml, diluted if needed) was added to 0.16 ml phosphate buffer, followed by 0.2 ml potassium cyanide solution, and the solution mixed rapidly. The sample was then added, together with 0.12 ml ferric nitrate solution, and the solution again mixed rapidly. The sample was made up to 1 ml with dH_2O prior to reading absorbance at 460 nm against a thionate-free blank.

2.4.5 Determination of Sulfate

Reagents

Conditioning reagent: 250 ml distilled water; 50 ml glycerol; 30 ml concentrated HCl; 10 ml 95% ethanol; 75 g NaCl (made up to 500 ml).

Crushed barium chloride

Procedure

Samples from pyrite leachate experiments were prepared and stored as for iron and copper (section 2.4.3)

One millilitre of sample solution (diluted when necessary) was centrifuged for 10 minutes and added to 1 ml of conditioning reagent. The sample and conditioning reagent was mixed thoroughly. Fine-grain barium chloride (*ca.* 60 mg) was added, and

the solution was mixed for 30 seconds. The absorbance was read at 420 nm against sulfate-free blank using the Hydrocheck (WPA Ltd., UK) system.

2.4.6 Determination of Protein: the Bradford Assay

Reagents

100 mg Coomassie Brilliant Blue G-250/l of 5% ethanol in 10% phosphoric acid

Standards

A standard curve was made using bovine serum albumin (BSA) over the range of 0–100 µg BSA/ml of 0.5 M NaOH (Figure 2.4).

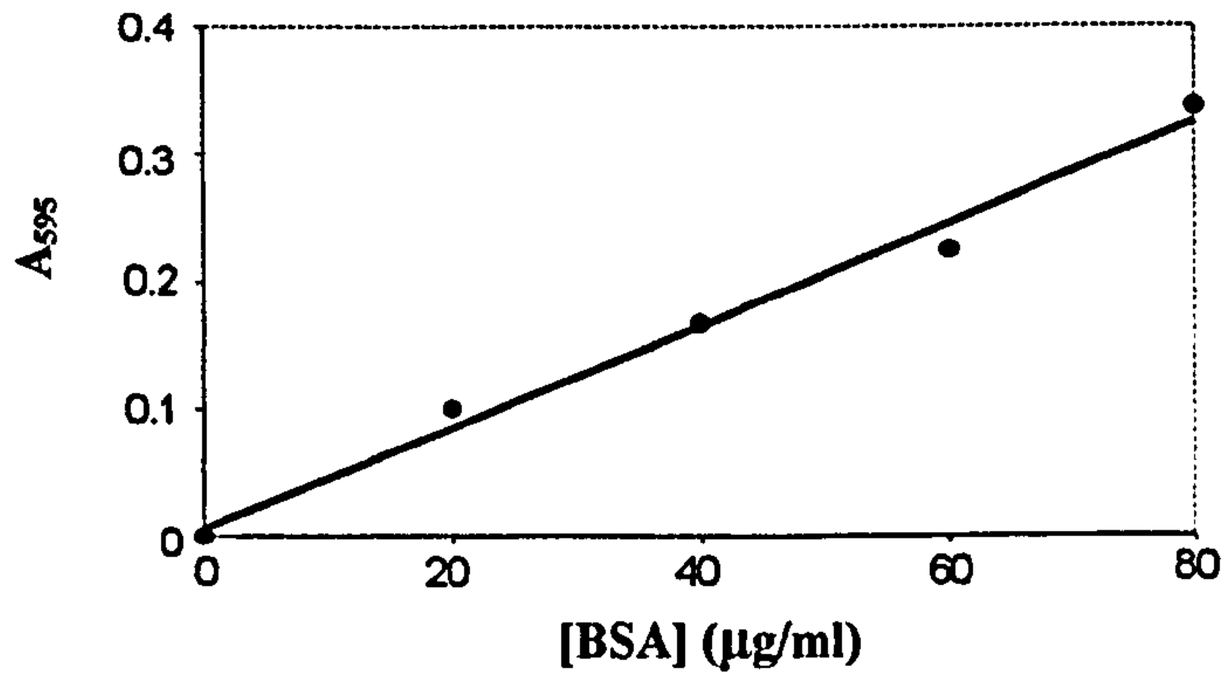

Figure 2.4: A typical standard curve for the Bradford assay. The equation of the fitted line is y=0.004x + 0.0064. Rval=0.9874.

Procedure

Cells were harvested by centrifugation (13,000 rpm, 3 min), resuspended in 0.5 ml of 0.5 M NaOH, and held for 15 minutes at room temperature. One hundred microlitres of this extract (diluted in 0.5 M NaOH if necessary to get into the range of the standards) was mixed with 1 ml of the Bradford reagent and incubated for 2 minutes in the dark before measuring the absorbance at 595 nm.

2.4.7 Determination of Dissolved Organic Carbon (DOC)

Standards

Standard curves were made using KHP standard (anhydrous potassium hydrogen phthalate; $C_8H_5O_4K$) (Pollution & Process Monitoring Ltd., UK) over the range of 0–10 ppm (Figure 2.5).

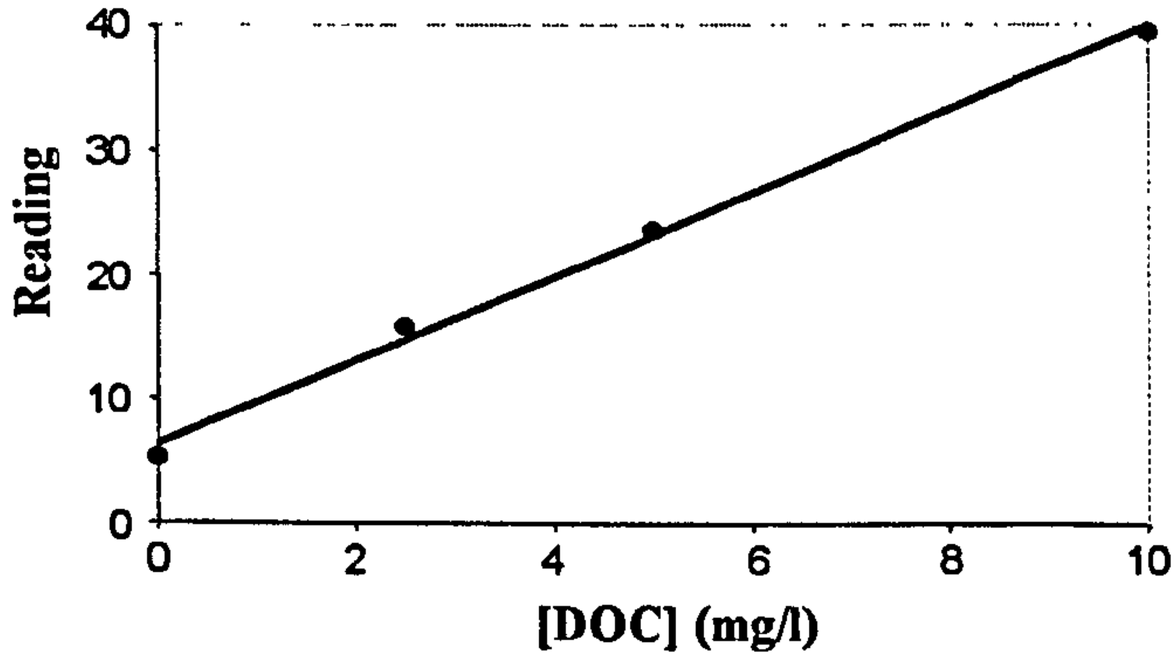

Figure 2.5: A typical standard curve for the DOC assay. The equation of the fitted line is y=3.3787x + 6.288. Rval= 0.9959.

Procedure

Approximately 2 ml of sample was filter-sterilised through 0.2 µm cellulose nitrate membranes and kept at –20°C until needed. The samples were diluted with dH_2O if necessary and DOC concentration determined using PROTOC DOC analyser (Pollution & Process Monitoring Ltd., UK).

2.5 Biomolecular Techniques

2.5.1 Polymerase Chain Reaction (PCR)

The 16S rRNA gene of eubacteria/archaea was amplified by PCR using "27f primer" (5'-AGAGTTTGATCMTGGCTCAG-3')/"20f primer" (5'-TCCGGTTGATCCYGCCR G-3') and "1492r primer" (5'-TACGGYTACCTTGTTACGACTT-3'), complimentary to positions 8 to 27/1 to 20 and 1510 to 1492 of *Escherichia coli* 16S rRNA gene, respectively (Lane *et al.*, 1992; Orphan *et al.*, 2000).

Reagents

All reagents that were not supplied with the *Taq* enzyme were made with autoclaved deionised water (pH 7.0 with NaOH).

Taq polymerase:	usually supplied as a 5 unit/µl stock.
10X reaction buffer:	supplied with enzyme.
25 mM $MgCl_2$:	supplied with enzyme.
dNTP solution:	containing 2 mM each dATP, dCTP, dTTP and dGTP.
"27f"/"20f" primer:	100 ng /µl deionised water.
"1492r" primer:	100 ng /µl deionised water.

DNA Preparation

Bacteria were grown in appropriate medium until late exponential phase to early stationary phase, and cells harvested by centrifuging. When using iron-oxidisers or iron-reducers, cells were washed first in 10 mM H_2SO_4 first to remove ferric precipitates, and then in TE buffer (pH 8.0). The cell pellet was resuspended in 20 µl of the PCR lysis solution (0.05 M NaOH + 0.25% SDS) and was heated to 95°C for 10 minutes, and 180 µl of deionised water added.

PCR Amplification

'Touchdown PCR' was first described by Don *et al.* (1991) to redress the imbalance between correct and spurious annealing. The 16S rRNA gene of most of the acidophilic organisms (e.g. *Leptospirillum* spp., *Sulfobacillus* spp., *Acidithiobacillus* spp., *Am. ferrooxidans*, and *Ferroplasma* spp.) were successfully amplified by 'Touchdown PCR' in the presence of 2% DMSO.

Fifty microlitres of reaction master mix was prepared for "N" number of reactions by adding the following:

5 x N µl	10X reaction buffer
5 x N µl	25mM $MgCl_2$
5 x N µl	dNTPs
1 x N µl	forward primer
1 x N µl	reverse primer
1 x N µl	DMSO (dimethylsulfoxide)
0.5 x N µl	*Taq* polymerase
30.5 x N µl	deionised water (pH 7.0 with NaOH)

Master mix (49 µl aliquots) was placed in each reaction tube and 1 µl of cell lysate added. The PCR reaction was run as follows: an initial denaturation at 95°C for 5 minutes, followed by 20 cycles (95°C for 30 s, 57°C (-0.5°C per cycle) for 30 s and 72°C for 1.5 min) and by 15 cycles (95°C for 30 s, 47°C for 30 s and 72°C for 1.5 min) and a final 10 minutes incubation at 72°C. The reaction was carried out in a thermocycler (Progene Techne, Cambridge). Following PCR, the amplified 16S rRNA gene was confirmed by analysing 5 µl of the PCR reaction on a 0.7% (w/v) agarose gel (section 2.5.2). When used for sequencing, PCR products were purified using PCR purification kit (QIAGEN) according to the manufacture's instructions (section 2.5.7).

2.5.2 Agarose Gel Analyses of DNA

Reagents

0.5 M EDTA:	46.53 g EDTA (sodium ethylenediaminetetra-acetic acid) adjusted to pH 8.0 with 10 M NaOH and made up to 250 ml with dH_2O, autoclaved before use
5X TBE:	54 g Tris (tris(hydroxymethyl)methylamine) 27.5 g boric acid 20 ml 0.5 M EDTA stock made up to 1 litre with dH_2O and autoclaved before use
6X DNA loading buffer:	0.25% (w/v) bromophenol blue in 30% (v/v) glycerol.

Procedure

To prepare a 0.7% agarose gel (typically for analysing the PCR products), electrophoresis grade agarose was added to 0.5X TBE. To prepare >2% agarose gel (typically for analysing the restriction DNA fragments), high-resolution blend agarose (type 3:1; Amresco) was added to 1X TBE. The agarose solution was melted by heating in a microwave and cooled (to ~50°C) before pouring it into the mould to polymerise. The DNA samples were mixed with the DNA loading buffer prior to loading into respective wells in the agarose gel. The gel was run until the blue dye had migrated to the desired position. The gel was stained in an ethidium bromide bath (~10 min) prior to analysing the DNA pattern under ultraviolet (UV) light.

2.5.3 Cloning of the 16S rRNA Gene

Ligation Reaction

Ligation was carried out using the pGEN®-T Easy Vectors Systems (Promega) according to the manufacture's instructions.

Ligation reactions were set up by mixing 5 µl of 2X Rapid Ligation Buffer, 1 µl pGEN®-T Easy Vector, 3 µl PCR product and 1 µl of T4 DNA Ligase. The reactions were incubated for 1 to 2 hours at room temperature.

Transformation

Tubes of frozen DH5α competent cells were removed from –70°C storage and were placed in an ice bath until just thawed. Five microlitres of the ligation reaction were added into the tube containing 100 µl of DH5α and the tube was gently flicked to mix. The tube was placed on ice for 30 minutes. Cells were heat-shocked for 45-50 seconds in a water bath at 42°C. The tube was immediately returned to ice for 2 minutes. Nine hundred microlitres of (room temperature) SOC medium* was added to the tube before

incubating for 1 to 2 hours at 37°C, shaken at 130 rpm. Next, 100 µl of transformation culture was spread onto a LB/ampicillin/X-Gal plate and the plate was incubated overnight at 37°C.

*SOC medium contained 20 g tryptone, 5 g yeast extract and 0.5 g NaCl in 1L dH_2O (pH adjusted to 7.0 with NaOH). Sterile stock glucose solution (1M) was added to the final concentration of 20 mM.

Screening of positive clones was then carried out by PCR (section 2.5.4) and finally confirmed by RFLP (section 2.5.5) analyses.

2.5.4 PCR Screening of Cloned 16S rRNA Genes

This PCR method was used to screen for positive clones of the 16S rRNA gene previously amplified by PCR. Following amplification using primers specific for the cloning vector, the resulting product was confirmed as positive by RFLP analysis alongside the original PCR product used in the cloning.

Reagents

Taq polymerase:	usually supplied as a 5U *Taq*/µl stock.
10X reaction buffer:	supplied with enzyme.
25 mM $MgCl_2$:	supplied with enzyme.
dNTP:	stock solution containing 2 mM each dATP, dCTP, dTTP and dGTP made in deionised water (pH 7.0 with NaOH, autoclaved).
"M13 forward" primer:	100 ng/µl deionised water (pH 7.0 with NaOH, autoclaved). This primer has the sequence of 5'-GTA AAA CGA CGG CCA G-3'.
"M13 reverse" primer:	100 ng/µl deionised water (pH 7.0 with NaOH, autoclaved). This primer has the sequence of 5'-CAG GAA ACA GCT ATG AC-3'.

Procedure

Twenty microlitres of reaction master mix was prepared for "N" number of reactions by adding the following:

2 x N µl	10X reaction buffer
2 x N µl	25mM $MgCl_2$
2 x N µl	dNTPs
0.5 x N µl	forward primer
0.5 x N µl	reverse primer
0.5 x N µl	*Taq* polymerase
12.5 x N µl	deionised water (pH 7.0 with NaOH)

Aliquots of 20 µl of master mix were placed into each reaction tube. A small amount of a large, well-separated white colony was toothpicked directly into each individual PCR mix. PCR was run as follows: 95°C for 10 minutes, followed by 30 cycles of: 95°C for 30 sec, 55°C for 30 sec and 72°C for 2 minutes, followed by a 10 minutes incubation at 72°C. Following PCR, to confirm that the correct 16S rRNA gene was cloned, 5 µl of this PCR reaction was analysed using RFLP (section 2.5.5).

2.5.5 RFLP Analysis of Cloned 16S rRNA Genes

The purpose of this RFLP analysis was to confirm that the cloned DNA was the 16S rRNA gene of interest. This was determined by comparing the RFLP patterns of the cloned inserts to that of the authentic 16S rRNA gene obtained from the original PCR reaction.

For "N" number of reactions, the reaction mix was set up as follows:

5x N	µl	10X bufferB
2.5x N	µl	*Eco*RI
2.5x N	µl	*Msp*I
15x N	µl	deionised water (pH 7.0 with NaOH)

Five microlitres of the reaction mix was aliquot out to individual tubes and added to 5 µl of the newly made PCR reactions. Also, 5 µl of the original PCR reaction was added

to the last tube. The tubes were incubated at 37°C for 1-2 hours. The resulting products were analysed on a 3% agarose gel (section 2.5.2). The clone that yielded an identical RFLP to the product from the original PCR reaction was grown overnight in 3 ml LB (Luria-Bertani) medium containing 100 μg ampicillin/ml, and the plasmid DNA was purified using the Miniprep method (section 2.5.6). LB medium contained 10 g tryptone, 5 g yeast extract, and 10 g NaCl (in 1L dH_2O, pH adjusted to 7.0 with NaOH).

2.5.6 Miniprep of Plasmid DNA

The purification of plasmid DNA was carried out using the CONCERT™ Rapid Plasmid Purification Systems (GIBCO BRL®) according to the manufacturer's instruction. The following reagents were used in the purification procedures:

Reagent (all supplied with kit)

Cell Suspension Buffer (G1):	50 mM Tris-HCl (pH 8.0); 10 mM EDTA
RNase A:	20 mg/ml in Cell Suspension Buffer
Cell Lysis Solution (G2):	200 mM NaOH; 1% SDS
Neutralisation Buffer (G3):	Contains acetate and guanidine hydrochloride
Wash Buffer (G4):	Contains NaCl, EDTA and Tris-HCl (pH 8.0)
TE Buffer (TE):	10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA

Method

The positive transformant was grown overnight in an LB liquid medium supplemented with ampicillin (100 μg/ml), at 37°C. One to 5 ml of the culture was centrifuged and all medium was thoroughly removed. Two hundred and fifty microlitres of cell suspension buffer (G1) (containing RNase A) was added to the pellet and the cells were suspended until homogeneous. Two hundred and fifty microlitres of cell lysis solution (G2) was added and mixed gently by inverting before incubating at room temperature for 5 minutes. Three hundred and fifty microlitres of neutralization buffer (G3) was added and mixed immediately by inverting the tube, and then the mixture was centrifuged at

13,000 rpm (15,800 rcf) for 10 minutes. A cartridge was placed in a 2 ml wash tube, the supernatant loaded into a spin cartridge, which was then centrifuged at 13,000 rpm for 1 minute. The cartridge was placed back into the 2 ml wash tube and 700 µl of wash buffer (G4) was added, and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded before centrifuging the cartridge again at 13,000 rpm for 1 minute to remove the residual wash buffer. The cartridge was placed in a 1.5 ml recovery tube, 75 µl of warm TE buffer (TE) added directly to the centre of the spin cartridge, followed by incubation at room temperature for 1 minute, and finally centrifugation at 13,000 rpm for 2 minutes.

2.5.7 Sequencing of Cloned 16S rRNA Gene or PCR-Amplified 16S rRNA Gene

The plasmid DNA or PCR products were purified (sections 2.5.1 and 2.5.6) and sent to MWG-Biotech (Ebersberg, Germany) for sequencing.

2.5.8 Sequence Analyses and Phylogenetic Tree Assembly

The sequence data were compared with 16S rRNA gene sequences deposited in the Genbank database using the BLAST search program (Altschul *et al.*, 1997). The 16S rRNA gene sequences of various bacteria (including those closely related to the unknown sequences, as indicated from the BLAST search) obtained from the GenBank database were aligned with those of the new sequence using ClustalW program (Thompson *et al.*, 1994). These alignments were then used to construct a distance matrix (Jukes and Cantor, 1969), followed by phylogenetic tree construction by neighbour joining (Saitou and Nei, 1987). DNA parsimony analyses was also used for comparison. These algorithms were provided in PHYLIP version 3.5c (Felsenstein, 1993). Phylogenetic trees were viewed using Treeview software (Page, 1996).

2.5.9 Chromosomal DNA Extraction and Purification

The protocol used for the extraction of chromosomal DNA was that described by Wilson (1987).

Reagents

TE buffer:	10 mM Tris-HCl + 1 mM EDTA, pH 8.0.
SDS:	10% sodium dodecyl sulfate in deionised water.
Proteinase K:	20 mg proteinase K /ml deionised water.
RNAase:	10 mg RNAase A / ml 0.1 M sodium acetate (pH 5.2) heated to 100°C for 15 minutes to inactivate DNAases and allowed to cool slowly to room temperature before adding 0.1 volume of 1 M Tris-HCl (pH 7.4).
Sodium acetate:	3 M sodium acetate in deionised water, adjusted to pH 5.2 with glacial acetic acid
Phenol/Chloroform:	25 phenol: 24 chloroform: 1 isoamyl alcohol
Chloroform:	24 chloroform: 1 isoamyl alcohol
Isopropanol:	
70% Ethanol:	

Procedure

Cells in late exponential phase were harvested by centrifugation (15,000 rpm, 15 min, 4°C). The pellet was washed firstly with 10 mM H_2SO_4 to remove any ferric iron precipitates and then with TE buffer. The cell pellet was then resuspended in 567 µl TE buffer and added to 30 µl SDS solution and 3 µl proteinase K solution, followed by incubation for 1 hour (or until the solution was observed to clear) at 37°C without shaking.

Five hundred microlitres of the phenol: chloroform solution was added to the mixture and mixed gently but thoroughly by inverting the tube, followed by centrifugation (10,000 rpm, 5 min) to separate the phases. The upper aqueous layer containing DNA was removed to a new tube avoiding taking any of the white precipitate at the interphase (using a wide-bore pipette tip to avoid shearing the DNA.). Five hundred microlitres of the chloroform solution was added and mixed gently but thoroughly by inverting the tube, which was repeated until there was no white precipitate left at the interphase. The

DNA was precipitated with 70 µl sodium acetate and 500 µl isopropanol, by gently flicking the mixture until a stringy precipitate formed. The DNA was placed into a test tube with 1 ml 70% chilled ethanol. The tube was inverted several times, making sure that the pellet floated free of the bottom of the tube. The tube was centrifuged (10,000 rpm, 15 min) to recover the DNA as a pellet. The pellet was resuspended in 1 ml TE buffer and added to the RNAase solution (final concentration of 10 µg/ml) for several hours. The concentration of the DNA was measured spectrophotometrically at 260 nm, where an absorbance value of 1.0 is equivalent to 50 µg DNA/ ml dH_2O. This DNA solution was adjusted to the desired concentration by addition of TE buffer before further use.

2.5.10 DNA Purification by Caesium Chloride Gradient Centrifugation

Reagents

DNA Solution:	A previously prepared DNA solution (section 2.5.9) at a concentration of 50 –100 µg/ml TE buffer.
TE Buffer:	10 mM Tris-HCl + 1 mM EDTA (pH 8.0)
Sodium acetate:	3 M sodium acetate in deionised water, adjusted to pH 5.2 with glacial acetic acid.
Ethidium bromide:	10 mg/ ml dH_2O.
Saturated butanol:	deionised water was added to butanol until it was seen as a separate phase after mixing well.

Preparation of dialysis tubing

A length of tubing was heat sterilised (120°C, 20 min) in a beaker containing 1 mM EDTA, and was rinsed and stored at 4°C in a sterile bottle containing autoclaved deionised water.

Procedure

Caesium chloride (3.9 g) was added to 4 ml of the DNA solution in a plastic test tube and mixed gently until dissolved. Twenty microlitres of the ethidium bromide solution was added and mixed gently. The above solution was carefully put into a 4 ml sealable centrifuge tube using a Pasteur pipette to avoid any bubbles, until the tube was completely filled. The tubes were balanced using a caesium chloride solution (1.05 g CsCl/ ml dH_2O) and centrifuged in a Beckman VTi80 rotor overnight (55,000 rpm, 15°C). The DNA band was visible in plain light as a reddish orange band (due to the bound ethidium bromide). The DNA was removed without shearing by making a hole in the top of the tube and covering it with a finger, and then making another hole in the bottom of the tube under which a beaker was placed. By releasing the hole at the top, the DNA was collected in an Eppendorf tube. The ethidium bromide was extracted with an equal volume of water-saturated butanol until the butanol was no longer pink. The DNA was put into dialysis tubes and placed into a beaker of 150 ml of 0.1X SSC for about 1 hour, or until no more caesium chloride was seen running down from the dialysis bag. The tubes were then put into 250 ml 0.1X SSC overnight with gentle stirring. The DNA solution was transferred to a test tube and its concentration was measured on a spectrophotometer at 260 nm. An absorbance value of 1.0 at 260 nm was equivalent to 50 µg DNA/ ml dH_2O.

This DNA solution was adjusted to the desired concentration by addition of 0.1X SSC, or precipitated and dissolved in 0.1X SSC before further use. To precipitate the DNA, 1/10 volume sodium acetate solution and 1 volume isopropanol were added, and the solution gently mixed. The DNA was recovered by centrifuging. The DNA was then rinsed with 1 ml of 70% ethanol (chilled to 4°C) and recovered by centrifugation.

2.5.11 Determination of DNA Base Composition

The determination of G+C (mol%) composition of chromosomal DNA was carried out using the melting profile method adapted by Dr. P. R. Norris (University of Warwick, U.K).

Reagent

0.1X SSC: 15 mM NaCl, 1.5 mM trisodium citrate in dH_2O, autoclaved before use

Procedure

Caesium chloride gradient-purified and dialysed chromosomal DNA (section 2.5.10) was diluted to 20-30 µg DNA/ml 0.1X SSC. Using 0.5 ml quartz cuvettes (stoppered to prevent evaporation), the melting temperature was determined on a Hewlett Packard (HP) 8453A UV-visible spectrophotometer connected to a HP 89090A Peltier temperature controller. The DNA melting temperature was the temperature at which a 50% increase in A_{260} nm was obtained, and was calculated using a DNA melt software package (HP 845x). The base composition of the DNA was determined from its melting point, using the DNA melt software program of Marmur and Doty (1962):

$$G + C \text{ (mol\%)} = 2.44 [(T_m - 81.5) - (16.6 \log M)]$$

T_m = melting temperature of the DNA

M = molar concentration of the cations in 0.1X SSC (= 0.0195 M)

A standard curve for the measurement of G+C contents of microbial DNA samples was made using control DNA of other microorganisms (*Micrococcus luteus, Acidocella facilis*^{T}, *Acidocella aminolytica*^{T}, *Escherichia coli* strain B and Calf Thymus DNA). All the DNA except that from *Acidocella* spp. were obtained from Sigma Chemicals Ltd.. *Acidocella* DNA was prepared within the laboratory. The determined standard curve

was: Y (measured G+C contents) = 1.248X(expected G+C contents) – 7.528, Rval=0.99594.

2.5.12 Microbial Population Analysis by FISH (Fluorescent *In Situ* Hybridisation)

As *in situ* hybridisation is a very sensitive technique, it was important that all reagents used for FISH were free from all microbes. To ensure this, all reagents were filtered through 0.2 μm membrane filters into clean bottles prior to autoclaving.

Fixation of cells for FISH:

Reagents and solutions:

PBS (1X): 130 mM NaCl (7.6 g per litre)
10 mM $Na_2HPO_4 \cdot 12H_2O$ (3.58 g per litre)
3 mM $NaH_2PO_4 \cdot H_2O$ (0.46 g per litre)
pH 7.2 (adjusted with NaOH or HCl as necessary)
The solution was filtered through 0.22 μm membranes into a sterile container, autoclaved and stored at 4°C.

4% paraformaldehyde ("PFA") in PBS:

Thirty three millilitres of deionised water (filtered and autoclaved) was heated (to ~ 60°C), and 2 g of paraformaldehyde and one drop of 2 M NaOH were added. After stirring for about 2 minutes (when the PFA went into solution), 16.5 m1 of 3 x PBS was added and the resulting solution cooled. The solution pH was adjusted to 7.2 with NaOH or HCl. The solution was kept in small volumes (750 μl) at –20°C.

Absolute Ethanol

Oxalic acid: 500 mM (63.04 g per litre deionised water) solution, filtered through 0.22 μm membranes).

Fixation of samples containing bacteria and archaea with paraformaldehyde:

When using pyrite cultures, 1.5 ml pyrite culture was removed from bioreactor after approximately 5 minutes of vigorous stirring (300 rpm) for equal distribution of pyrite, put into an Eppendorf tube and immediately vortexed for 1 minute to detach microbes

from the pyrite surface. (As shown in Table 2.4, detached cell number was the greatest when the sample was vortexed for 1 minute without addition of Triton-X). Next, pyrite was removed from the solution by centrifuging the tube very gently at 5000 rpm (2300 rcf) for 10 seconds. One millilitre of the supernatant was transferred to another Eppendorf tube, and cells were harvested by centrifugation (13,000 rpm (15,800 rcf), 5 min). Cells were resuspended in 250 µl of ice-cold PBS and mixed with 750 µl PFA, and incubated at 4°C for 1-3 hours. In cases where iron phosphate precipitates formed after fixing the cells, a small amount of 500 mM oxalic acid solution was added until the solution once again cleared. Cells were harvested (twice) and washed in 1 ml PBS (ideally to a concentration of 10^8-10^9 cells/ml), and one volume of ice-cold ethanol added. Fixed cells were stored at –20°C until needed.

Table 2.4: Effect of vortex time and Triton-X concentration on number of detached microbes from pyrite surface using bioreactor culture containing *Leptospirillum* MT6 and *Sulfobacillus* Y004 (chapter 5).
The sample was taken from bioreactor after approximately 5 minutes of vigorous stirring (300 rpm) for equal distribution of pyrite. Aliquots (450 µl) of the pyrite cultures were put into Eppendorf tubes and 50 µl of Triton-X solutions (0, 0.01 and 0.05%) were added. The tubes were immediately vortexed (10 sec, 1 min or 2 min) and then centrifuged very gently (5000 rpm (2300 rcf) for 10 sec) prior to Thoma cell counting (N. Okibe, unpublished data).

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Preparation of microscopic slides for FISH and application of cells to the slides:

Reagents and solutions:

Ethanolic KOH
95 ml ethanol
5 ml deionised water that has been filtered (0.2 µm membranes)
10 g KOH

Gelatine solution
0.15 g gelatine
0.02 g chromium potassium sulfate 12-hydrate
200 ml deionised water
Heated to 70°C prior to use.

Ethanol series:
3 ethanol solutions of 50% (v/v), 80% (v/v) and 95% (v/v).

Procedure:

Slides were soaked in ethanolic KOH for 1 hour and rinsed well in filtered deionised water. Air-dried slides were placed in the gelatine solution at 70°C and held horizontally for about 10 seconds to evenly coat the slides. The slides were allowed to dry and stored in a sealed slide box at 4°C until needed. Five to ten microlitres of fixed sample (diluted with filtered deionised water if necessary) were spread on to the gelatine- coated slide and allowed to air dry. The slides were rinsed well by immersing into filtered deionised water twice. The sample smears were dehydrated by immersing slide into a series of solutions of 50%, then 80% and then 95% ethanol (3 min in each). When completely dry, the slides were ready for hybridisation.

Fluorescent *in situ* hybridisation (FISH):

Reagents and solutions:

Hybridisation buffer (2ml): 40 µl 1 M Tris/HCl (pH 7.4); 2 µl 10% SDS;
360 µl 5 M NaCl; deionised formamide; deionised water

Washing buffer (50 ml):
39.5 ml deionised water; 50 µl 10% SDS; 1 ml 1M Tris-HCl (pH 7.4); 0.5 ml EDTA (pH 8.0 with NaOH); 5M NaCl; deionised water

Stock DAPI solution: 1 mg DAPI/ml deionised water that had been filtered and autoclaved (stored at –20°C).

Mounting medium: 8.75 g (7 ml) glycerol was put into a small beaker and added to 0.38 g sodium tetraborate. When dissolved, the solution pH was adjusted to 9.2. 30 mg N-propyl gallate was then added, making sure that pH was still above 9.0. Finally, 3 ml deionised water was added. Mounting medium was stored for no more than 2 weeks in the dark at room temperature.

Procedure:

Two millilitres of hybridisation buffer was prepared according to the required stringency (Table 2.5) by adding the appropriate amount of deionised formamide. A tissue, soaked with about 1.8 ml of the hybridisation buffer was placed in a 50 ml conical test tube, which was allowed to equilibrate in a hybridisation oven (HIR4M, Grant Instruments, Cambridge) at 46°C for about 30 minutes. Each sample smear was added with 10 µl of hybridisation buffer containing 25 ng of each labelled probe and was covered with a coverslip. Slides were transferred, with smear side up, to the equilibrated tube in the hybridisation oven and incubated at 46°C for 2 hours. The cover slip was gently removed and the slide was immersed in pre-warmed (48°C) wash buffer of appropriate stringency (Table 2.5) at 48°C for 15 minutes. The slide was gently rinsed

with filtered deionised water and was allowed to air-dry. Ten microlitres of DAPI solution (1 µg/ml filtered deionised water) were added to the smear, and incubated in the dark at room temperature for 10 minutes. The slide was then washed twice by dipping into deionised water and air-dried in the dark. Ten microlitres of mounting medium was added, and a coverslip was placed on top of the smear.

The number of microbes on the slides was counted using a fluorescent microscope, ECLIPSE E600 (Nikon, Japan) (section 2.3.3). Photographs were taken using an attached digital camera, COOLPIX (MDC Lens 0.82-0.29x) (Nikon, Japan).

Table 2.5: Acidophile-specific oligonucleotide probes used in this study that target the 16S rRNA.

Probe Name	Target Organism	Sequence (5'-3')	T*m* (°C)	Formamide (%)	Ref.
EUB338Cy3*	Eubacteria	GCTGCCTCCCGTAGGAGT	60.5	10	Amann *et al.*, 1990
EUB338Fl*	Eubacteria	GCTGCCTCCCGTAGGAGT	60.5	10	Amann *et al.*, 1990
FER656	*Ferroplasma* spp.	CGTTTAACCTCACCCGATC	56.7	25	Edwards *et al.*, 2000b
LF655	*Leptospirillum* groups I, II and III[#]	CGCTTCCCTCTCCCAGCCT	63.0	35	Bond and Banfield, 2001
ACM995	*Am. ferrooxidans*	CTCTGCGGCTTTTCCCTCCATG	64.0	10	P. R. Norris, unpublished
THC642	*At. caldus*	CATACTCCAGTCAGCCCGT	58.8	25	Edwards *et al.*, 2000a

Table 2.5 foot notes:

* Two labelled versions of this probe, one with Cy3 and the other with FAM-6 (a fluorescein derivative that matches the GFP filter on the Nikon microscope) were used. The latter probe was used simultaneously with any other Cy3 labelled probe.

[#] The *Leptospirillum* grouping is based on phylogenetic analysis and group III is represented only by environmental clones from the Iron Mountain site (Bond *et al.*, 2000a).

Chapter 3

Characterisation of Novel Acidophilic Microorganisms Isolated from a Commercial Bioleaching Operation

3.1 Introduction

Moderately thermophilic microorganisms are attractive biological agents for mineral bioprocessing due to their often faster growth rates and oxidation of minerals, compared with mesophiles. In this study, several moderately thermophilic acidophiles isolated from a pilot plant aerated tank operation (Mintek, Randberg, South Africa; section 2.1) were characterised. Some of the Mintek isolates described in this chapter were also used in pure and mixed culture leaching experiments (chapters 4 and 5).

3.2 Isolation of Mintek isolates

To elucidate the microbial population in a commercial bioleaching operation, samples from a pilot plant aerated tank operation (Mintek, South Africa) using three in-line reactors were analysed by plating samples onto selective media (Johnson, unpublished data; Figure 3.1). The system consisted of a feed pulp tank and three reactors in series, with a container at the end for product collection. The concentrate was added at a pulp density of 7.5% at an overall 6-day residence time. The operation temperature was 45°C and the pH was controlled at levels below 1.8. The air supply to the reactors was enriched with CO_2 and supplied to the reactors by means of a sparger situated below the impeller. The ore being leached was a polymetallic concentrate containing Cu (22%), Fe (23%), Zn (8%), Pb (6%), Ag (3100ppm) and S^{2-} (30%) (Mariekie Gericke, Mintek; personal communication).

The designations of the six isolates studied, their characteristics, and routes of isolation are summarised in Table 3.1.

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Figure 3.1: Microbial populations in a pilot plant aerated tank operation using three in-line reactors (Mintek, South Africa).

Table 3.1: Description of Mintek isolates.

Isolate	Characteristics	Route of Isolation
MT1, MT2	Sulfur-oxidisers Moderately thermophilic rods *At. caldus*-like	Sample from Tank 1 (Figure 3.1) was serially diluted and directly spread on ferrous iron/tetrathionate overlay plates (section 2.2.1.2.1.2). Colonies appeared after 7 days incubation at 45°C.
MT6	Iron-oxidiser Moderately thermophilic, motile spirilla *Leptospirillum*-like	Sample from Tank 1 (Figure 3.1) was serially diluted and directly spread on ferrous iron overlay plates (section 2.2.1.2.1.1). Colonies appeared after 7 days incubation at 45°C.
NC	Iron-oxidiser Moderately thermophilic, spore-forming rods *Sulfobacillus*-like	Supposedly pure culture of *Leptospirillum* MT6 in pyrite concentrate media (section 4.2) was directly spread on ferrous iron/tetrathionate overlay plates (section 2.2.1.2.1.2). Colonies appeared after 3 days incubation at 45°C.
MT16, MT17	Iron-oxidisers Mesophilic-moderate thermophilic, irregular cocci *Ferroplasma*-like	Samples from Tank 3 (MT16) and Tank 2 (MT17) (Figure 3.1) were serially diluted and directly spread on ferrous iron/tetrathionate overlay plates (section 2.2.1.2.1.2). Colonies appeared after 14 days incubation at 45°C.

3.3 Determination of 16S rRNA gene sequences of Mintek isolates, and their phylogenetic affiliations

3.3.1 Methods

The isolates were grown in appropriate media (section 2.2.1) and harvested at early stationary phase. Amplification, cloning and sequencing of their 16S rRNA genes were carried out using methods described in sections 2.5.1-2.5.7. A phylogenetic tree was constructed using the determined 16S rRNA gene sequences of Mintek isolates and other published 16S rRNA gene sequences of representative microorganisms (section 2.5.8).

3.3.2 Results

The length of the 16S rRNA gene sequences determined, the most homologous organisms (%), and the Gen Bank submission numbers are listed in Table 3.2.

Table 3.2: 16S rRNA genes from the Mintek isolates.

Isolate	The length of the determined 16S rRNA gene	Accession numbers	Most homologous organism (accession number: homology %)
MT1	1462 bp	AF513711	*At. caldus*^{T} (Z29975: 99.5%)
MT2	496 bp, 559 bp, 468 bp (3 partial fragments)		99.6% with MT1 (1st 496bp)
MT6	1484 bp	AF513709	*L. ferriphilum*^{T} (AF356829: 99.5%)
NC	1438 bp	AY121610	*"Sb. yellowstonensis"* strain YTF1 (AY007665: 98.9%)
			[*Sulfobacillus* YTF3 (chapter 7) (AF507964: 99.6%)]
MT16	1401 bp	both isolates	*Fp. acidiphilum*^{T} (AJ224936: 99.6%)
MT17	1400 bp	AF513710	*"Fp. acidarmanus"* (849bp determined) (AF145441: 99.2%)

Isolates MT1 and MT2 were most closely related to *At. caldus*, with the homology of the 16S rRNA gene sequence (1462bp) of isolate MT1 being 99.5% to that of the type strain of *At. caldus* (strain KU). The 16S rRNA gene of isolate MT6 (1484 bp) and isolate NC (1438 bp) showed the highest homology to that of the type strain of *L. ferriphilum* (99.5%) and *"Sb. yellowstonensis"* YTF1 (98.9%), respectively. The 16S rRNA gene from the archaeon-like isolates MT16 and MT17 both had the highest homology with that of *Fp. acidiphilum*^{T} (99.6%). In addition, isolates MT16 and MT17 had 99.6% homology to each other.

The phylogenetic relationship of the novel isolates was determined using 16S rRNA gene sequences of other known acidophiles (Figure 3.2).

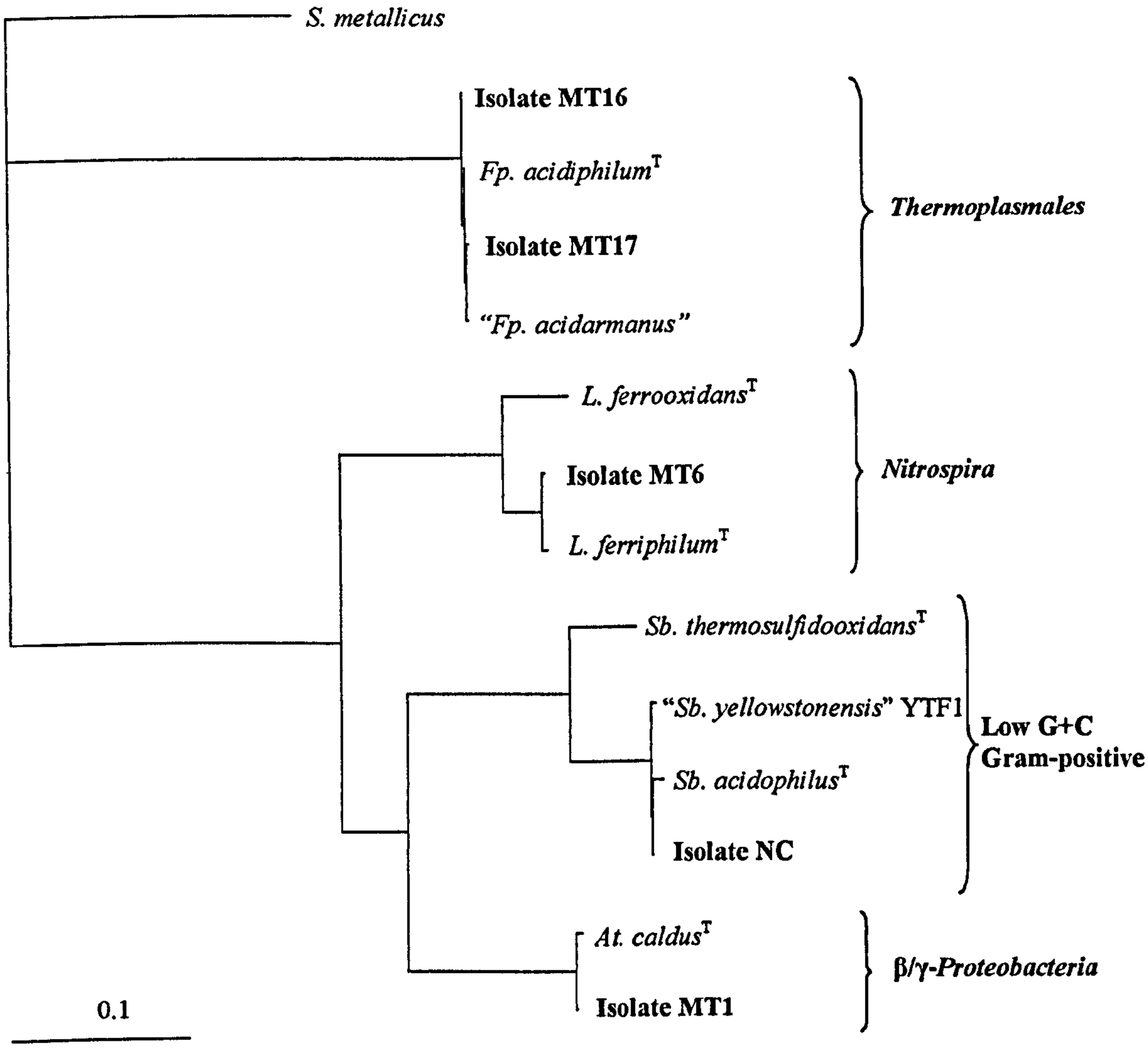


Figure 3.2: Phylogenetic relationships of the novel "Mintek" isolates (in bold) to known acidophilic prokaryotes. The phylogenetic tree was rooted with *S. metallicus*. The bar represents 0.1 nucleotides substitution per 100 for the horizontal branch lengths.

3.4 Determination of optimal pH and temperature of iron-oxidising isolates, *Leptospirillum* MT6 and *Ferroplasma* MT17

3.4.1 Methods

In order to determine the optimum pH and temperatures of Mintek isolates MT6 and MT17, bioreactors (section 2.2.1.3) containing either 1.5 L of ferrous iron medium (25 mM ferrous sulfate, pH 1.8; section 2.2.1.1.1) or "*Ferroplasma*" medium (including 0.0125% (w/v) TSB; section 2.2.1.1.5), were set up. Bioreactor cultures were maintained at an aeration rate of 0.5 l/min, and stirred at 100 rpm. To monitor growth of isolate MT6, the bioreactor culture was maintained at pH 1.8 for the optimum temperature analysis, and at 43°C for the optimum pH analysis. For isolate MT17, the bioreactor culture was maintained at pH 1.5 for the optimal temperature analysis, and at 37.5°C for the optimal pH analysis.

Samples were removed every 1-3 hours and ferrous iron concentrations in the cultures were determined using the ferrozine assay (section 2.4.2.2). Changes in the optical densities of culture were also monitored (section 2.2.2.1) in the case of isolate MT17. Following completion of iron oxidation, cultures were drained, leaving ~200 ml of spent medium, and fresh medium was added to make up the culture volume to ~1.5 L, prior to starting new experimental run. For isolate MT6, growth rates at temperatures between 35°C and 48°C were tested, and pH between 0.8 and 2.0. For isolate MT17, the temperature range was 30 to 50°C, and the pH range 0.55 to 1.85. In addition, the effect of dissolved solids on the growth of isolate MT17 was tested by changing the conductivity range of from 15.8 mS cm^{-1} to 61 mS cm^{-1} (at optimum pH and temperature). The conductivity of the medium was modified by addition of K_2SO_4 (0 mM to 300 mM) to the standard medium. Culture doubling times were calculated for

each experiment from semi-logarithmic plots of ferrous iron oxidised (or optical density) against time. Experiments were carried out in duplicate.

3.4.2 Results

The culture doubling times of isolates MT6 and MT17 at different pH and temperatures are shown in Figure 3.3 and Figure 3.4, respectively. The optimum temperature and pH of *Leptospirillum* MT6 were found to be 43°C and 1.5, respectively. Isolate MT6 was able to grow at pH 0.8 (pH <0.8 was not tested). The maximum temperature of the growth of isolate MT6 was 50°C. In the case of *Ferroplasma* MT17, the optimum temperature was 39°C and optimum pH was 1.5. Growth of isolate MT17 was observed at temperatures up to 47°C, where ferrous iron oxidation and biomass (OD_{600}) both increased exponentially (Figure 3.5). Although isolate MT17 did not grow at 50°C, ferrous iron oxidation was still observed, at the rate of 0.76 mM/h, but iron oxidation was no longer coupled to growth (Figure 3.5). Similarly, ferrous iron oxidation was found not to be coupled to growth of isolate MT17 at pH 0.55. In the medium conductivity range from 24 mS cm^{-1} to 46.2 mS cm^{-1}, there were no observable effects of soluble potential on culture doubling times (Figure 3.6). However, at 15.8 mS cm^{-1} (0 mM K_2SO_4) and at 61 mS cm^{-1} (300 mM K_2SO_4), it appeared that the cell growth was not coupled to ferrous iron oxidation and the doubling times determined from optical densities were much slower than that measured by ferrous iron oxidation (Figure 3.6).

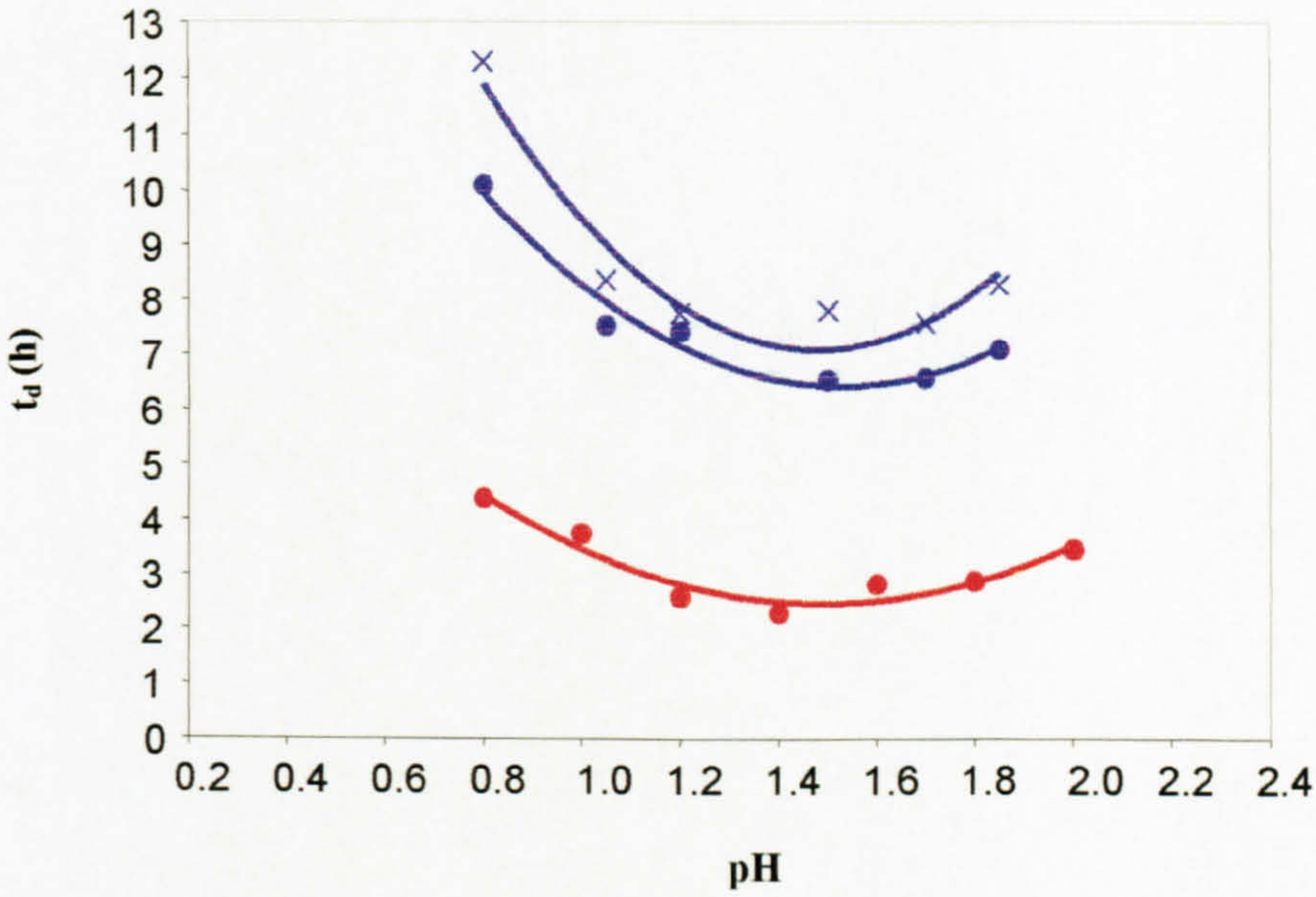


Figure 3.3: Effect of pH on the culture doubling times (t_d's) of *Leptospirillum* MT6 (at 43°C) and *Ferroplasma* MT17 (at 37.5°C). Key: •, isolate MT17 (based on ferrous iron oxidation); ×, isolate MT17 (based on OD_{600} measurements); •, isolate MT6 (based on ferrous iron oxidation).

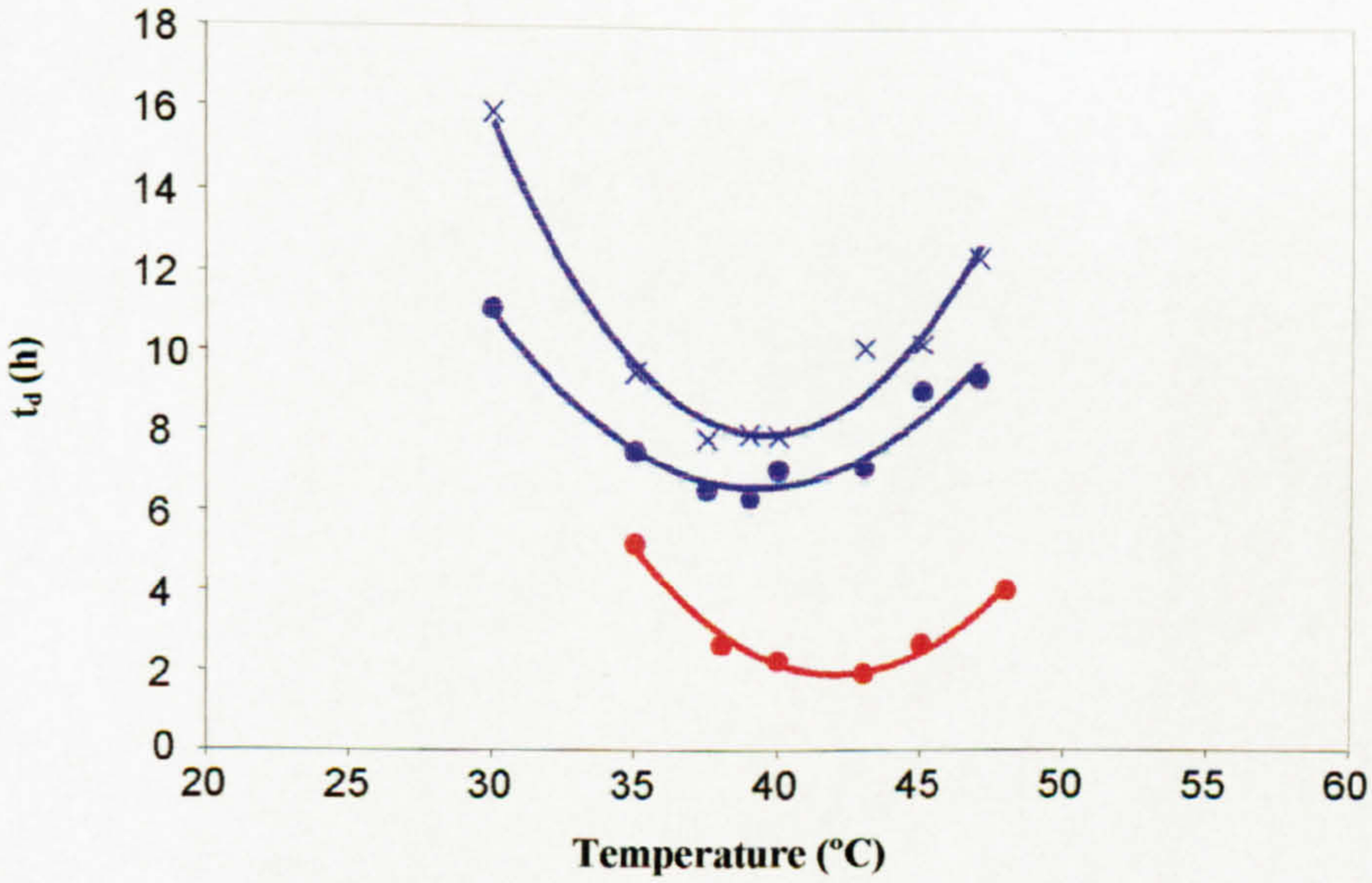


Figure 3.4: Effect of temperature on the culture doubling times (t_d's) of *Leptospirillum* MT6 (at pH 1.8) and *Ferroplasma* MT17 (at pH 1.5). Key: •, isolate MT17 (based on ferrous iron oxidation); ×, isolate MT17 (based on OD_{600} measurements); •, isolate MT6 (based on ferrous iron oxidation).

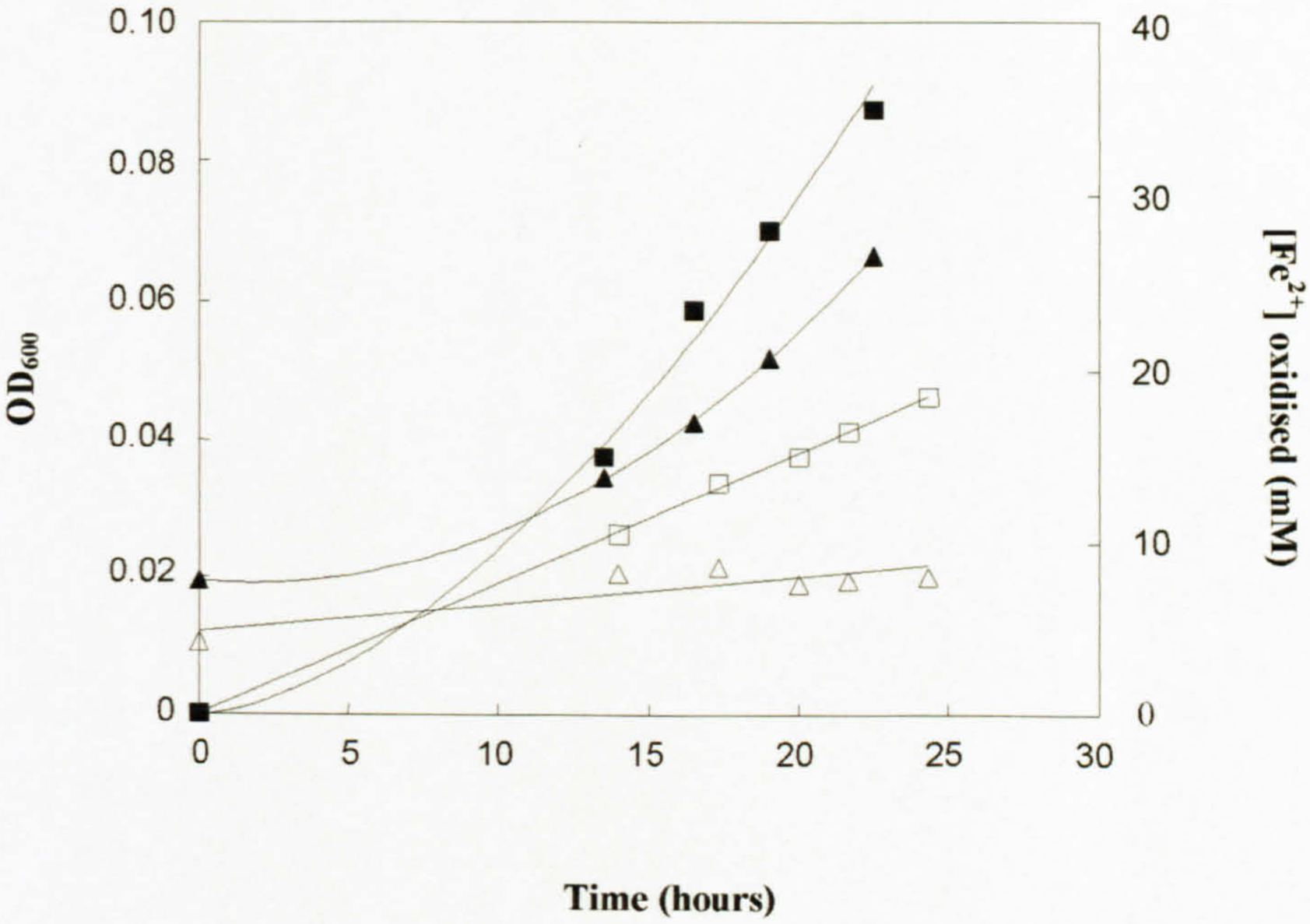


Figure 3.5: Growth (▲, △) and oxidation of ferrous iron (■, □) by *Ferroplasma* MT17 at 45°C (▲, ■) and at 50°C (△, □) (pH 1.5).

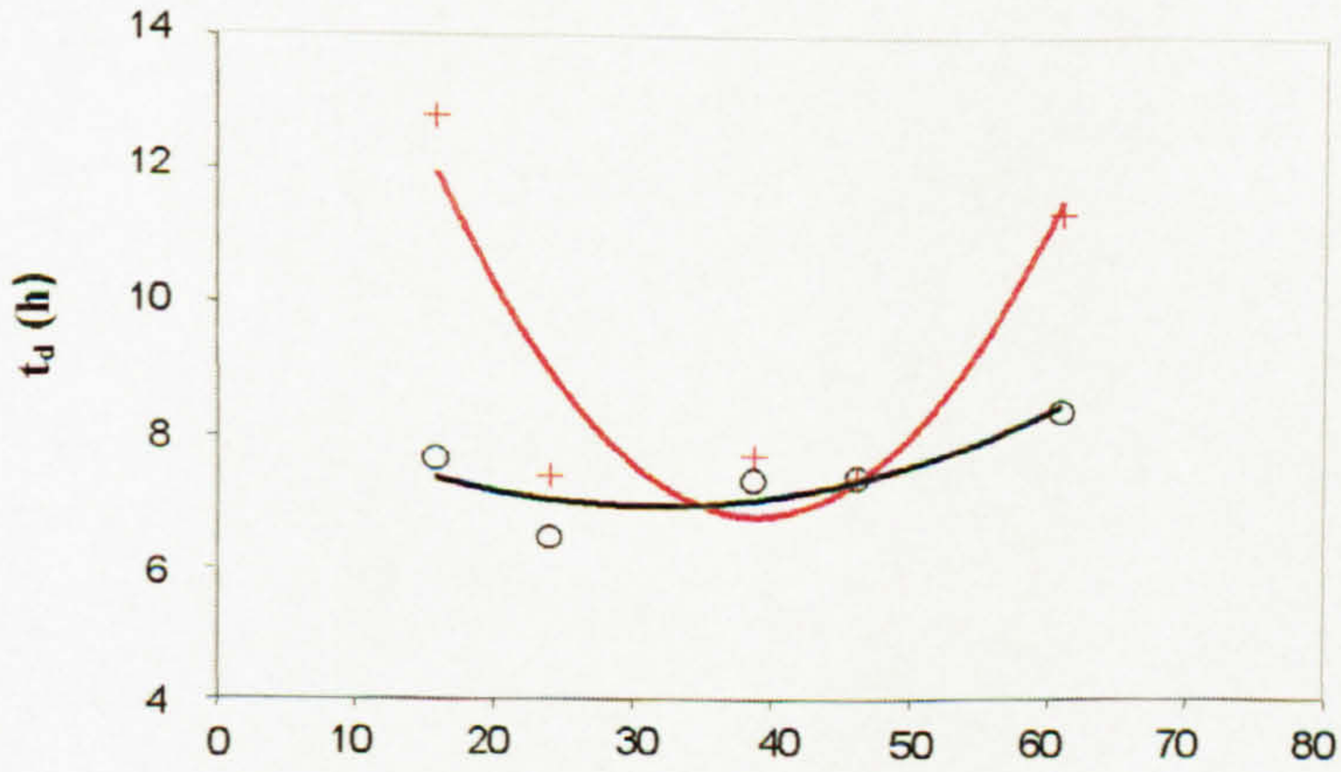


Figure 3.6: Effect of culture conductivity on the culture doubling times (t_d's) of *Ferroplasma* MT17 (at 39°C, pH 1.5). Key: +, as determined by OD_{600} measurements; ○, as determined by ferrous iron oxidation.

3.5 Analysis of chromosomal G+C contents of *Leptospirillum* MT6 and *Ferroplasma* MT17

3.5.1 Methods

Isolates MT6 and MT17 were grown in appropriate media (section 2.1.1) and cells were harvested. The chromosomal DNA was purified (section 2.5.9 and 2.5.10) and the G+C content of their chromosomal DNAs was determined (section 2.5.11).

3.5.2 Results

The G+C contents of the chromosomal DNA from *Leptospirillum* MT6 and *Ferroplasma* MT17 were determined as 55.2±0.3 mol% and 37.5±0.2 mol%, respectively.

3.6 Evaluation of liquid media for growth of *Ferroplasma* isolates MT16 and MT17

3.6.1 Introduction

The liquid medium used originally for subculturing isolates MT16 and MT17, referred as "SLM" (Standard Liquid Medium), contained 1X heterotrophic basal salts, trace elements, 0.02% yeast extract, 0.0125% tryptone soya broth (TSB), 50 mM $FeSO_4$ and 50 mM K_2SO_4 (final pH 1.5 adjusted with H_2SO_4); this medium was used for optimum pH, temperature and conductivity experiments (section 3.4). In further experiments, a number of variations of the standard liquid medium were prepared and tested for growth of these archaea.

3.6.2 Methods

One hundred millilitre flasks, each containing 50 ml of the following media (variations 1 and 2) were prepared and inoculated with isolates MT16 and MT17, pre-grown in SLM.

Variation 1

a. SLM (control)
b. SLM ($FeSO_4$ replaced with 5mM tetrathionate)
c. SLM (+ 10mM glucose)
d. SLM (+ 10mM glycerol)
e. SLM ($FeSO_4$ replaced with $Fe_2(SO_4)_3$)
f. SLM (-$FeSO_4$)

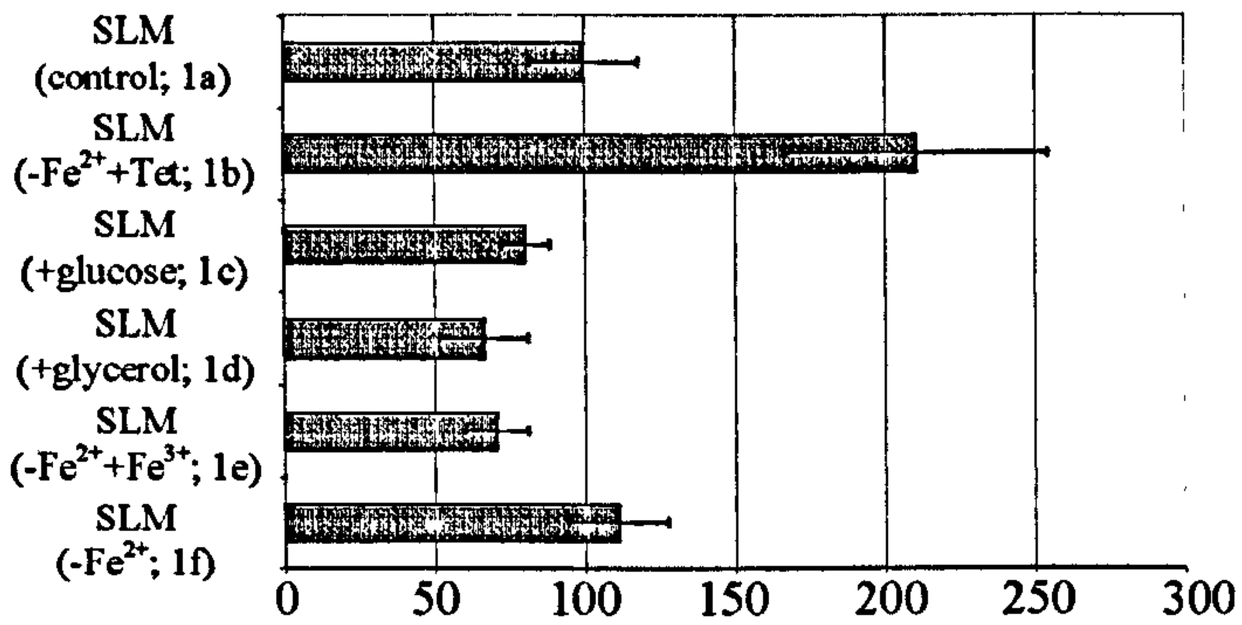
Variation 2

a. SLM (control)
b. SLM (-yeast extract, -TSB)
c. SLM (-TSB)
d. SLM (-yeast extract)

Flasks were incubated, shaken, at 37°C. Samples were removed and tested for growth by measuring protein concentration of cultures (section 2.4.6). Experiments were carried out in duplicate. Statistical analysis (ANOVA and Student-Newman-Keuls method) was made using the InStat programme (GraphPad, USA) and SPSS programme (SPSS Science, USA), respectively.

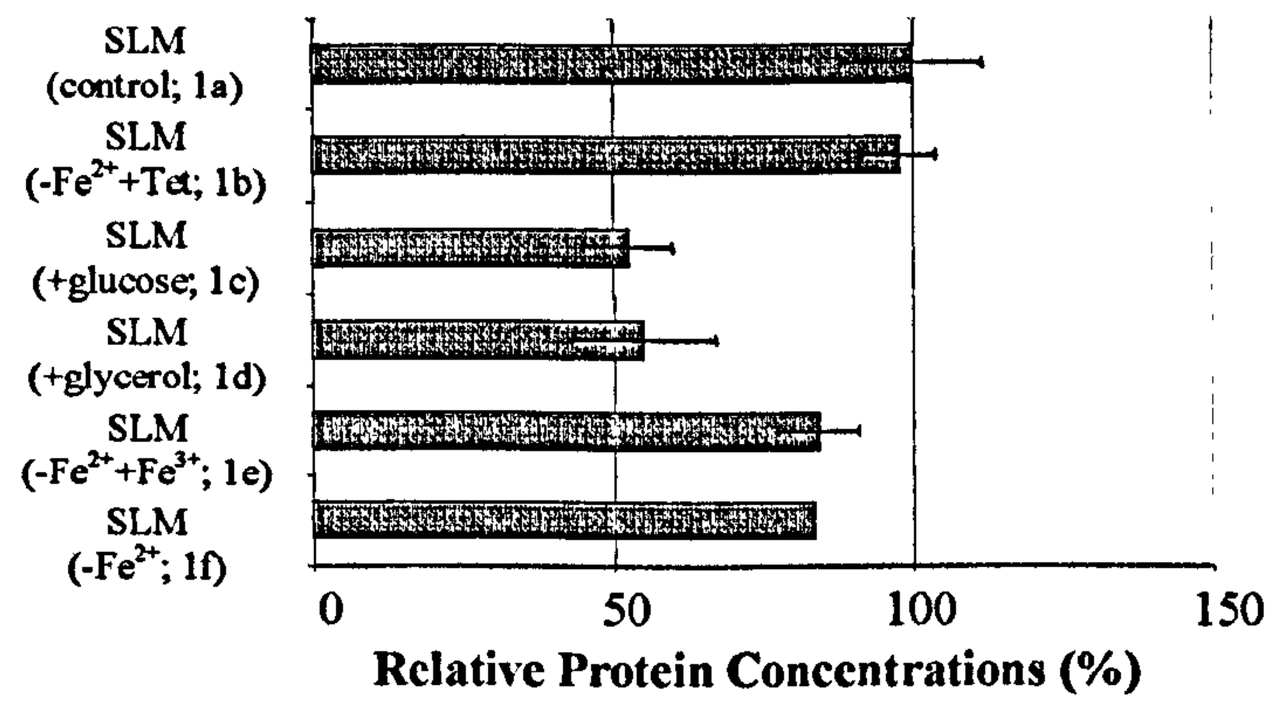
3.6.3 Results

Growth of isolates MT16 and MT17 in different media are shown in Figures 3.7–3.10. In these figures, the protein concentration of each medium was translated to a relative percentage of protein concentration (compared to those in standard SLM medium which were denoted as 100%). Figures 3.7 and 3.8 show relative protein concentrations of MT16 and MT17 cultures at 87 hours and Figures 3.9 and 3.10 show relative protein concentrations at 70 hours, where, in each case, cells were in early-stationary phase.



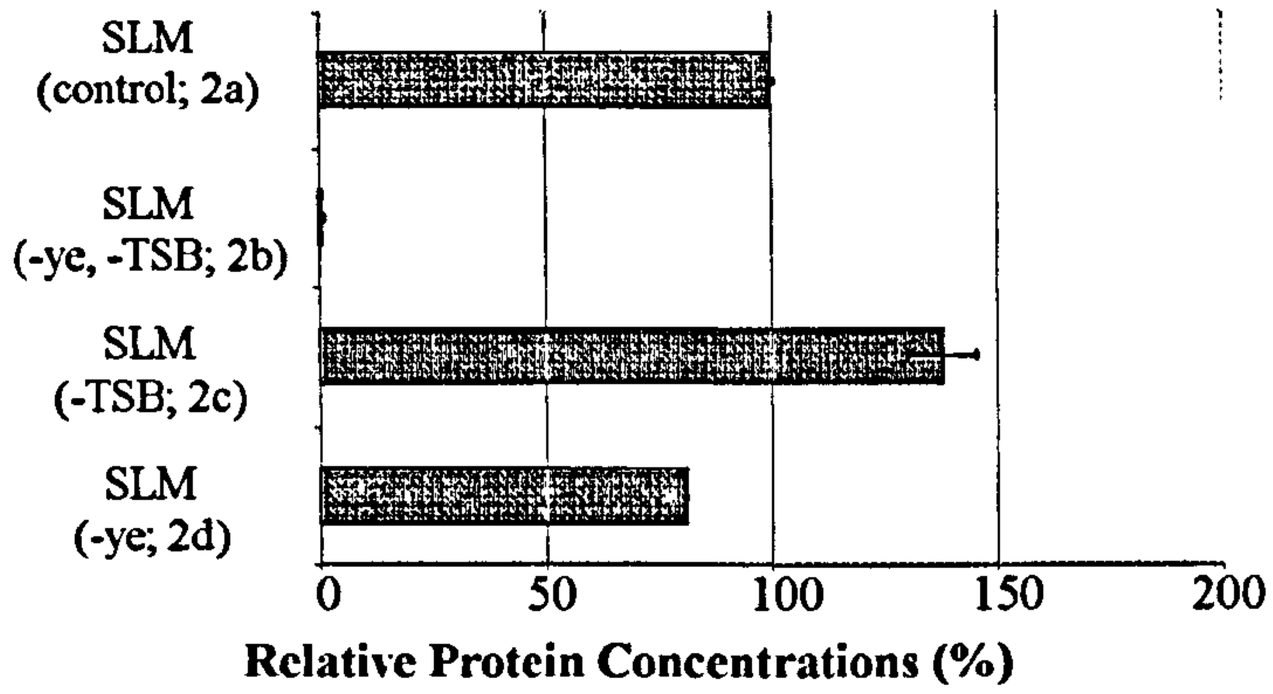
	1a	1b	1c	1d	1e	1f
1a		*	-	-	-	-
1b			*	*	*	*
1c				-	-	-
1d					-	-
1e						-
1f						

Figure 3.7: Comparison of growth yields of *Ferroplasma* MT16 in different liquid media. * indicate two values that are significantly different ($P<0.05$) whereas – indicate those that are not significantly different ($P>0.05$: Student-Newman-Keuls method).



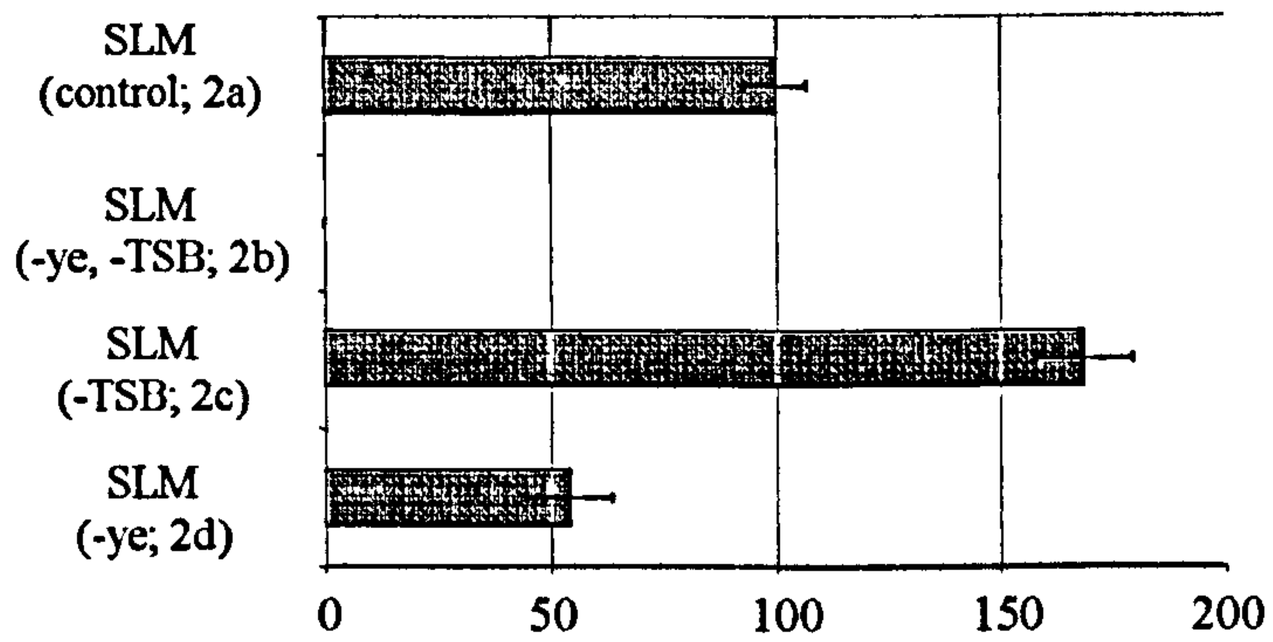
	1a	1b	1c	1d	1e	1f
1a		-	*	*	-	-
1b			*	*	-	-
1c				-	-	-
1d					-	-
1e						-
1f						

Figure 3.8: Comparison of growth yields of *Ferroplasma* MT17 in different liquid media. * indicate two values that are significantly different ($P<0.05$) whereas – indicate those that are not significantly different ($P>0.05$: Student-Newman-Keuls method).



	2a	2b	2c	2d
2a		*	*	-
2b			*	*
2c				*
2d				

Figure 3.9: Comparison of growth yields of *Ferroplasma* MT16 in different liquid media. * indicate two values that are significantly different ($P<0.05$) whereas – indicate those that are not significantly different ($P>0.05$: ANOVA).



	2a	2b	2c	2d
2a		*	*	*
2b			*	*
2c				*
2d				

Figure 3.10: Comparison of growth yields of *Ferroplasma* MT17 in different liquid media. * indicate two values that are significantly different ($P<0.05$: ANOVA).

Growth of isolates MT16 and MT17 occurred in all of the media tested, expect those where organic materials were omitted (Figure 3.7-3.10). Both isolates were able to grow heterotrophically without ferrous iron; replacing ferrous iron with ferric iron had little effect on biomass yields. Addition of glucose and glycerol had negative impacts on

growth yields of isolate MT17 ($P<0.05$). Addition of tetrathionate to SLM resulted in enhanced growth yields of isolate MT16, but not isolate MT17. Both isolates MT16 and MT17 showed significantly ($P<0.05$) greater growth yields in the absence of TSB and lesser growth yields in the absence of yeast extract. In the absence of organic materials, protein concentrations did not increase during culture incubation.

3.7 Oxidation of tetrathionate by *Ferroplasma* MT16 and MT17

3.7.1 Methods

One hundred millilitre flasks (in duplicate), each containing 50 ml SLM (with 5 mM tetrathionate instead of $FeSO_4$), were prepared. Flasks were inoculated with isolates MT16 or MT17 and incubated shaken at 37°C. Samples were removed and analysed for tetrathionate concentrations (section 2.4.4) and OD_{600} (section 2.2.2.1).

3.7.2 Results

It was found that, although ODs increased after ~20 hours, there were lag periods of 120 hours before both isolates started to oxidise tetrathionate (Figure 3.11 and 3.12). However, by 300 hours, almost all of the tetrathionate added had been oxidised by both isolates (though sulfate production was not measured and disappearance of tetrathionate may be due to production of thiosulfate); no decrease of tetrathionate concentration was observed in cell-free controls (data not shown).

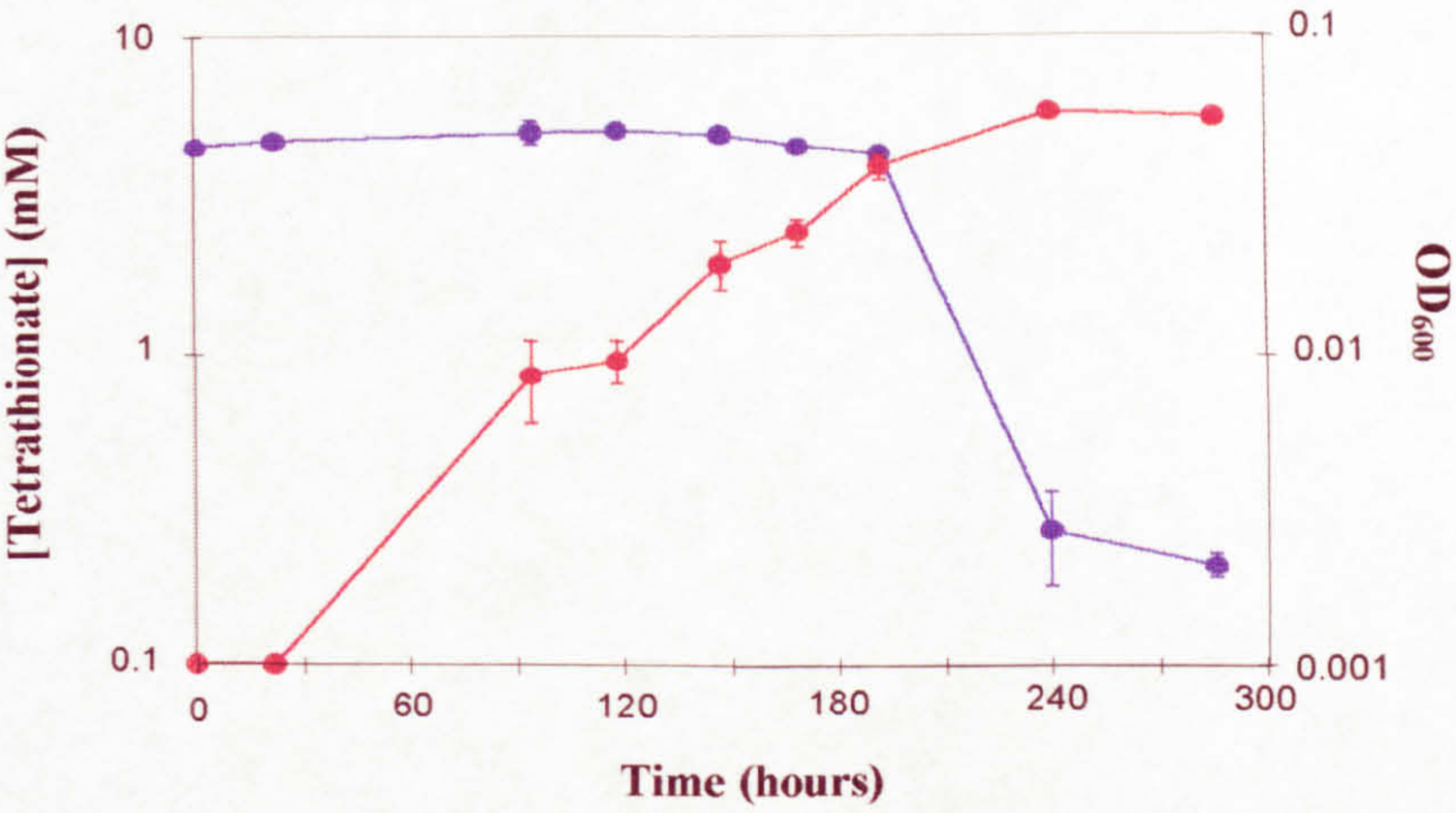


Figure 3.11: Growth (●) and oxidation of tetrathionate (●) by *Ferroplasma* MT16.

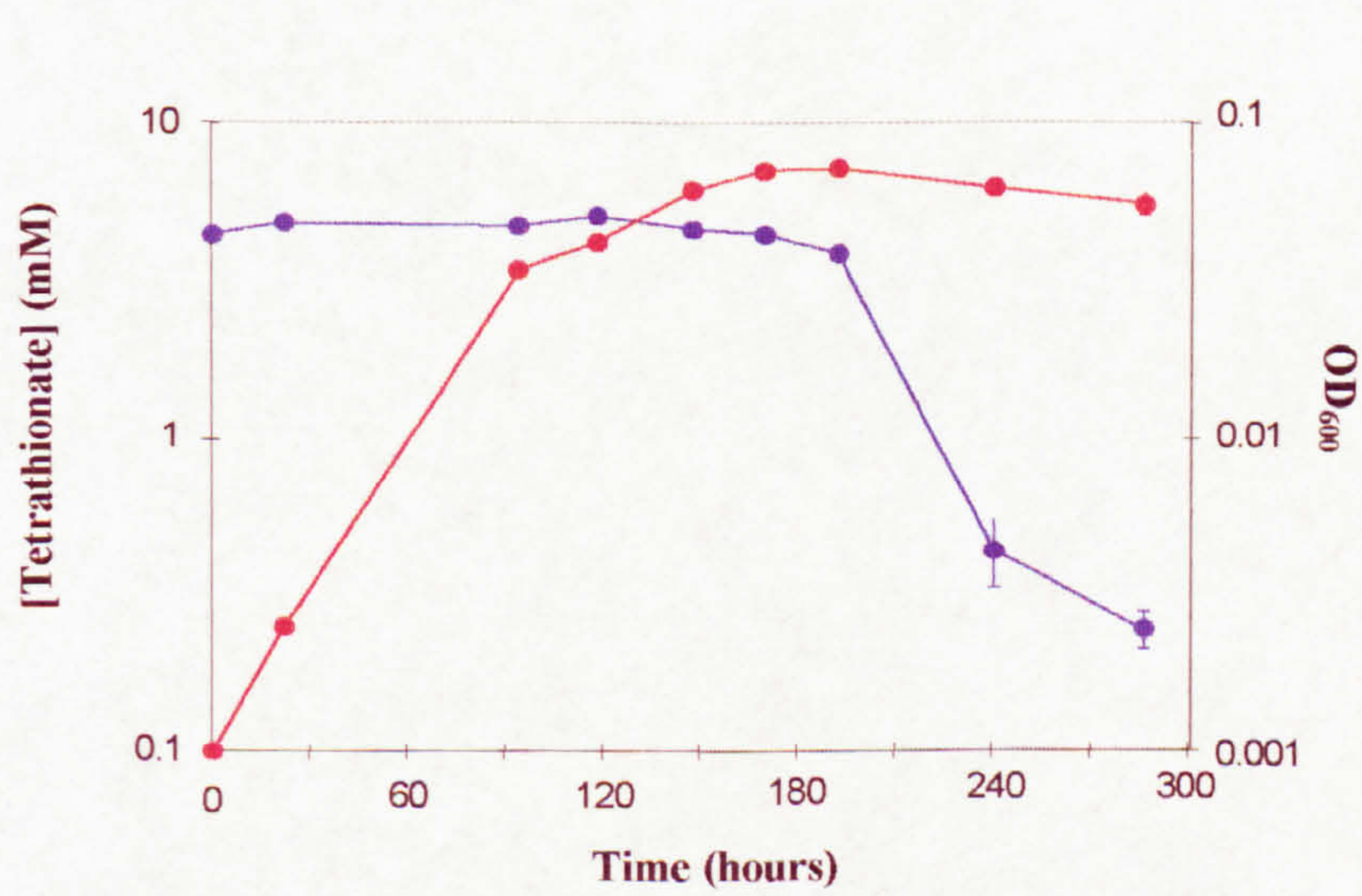


Figure 3.12: Growth (●) and oxidation of tetrathionate (●) by *Ferroplasma* MT17.

3.8 Effect of yeast extract on the growth of *Ferroplasma* MT16 and MT17

3.8.1 Methods

One hundred millilitre flasks, each containing 50 ml of "*Ferroplasma*" medium (section 2.2.1.1.5) amended with different concentrations of yeast extract (0.005, 0.01, 0.02, 0.05 and 0.1% w/v) were prepared. Flasks were inoculated and incubated, shaken, at 37°C. Samples were removed periodically and analysed for protein (section 2.4.6) and ferrous iron concentrations (section 2.4.2.2).

3.8.2 Results

The effects of yeast extract on the growth and iron oxidation by isolates MT16 and MT17 are shown in Table 3.3 and Figures 3.13 and 3.14.

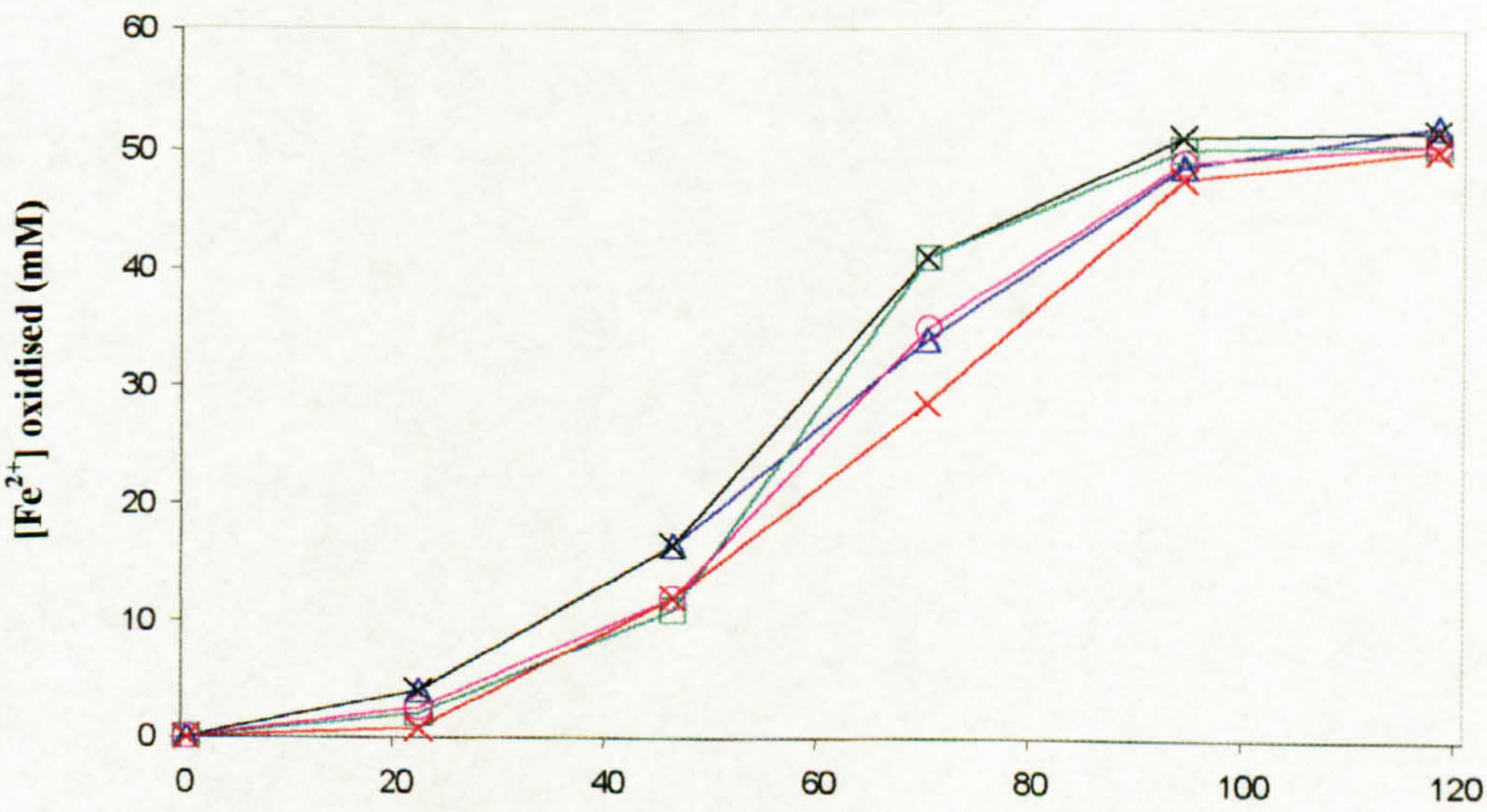


Figure 3.13: Effect of yeast extract on iron oxidation by *Ferroplasma* MT16. Key: △, 0.005%; ✕, 0.01%, ❑, 0.02%; ○, 0.05%; ✕, 0.1% yeast extract (w/v).

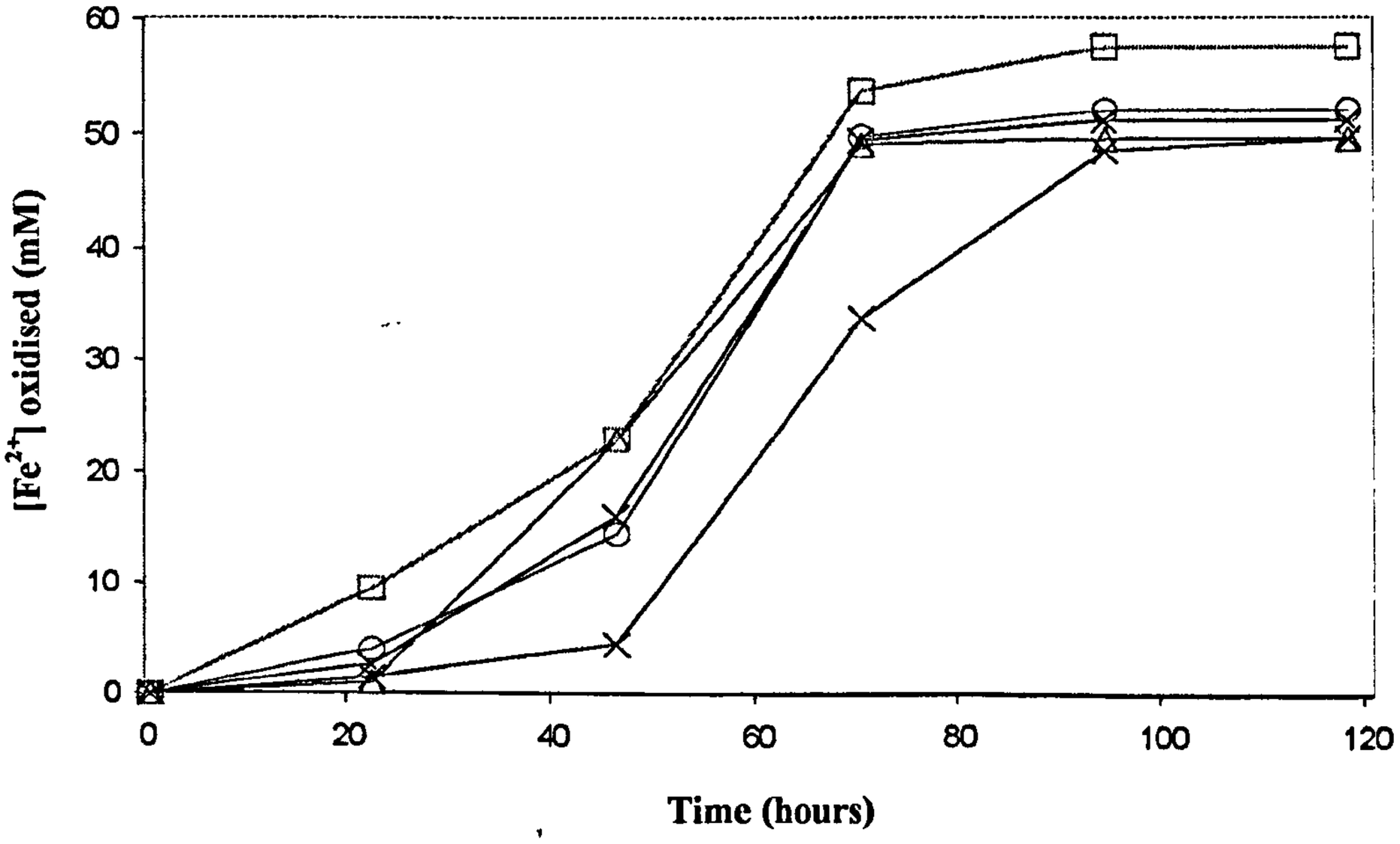


Figure 3.14: Effect of yeast extract on iron oxidation by *Ferroplasma* MT17. Key: △, 0.005%; ✕, 0.01%, ❑, 0.02%; ○, 0.05%; ✕, 0.1% yeast extract (w/v).

Table 3.3: Effect of yeast extract on growth yields of isolates MT16 and MT17.

[yeast extract] (%) (w/v)	[protein] (μg/ml)* MT16	MT17
0.005	4.0 ± 2.0	6.6 ± 0.4
0.01	5.5 ± 0.7	8.9 ± 0.3
0.02	12.6 ± 0.2	12.2 ± 1.2
0.05	10.8 ± 0.2	9.6 ± 0.6
0.1	13.3 ± 1.3	10.5 ± 0.9

* determined after 94 hours incubation.

There was no noticeable effect of yeast extract concentrations between 0.005 and 0.05% on ferrous iron oxidation by isolate MT16 (Figure 3.13). Iron oxidation by isolate MT17 was slower at the highest yeast extract concentration tested (0.1%; Figure 3.14). After 94 hours of incubation (late-exponential phase), protein concentrations in cultures varied depending on the initial yeast extraction concentrations. Growth yields of isolates MT16 and MT17 increased with yeast extract concentrations up to 0.02%. However, growth yields did not increase further when yeast extract was provided at >0.05% (w/v).

3.9 Potential utilisation of glucose and glycerol by *Ferroplasma* MT16 and MT17

3.9.1 Methods

One hundred millilitre flasks, each containing 50 ml of "*Ferroplasma*" medium (section 2.2.1.1.5) amended either with glucose, glycerol or unamended controls, were prepared. The final concentrations of glucose and glycerol were 5 mM and 10 mM, respectively. The flasks were inoculated with isolates MT16 or MT17, which had undergone several transfers through the same media. Flasks were incubated, shaken, at 37°C. Samples were removed at regular intervals and optical densities (OD_{600}; section 2.2.2.1) and ferrous iron concentrations (section 2.4.2.1) were determined.

3.9.2 Results

The effects of glycerol and glucose on growth of *Ferroplasma* MT16 and MT17 are shown in Figures 3.15 and 3.16. Compared to control flasks, growth yields of both isolates were greater in the presence of glucose, especially in the case of isolate MT16. Isolates MT16 and MT17 showed different responses to the addition of glycerol; growth of isolate MT16 was inhibited to some extent (ferrous iron oxidation slowed down) while the presence of glycerol slightly enhanced the rate of iron oxidation and growth yields of isolate MT17.

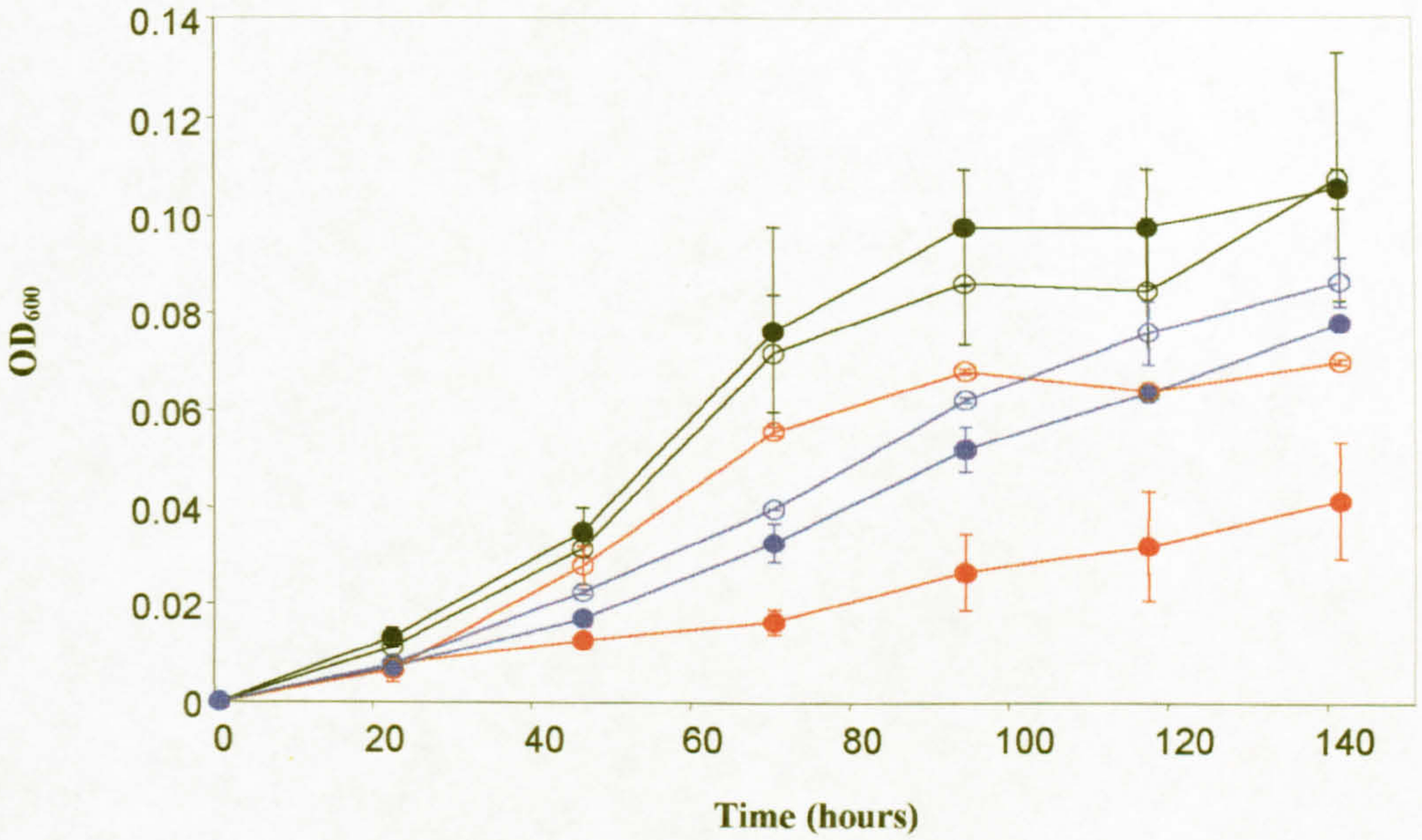


Figure 3.15: Effect of glycerol and glucose on the growth of *Ferroplasma* MT16 and MT17 (analysed by OD_{600}). Key: ●, MT16 +glucose; ❍, MT17 +glucose; ●, MT16 +glycerol; ❍, MT17 +glycerol; ●, MT16 control; ❍, MT17 control.

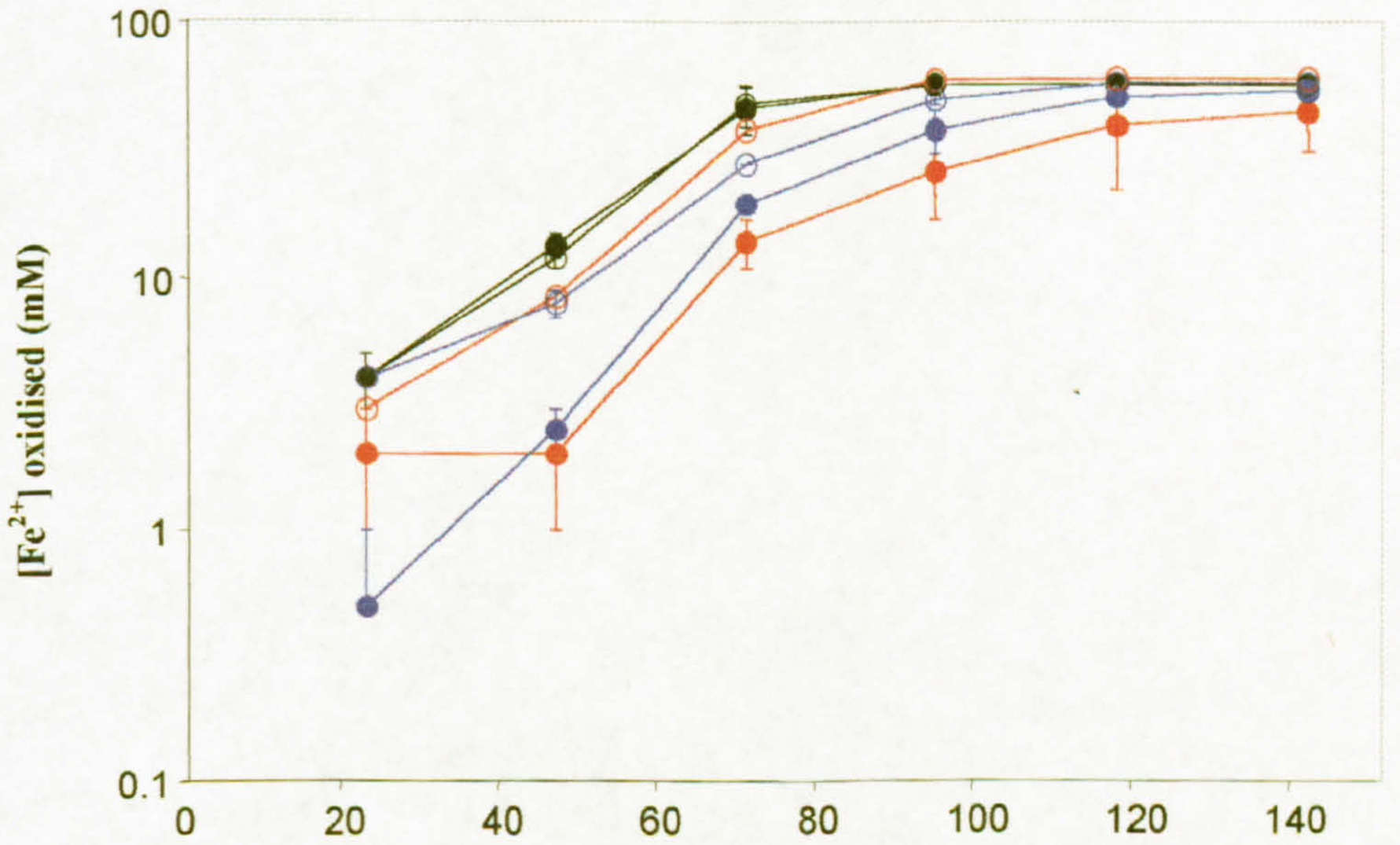


Figure 3.16: Effect of glycerol and glucose on iron oxidation by *Ferroplasma* MT16 and MT17 (analysed by Fe^{2+} oxidation). Key: ●, MT16 +glucose; ❍, MT17 +glucose; ●, MT16 +glycerol; ❍, MT17 +glycerol; ●, MT16 control; ❍, MT17 control.

3.10 Anaerobic growth of *Ferroplasma* MT16 and MT17 in the presence of glucose and ferric iron

3.10.1 Methods

"Ferroplasma" medium (section 2.2.1.1.5) containing 50 mM $Fe_2(SO_4)_3$ in place of $FeSO_4$ and amended with 5 mM glucose, was deoxygenated with N_2 gas and filtered through 0.2 μm cellulose nitrate membranes into sterile 20 ml anaerobic bottles (in triplicate). The bottles were then topped up with the inoculum until they were completely filled, and then sealed tightly with suba-seals. The inocula of isolate MT16 and MT17 used were pre-grown in the same medium in universal bottles under microaerobic condition (37°C, unshaken). The bottles were incubated, unshaken, at 37°C. Samples were withdrawn using a hypodermic syringe and tested for ferrous iron concentrations (section 2.4.2.2). After 75 hours, yeast extract (to 0.02% w/v) or glucose (to 5 mM) was added to two of the anaerobic cultures, whilst the third was unamended.

3.10.2 Results

Changes in ferrous iron concentrations during anaerobic incubation of these cultures are shown in Figure 3.17. Isolates MT16 and MT17 were both able to reduce ferric iron to ferrous iron, although at different rates. Isolate MT16 reduced ferric iron to a greater extent than isolate MT17. Addition of yeast extract or glucose (at 75 hours) did not affect ferric iron reduction by isolate MT17, whereas ferric iron reduction by isolate MT16 appeared to be stimulated by additional 0.02% (w/v) yeast extract.

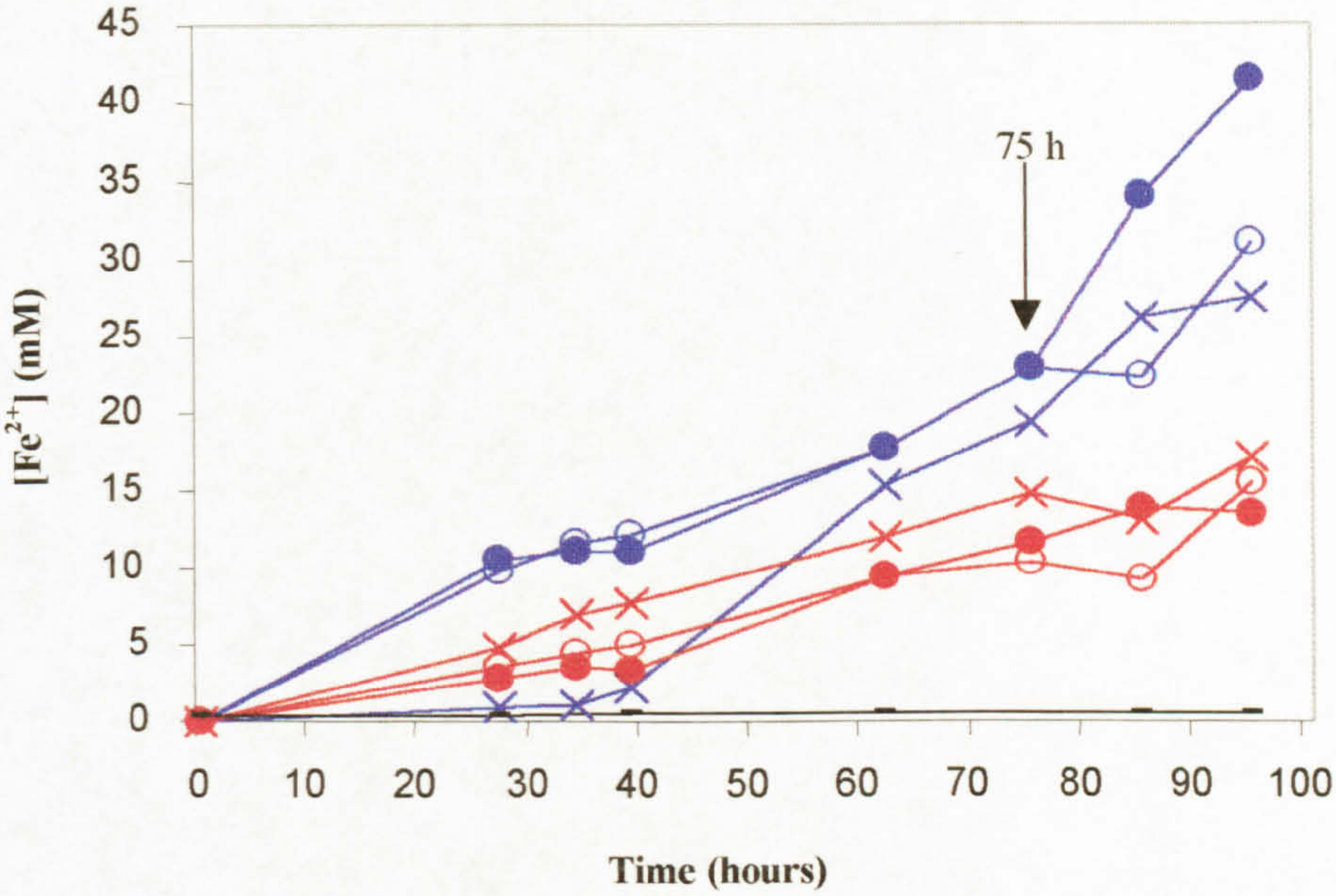


Figure 3.17: Changes in ferrous iron concentrations during anaerobic incubation of *Ferroplasma* MT16 (blue) and MT17 (red). Key: ● ●, 0.02 % yeast extract added at 75 hours; ○ ○, 5 mM glucose added at 75 hours; × ×, no additional organic carbon at 75 hours; -, cell-free control.

3.11 Pyrite oxidation by *Ferroplasma* MT17.

3.11.1 Methods

Isolate MT17 was pre-grown in "*Ferroplasma*" medium (section 2.2.1.1.5) at pH 1.5. Cells were harvested and resuspended in basal salts solution (pH 2.0) in order to remove residual organic materials. Six 100 ml flasks, each containing 50 ml of 2% pyrite medium (either Mintek pyrite concentrate or Cae Coch rock pyrite) adjusted to pH 2.0 were prepared (section 2.2.1.1.4). Two of the 3 flasks with each type of the pyrite were amended with yeast extract (0.02% (w/v), final concentration). The flasks were inoculated with pre-grown cells and incubated at 37°C, shaken. Samples were removed at regular intervals and soluble total iron was analysed (section 2.4.3).

In addition, *Ferroplasma* MT17 was tested for its ability to oxidise pyrite with an autotrophic sulfur-oxidising acidophile, *At. caldus*. Four 100 ml flasks, each containing 50 ml of 2% pyrite medium (either Mintek pyrite concentrate or Cae Coch rock pyrite) adjusted to pH 2.0 were prepared (section 2.2.1.1.4) and inoculated with a pre-grown mixed culture of *Ferroplasma* MT17 and *At. caldus* (strain KU). Samples were removed at regular intervals and soluble iron analysed (section 2.4.3).

3.11.2 Results

During 45 days of the experiment, no biologically-enhanced pyrite oxidation was observed in the yeast-extract amended *At. caldus* cultures (as expected), Mintek pyrite

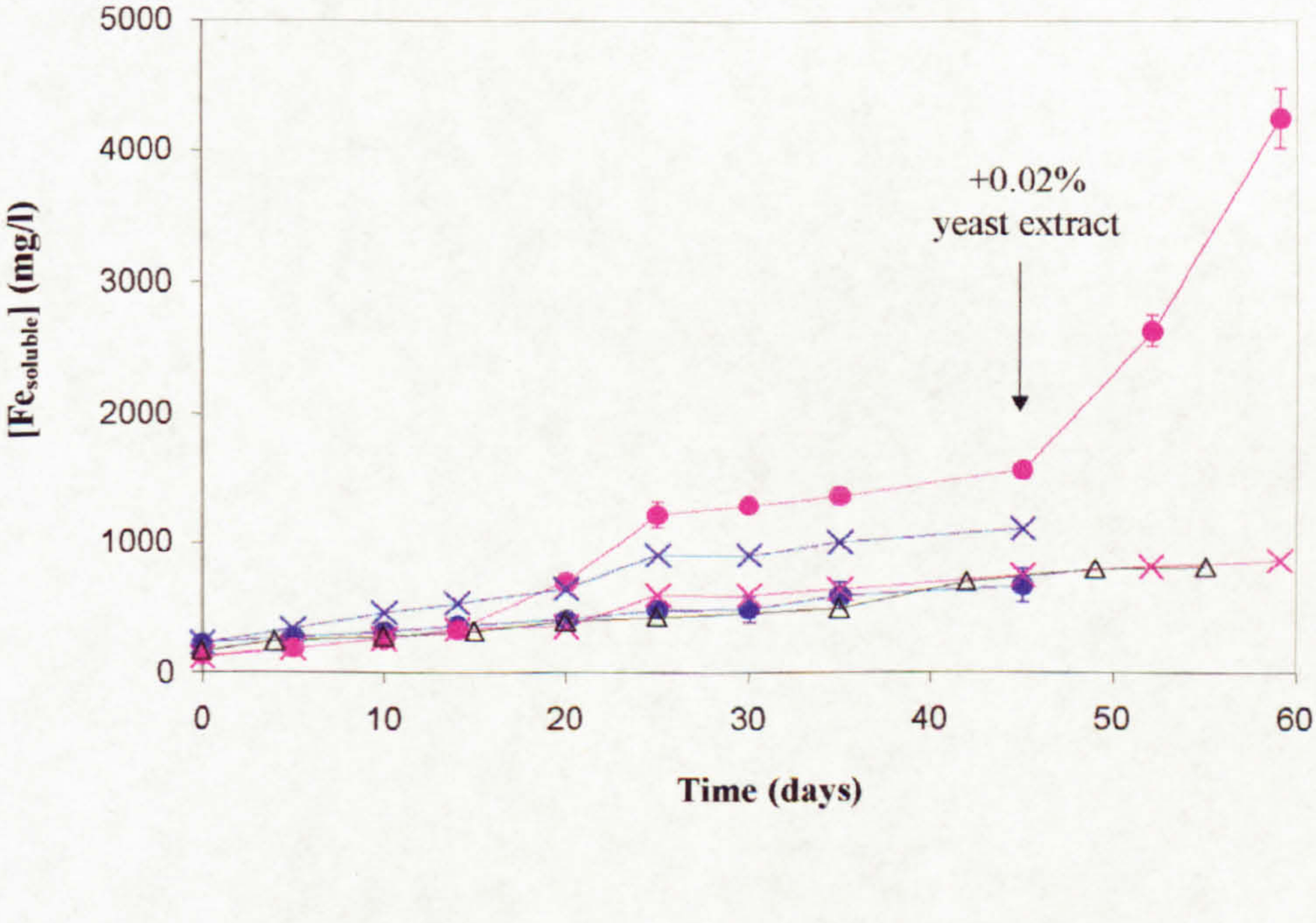


Figure 3.18: Oxidation of pyrite (Cae Coch rock pyrite and Mintek pyrite concentrate) by *Ferroplasma* MT17. Key: ●, Cae Coch rock pyrite (+0.02% yeast extract); × , Cae Coch rock pyrite (-yeast extract); ●, Mintek pyrite concentrate (+0.02% yeast extract); ×, Mintek pyrite concentrate (-yeast extract); △, Cae Coch rock pyrite (-yeast extract) "leached" by *At. caldus* (control). The arrow indicates addition of 0.02% yeast extract at day 45.

concentrate cultures and the single (yeast extract-free) Cae Coch rock pyrite culture (Figure 3.18). Although there appeared to be some oxidation of the Mintek pyrite concentrate by isolate MT17 in the absence of yeast extract, this particular culture was not replicated. Since pyrite oxidation was not apparent in either of the two replicate cultures of isolate MT17 grown on Mintek pyrite concentrates amended with yeast extract, it was concluded that this archaeon was unable to oxidise the pyrite concentrate.

With the Cae Coch rock pyrite cultures amended with yeast extract, oxidation started after about 15 days of lag period, but continued only for about 10 days (Figure 3.18). It was thought that this might have been due to limiting amounts of organic compounds. To examine whether additional yeast extract would re-stimulate pyrite oxidation, additional yeast extract (0.02% w/v) was added at day 45 to all of the Cae Coch rock pyrite cultures. This resulted in a stimulation of pyrite oxidation in the Cae Coch rock pyrite cultures that originally contained yeast extract, but not in those that, at the start of the experiment, did not contain yeast extract.

Mixed cultures of *Ferroplasma* MT17 and *At. caldus* KU did not show apparent enhanced oxidation of either the rock pyrite or the pyrite concentrate, relative to sterile controls (data not shown).

3.12 Discussion

Four apparently diverse moderately thermophilic microorganisms were isolated from an aerated tank bioleaching pilot plant operated by Mintek, South Africa. Sequence analysis of 16S rRNA gene revealed that isolates MT1, MT6, NC, and MT16/MT17 were most closely related to *At. caldus*^{T} (99.5%), *L. ferriphilum*^{T} (99.5%), "*Sb. yellowstonensis*" YTF1 (98.9%), and *Fp. acidiphilum*^{T} (99.6%), respectively. Isolates MT16 and MT17 had 99.2% homology also to "*Fp. acidarmanus*" although only a

partial (849bp) sequence is available (at the time of writing) for the 16S rRNA gene of this archaeon. In addition, isolates MT16 and MT17 had 99.6% homology to each other. Isolates MT6, MT16 and MT17 were characterised in greater detail, because of their novelty (isolate MT6 being a moderately thermophilic *Leptospirillum* sp., while isolates MT16 and MT17 were strains of the recently discovered archaeon genus, *Ferroplasma*).

Some characteristics of *Leptospirillum* MT6 and *Ferroplasma* MT17, in comparison to those of other known *Leptospirillum* spp. and *Ferroplasma* spp. are shown in Tables 3.4 and 3.5.

Table 3.4: Some characteristics of *Leptospirillum* spp. and isolate MT6 (Golovacheva *et al*., 1992; Hippe, 2000; Coram and Rawlings, 2002).

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Table 3.5: Some characteristics of *Ferroplasma* spp. and isolate MT17 (Golyshina *et al.*, 2000; Edwards *et al.*, 2000b).

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There were apparent differences in some characteristics of isolate MT6 and other *Leptospirillum* spp.. From analysis of 16S rRNA gene sequence homology, G+C content and pH responses, isolate MT6 appears to be more closely related to *L. ferriphilum* than to *L. ferrooxidans* though the number of *rrn* gene copies of isolate MT6 (a useful means to distinguish between these species) was not determined. However, there were some distinct differences between isolate MT6 and *L. ferriphilum* in their temperature responses; isolate MT6 was more thermotolerant than *L. ferriphilum* with optimum and maximum temperatures of 43°C and 50°C, respectively.

Another interesting difference between isolate MT6 and both *L. ferrooxidans* and *L. ferriphilum* is its much faster growth: culture doubling time of isolate MT6 under optimal conditions (43°C, pH 1.5) was about 2 hours (and <4 hours at 37°C, pH 1.5). These values are significantly less than the culture doubling times of both *L. ferrooxidans* and *L. ferriphilum* of 12-15 hours at 37°C (Coram and Rawlings, 2002). At its optimum temperature (43°C), the culture doubling times of isolate MT6 did not vary greatly between pH 0.8 and pH 2.0, suggesting that this isolate has a wide pH range in which it grows well, and that it may well be able to grow at pH values <0.8 (the lowest value tested in the present work).

From its temperature-response characteristics, isolate MT6 is similar to *L. thermoferrooxidans*. However, isolate MT6 is much more acidophilic than *L. thermoferrooxidans* (minimum pH of isolate MT6 <0.8, whereas that of *L. thermoferrooxidans* was found to be 1.3). It would be interesting, from a classification view point, to carry out further work on *L. thermoferrooxidans*, but this bacterium has unfortunately been lost (Johnson, 2001).

Further work, for example examining DNA:DNA homologies, would be useful in elucidating the relatedness of isolate MT6 to other *Leptospirillum* spp..

Although the 16S rRNA gene sequence of isolate MT17 was 99.6% homologous to that of *Fp. acidiphilum*^{T}, there were some differences in their characteristics (e.g., their capacities for tetrathionate oxidation and heterotrophic growth). Also, isolate MT17 was slightly more thermophilic (with optimum and maximum temperatures of 39°C and 47°C, respectively) and much more acid-tolerant (with optimum and minimum pH of 1.5 and <0.8, respectively) than *Fp. acidiphilum*^{T}. The G+C content of the chromosomal

DNA from isolate MT17 was 37.5 mol%, which is similar to that (36.5 mol%) recorded for *Fp. acidiphilum*^{T} (Golyshina *et al.*, 2000).

In addition, the 16S rRNA gene sequence of isolate MT17 was 99.2% homologous to "*Fp. acidarmanus*", though only 849bp 16S rRNA gene has apparently been determined for this acidophile. Isolate MT17 is similar to "*Fp. acidarmanus*" (but not to *Fp. acidiphilum*) in being capable to grow heterotrophically on yeast extract. "*Fp. acidarmanus*" is even more acid-tolerant than isolate MT17 and growth has been observed in media as low as pH 0 (Edwards *et al.*, 2000b). Isolate MT17, *Fp. acidiphilum* and "*Fp. acidarmanus*" showed some differences in physiological traits, but they are phylogenetically very closely related and they may be strain variations of the same single species.

It was indicated in some preliminary experiments that isolates MT16 and MT17 were not capable of utilising either glucose or glycerol as carbon sources. However, after a series of subculturing in glucose/glycerol-containing media, growth yields of both isolates were enhanced by the presence of glucose, especially isolate MT16. However, differences in OD_{600} values for cultures grown with and without glucose suggest that only a small proportion of the glucose provided (5 mM) was actually utilised by the isolates. Growth of isolate MT16 was inhibited to some extent by glycerol. Although it was reported that *Fp. acidiphilum* does not utilise organic compounds, including glucose and glycerol (Golyshina *et al.*, 2000), these results suggest that isolate MT16 and isolate MT17 may be able to grow, after a series of subculturing, on defined organic compounds, such as glucose.

Isolates MT16 and MT17 were able to reduce ferric iron to ferrous iron in anaerobic cultures. However, since no measurements were made of microbial biomass in this experiment, it cannot be concluded at this stage that these *Ferroplasma* isolates can

grow anaerobically by ferric iron respiration. Likewise, although it was found that both Mintek *Ferroplasma* isolates can oxidise tetrathionate (in apparent contrast to *Fp. acidiphilum*) it was not ascertained that these archaea can gain energy from oxidising reduced sulfur compounds. Given the obligate heterotrophic nature of these isolates, it would be necessary to limit the amount of organic carbon in cultures if energy gains (in terms of increased biomass) from tetrathionate oxidation were to be assessed.

Ferroplasma MT17 displayed similar pyrite-oxidising characteristics to *Leptospirillum* MT6, in that it was able to oxidise the rock pyrite (from the Cae Coch mine) but not the pyrite concentrate obtained from Mintek. This may be due to the sensitivities of both isolates to residual flotation chemicals present in the mineral concentrate (chapter 6). Although *Ferroplasma* MT17 was able to oxidise pyrite in pure culture, this was only found in yeast extract-amended cultures, again confirming that (like *Fm. acidophilum*) *Ferroplasma* spp. are iron-oxidising heterotrophic acidophiles. Bacelar-Nicolau and Johnson (1999) found that mixed cultures of the sulfur oxidising mesophiles *At. thiooxidans* or *A. acidophilum* and *Fm. acidophilum* T-23 were able to oxidise pyrite, though pure cultures of these acidophiles did not. A hypothesis was presented whereby organic carbon originating from the sulfur-oxidisers (which utilised the reduced sulfur compounds formed as intermediate products of pyrite oxidation) was utilised by heterotrophic *Fm. acidophilum*, which then continued to generate ferric iron from ferrous iron, which in turn oxidised the pyrite. In theory, a similar mutualistic relationship might occur between *Ferroplasma* spp. and *At. caldus*; the fact that it did not might indicate that either insufficient or inappropriate organic materials (for *Ferroplasma* MT17) originated from the sulfur-oxidiser. Mixed cultures including *Ferroplasma* MT17, were also used in pyrite oxidising experiments in bioreactors (chapter 5).

Chapter 4

Biooxidation of Pyrite by Defined Mixed Cultures of Moderately Thermophilic Acidophiles: Shake Flask Experiments

4.1 Introduction

In recent years, interest in biomining has focused more sharply on the use of thermophilic acidophiles, due to their abilities to enhance rates of mineral leaching and also to more effectively oxidise more recalcitrant minerals such as chalcopyrite ($CuFeS_2$). For example, a moderately thermophilic culture is used commercially in the 'BacTech' process (Miller, 1997). Commercial bioleaching operations have traditionally utilised non-defined microbial populations: Indeed, the microbiological composition of ore leaching systems is mostly unknown, although, more recently, this anomaly has began to be addressed by some research groups (Pizarro *et al.*, 1996; Norris *et al.*, 2000).

Moderately thermophilic acidophiles include various Gram-positive and Gram-negative bacteria, archaea and the rhodophyte *Cyanidium caldarium* (Doemel and Brock, 1971). Some of these microorganisms display synergistic interactions when oxidising minerals, such as *Sb. thermosulfidooxidans* and *At. caldus* (Dopson and Lindström, 1999) and *Sulfobacillus* spp. and *Am. ferrooxidans* (Clark and Norris, 1996a). Enhanced mineral leaching by mixed moderately thermophilic populations may result from oxidation of sulfur layers on mineral surfaces (e.g. by *At. caldus*) or by carbon transfers/interactions (Clark and Norris, 1996a; Dopson and Lindström, 1999). The possibility exists, therefore, that by control of microbial populations in bioreactor tanks, enhanced rates and efficiencies of mineral oxidation may be achieved. In this chapter, pure and mixed

cultures of moderately thermophilic bacteria were tested for pyrite oxidation in shake flasks.

4.2 Biooxidation of pyrite by pure and mixed cultures of four moderately thermophilic acidophiles

4.2.1 Methods

The following bacteria were used in preliminary pyrite leaching experiments: (i) a thermotolerant *Leptospirillum* (MT6); (ii) *At. caldus* KU; (iii) isolate GSM; (iv) *Sulfobacillus* NC. Replicate conical flasks (250 ml), each containing 100 ml of 2% pyrite medium (Cae Coch rock pyrite or Mintek pyrite concentrate, pH 2.0; section 2.2.1.1.4) were autoclaved and inoculated (2%, v/v) with either cultures or various combinations of mixed populations of moderately thermophilic acidophiles, all of which had been pre-grown in 2% pyrite medium (Cae Coch rock pyrite, pH 2.0). Flasks were incubated at 45°C, shaken, at 130 rpm, and samples were removed at regular intervals for determination of total soluble iron (section 2.4.3), ferrous iron (section 2.4.2.2), dissolved organic carbon (DOC) (section 2.4.7), culture pH and redox potentials (section 2.4.1), and microbial populations by plate counts (sections 2.2.1.2.1.1 and section 2.2.1.2.1.2). For plating, serial dilutions were made using well-mixed cultures to ensure equal distribution of pyrite/pyrite concentrate.

4.2.2 Results

4.2.2.1 Oxidation of pyrite by pure cultures of moderate thermophiles

Oxidation of the pyrite concentrate by pure cultures of the four moderate thermophiles and of rock pyrite by *Leptospirillum* MT6 is shown in Figure 4.1. No biologically-enhanced oxidation was observed in cultures of *At. caldus* or *Leptospirillum* MT6 (cell-

free control cultures with rock pyrite or pyrite concentrate showed similar trends in concentrations of soluble iron to pure cultures of *At. caldus* or *Leptospirillum* MT6; data not shown). In contrast, after a lag period of about 15 days, *Leptospirillum* MT6 oxidised rock pyrite very effectively (Figure 4.1). The two Gram-positive bacteria, *Sulfobacillus* NC and isolate GSM displayed similar (though relatively slow) rates of oxidation of the pyrite concentrate.

4.2.2.2 Oxidation of pyrite by mixed cultures of moderate thermophiles

Oxidation of pyrite concentrate by various combinations of moderately thermophilic bacteria is shown in Figure 4.2. In this experiment, *Sulfobacillus* NC was inadvertently introduced into some mixed cultures as a contaminant. *Sulfobacillus* NC appeared in supposedly pure cultures of *Leptospirillum* MT6 that have been grown on "autoclaved" pyrite concentrate. The iron-grown cultures of *Leptospirillum* MT6 did not contain these *Sulfobacillus*, therefore the implication was that this Gram-positive acidophile had survived (presumably as endospores) the autoclaving process.

With the exception of the mixed cultures of *At. caldus* and *Leptospirillum* MT6, all of the mixed cultures catalysed the oxidative dissolution of pyrite concentrate, though at different rates. In this experiment, the most effective oxidative dissolution of pyrite was seen with the mixed cultures of *Leptospirillum* MT6 and *Sulfobacillus* NC. Where either *At. caldus* or isolate GSM (or both of these) were present with *Leptospirillum* MT6 and *Sulfobacillus* NC, slightly slower rates of pyrite dissolution were observed. In all mixed cultures that included isolate GSM (but excluded *Sulfobacillus* NC) rates of oxidation were essentially the same as each other and very similar to those of pure cultures of isolate GSM (and much slower than in mixed cultures containing *Sulfobacillus* NC; Figure 4.2).

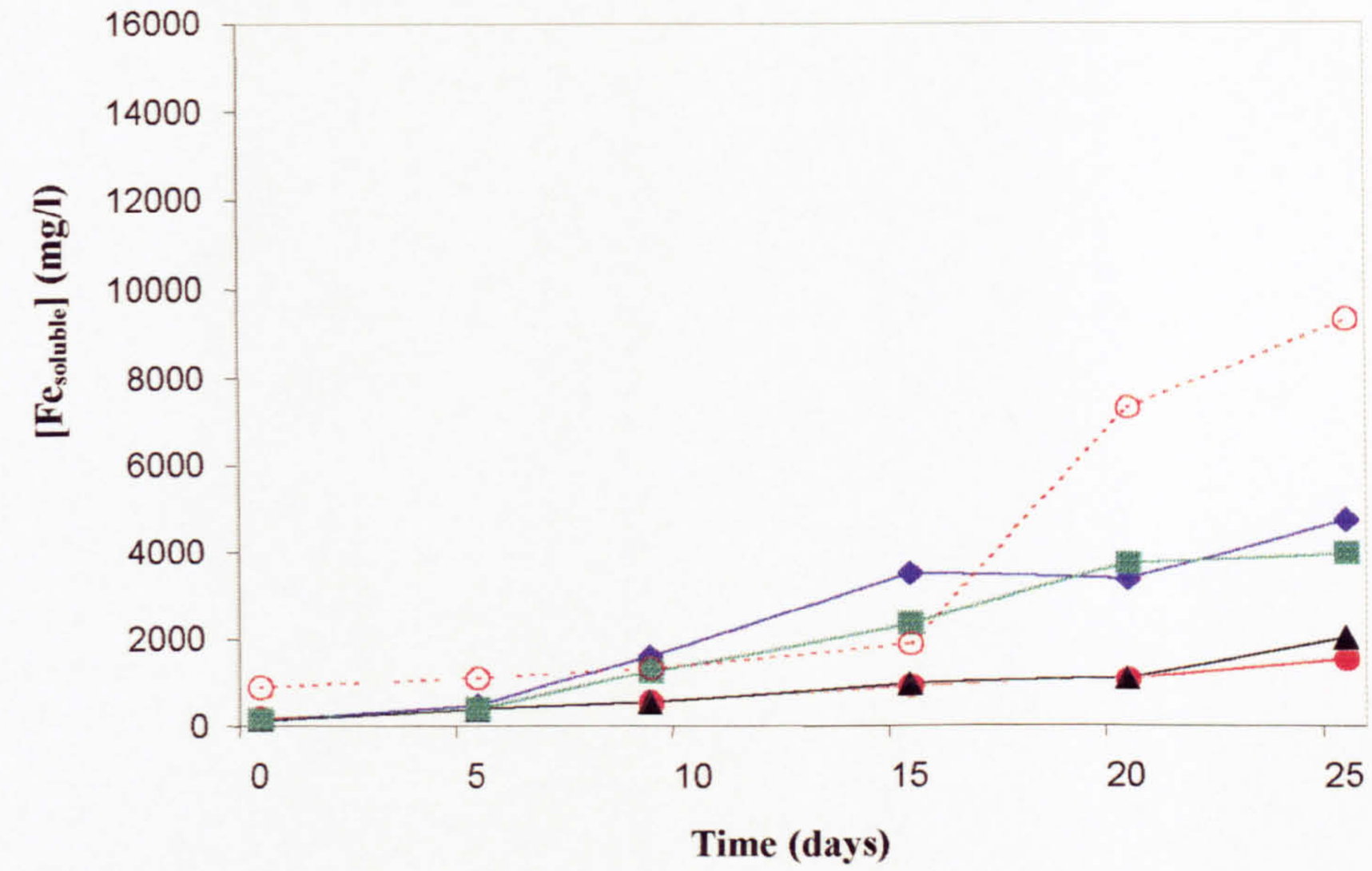


Figure 4.1: Oxidation of Mintek pyrite concentrate (solid lines) and Cae Coch rock pyrite (broken line) by pure cultures of moderate thermophiles. Key: ● and ○, *Leptospirillum* MT6; ▲, *At. caldus* KU; ◆, isolate GSM; ■, *Sulfobacillus* NC. (Evaporation of water was not taken into account.)

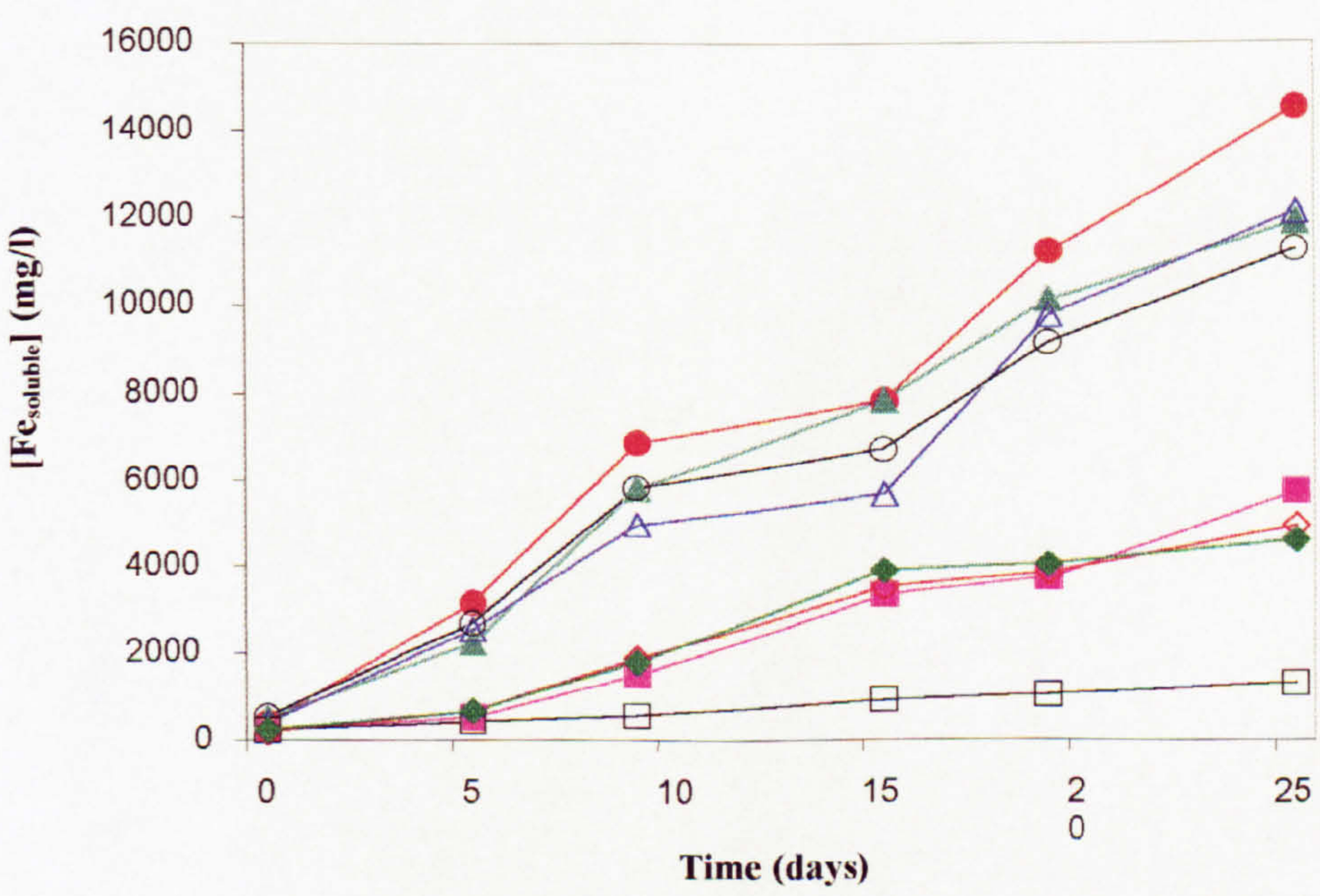


Figure 4.2: Oxidation of Mintek pyrite concentrate by mixed cultures of moderate thermophiles. Key: ●, *Leptospirillum* MT6+*Sulfobacillus* NC; ▲, *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM; △, *Leptospirillum* MT6+*Sulfobacillus* NC+*At. caldus* KU; ○, *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM+*At. caldus* KU; ■, *Leptospirillum* MT6+isolate GSM; ◇,isolate GSM+*At. caldus* KU; ◆, *Leptospirillum* MT6+isolate GSM+*At. caldus* KU; □, *Leptospirillum* MT6+*At. caldus* KU. (Evaporation of water was not taken into account.)

4.2.2.3 pH and redox potential trends in pyrite cultures of moderate thermophiles

Data showing changes in pH and redox potentials in pure and mixed cultures of moderate thermophiles oxidising pyrite concentrate are shown in Figure 4.3.

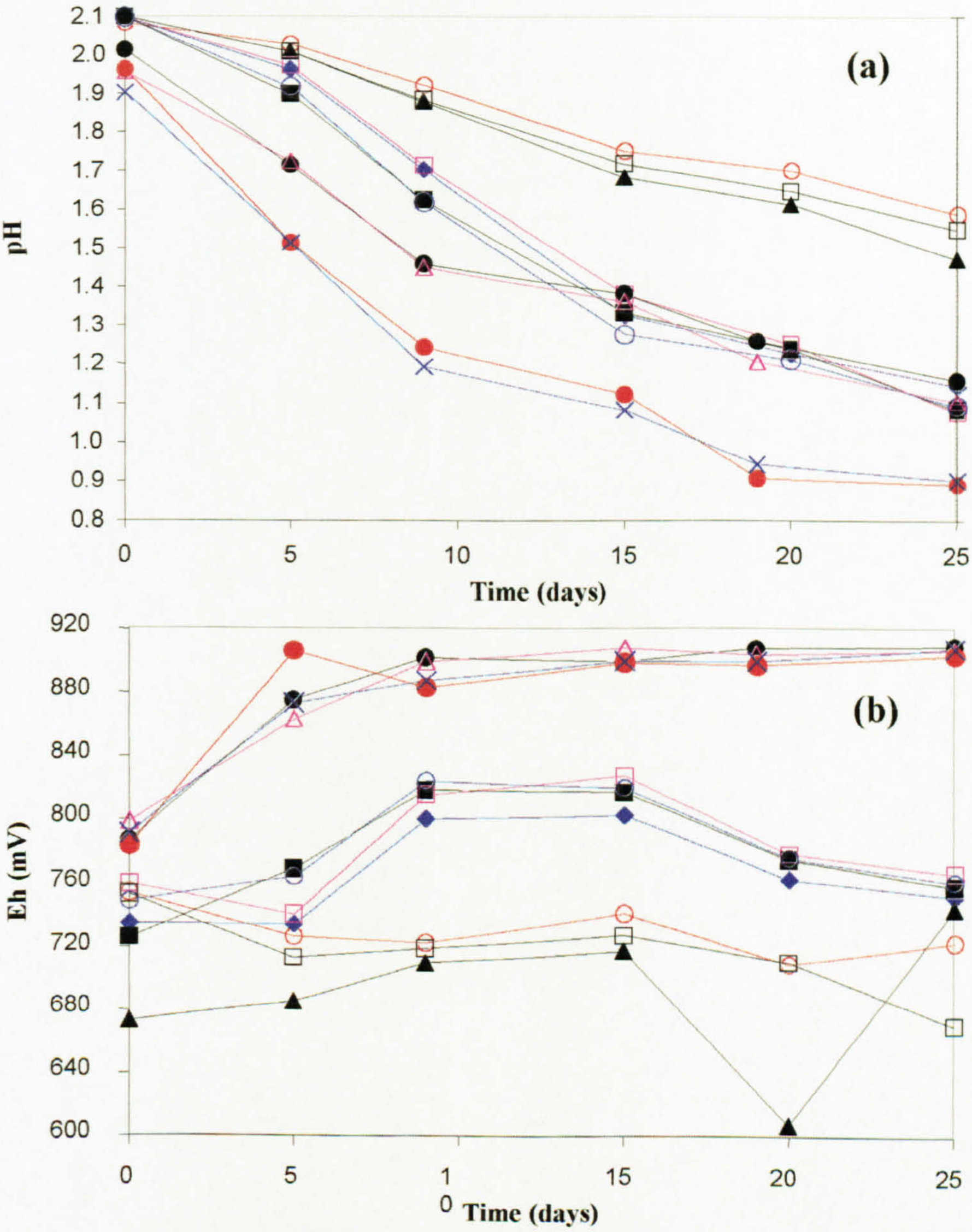


Figure 4.3: Changes in pH (a) and redox potentials (b) in cultures of moderate thermophiles oxidising Mintek pyrite concentrate. Key: ○, *Leptospirillum* MT6; ◆, isolate GSM; ▲, *At. caldus* KU; ■, isolate GSM+*At. caldus* KU; □, *Leptospirillum* MT6+isolate GSM; □, *Leptospirillum* MT6+*At. caldus* KU; ●, *Leptospirillum* MT6+*Sulfobacillus* NC; ○, *Leptospirillum* MT6+isolate GSM+*At. caldus* KU; △, *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM; ●, *Leptospirillum* MT6+*Sulfobacillus* NC+*At. caldus* KU; ×, *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM+*At. caldus* KU. (Evaporation of water was not taken into account.)

The pH declined in all cultures, though this was least in the pure cultures of *At. caldus*, *Leptospirillum* MT6, and in the mixed cultures of these two (where there was no biologically-enhanced oxidation of pyrite) and greatest in those mixed cultures that contained *Leptospirillum* MT6, *At. caldus* KU and at least one of the Gram-positive bacteria. Redox potentials also differed markedly between cultures, with the highest values (> +900 mV) being found in cultures containing *Leptospirillum* MT6 and *Sulfobacillus* NC. For those cultures in which the primary oxidising bacterium was isolate GSM, redox potentials averaged some 150 mV lower than these.

4.2.2.4 Microbial population changes in pyrite cultures

Numbers of moderate thermophiles (estimated from plate counts) in the cultures that contained all four bacteria are shown in Figure 4.4, and numbers of moderate thermophiles in all cultures are shown in Figure 4.5. Because colonies of isolate GSM and *Sulfobacillus* NC were not distinguishable on plates, numbers of these two acidophiles are indicated as total numbers of the two. Numbers of *Leptospirillum* MT6 in pure cultures, and also in the mixed cultures that excluded *Sulfobacillus* NC (and which did not oxidise) were < 10^2/ml (the limit of detection for the dilution range used). The dominant organism (by about an order of magnitude) in mixed cultures of the four acidophiles throughout incubation was *At. caldus* (Figure 4.4). This was also the case in all other mixed cultures that contained this sulfur-oxidiser (Figure 4.5b). In all mixed cultures that included both *Leptospirillum* MT6 and *Sulfobacillus* NC, the dominant iron-oxidiser was *Leptospirillum* MT6 (approx. 10^7/ml; Figures 4.4 and 4.5a), and the two Gram-positive bacteria (isolate GSM and *Sulfobacillus* NC) were present in smaller numbers (approx. 10^5/ml; Figures 4.4, 4.5c and 4.5d).

Numbers of isolate GSM were as high as 10^7/ml, but only in those mixed cultures that did not also contain both *Leptospirillum* MT6 and *Sulfobacillus* NC (i.e. where isolate GSM was the prime iron-oxidiser; Figure 4.5c).

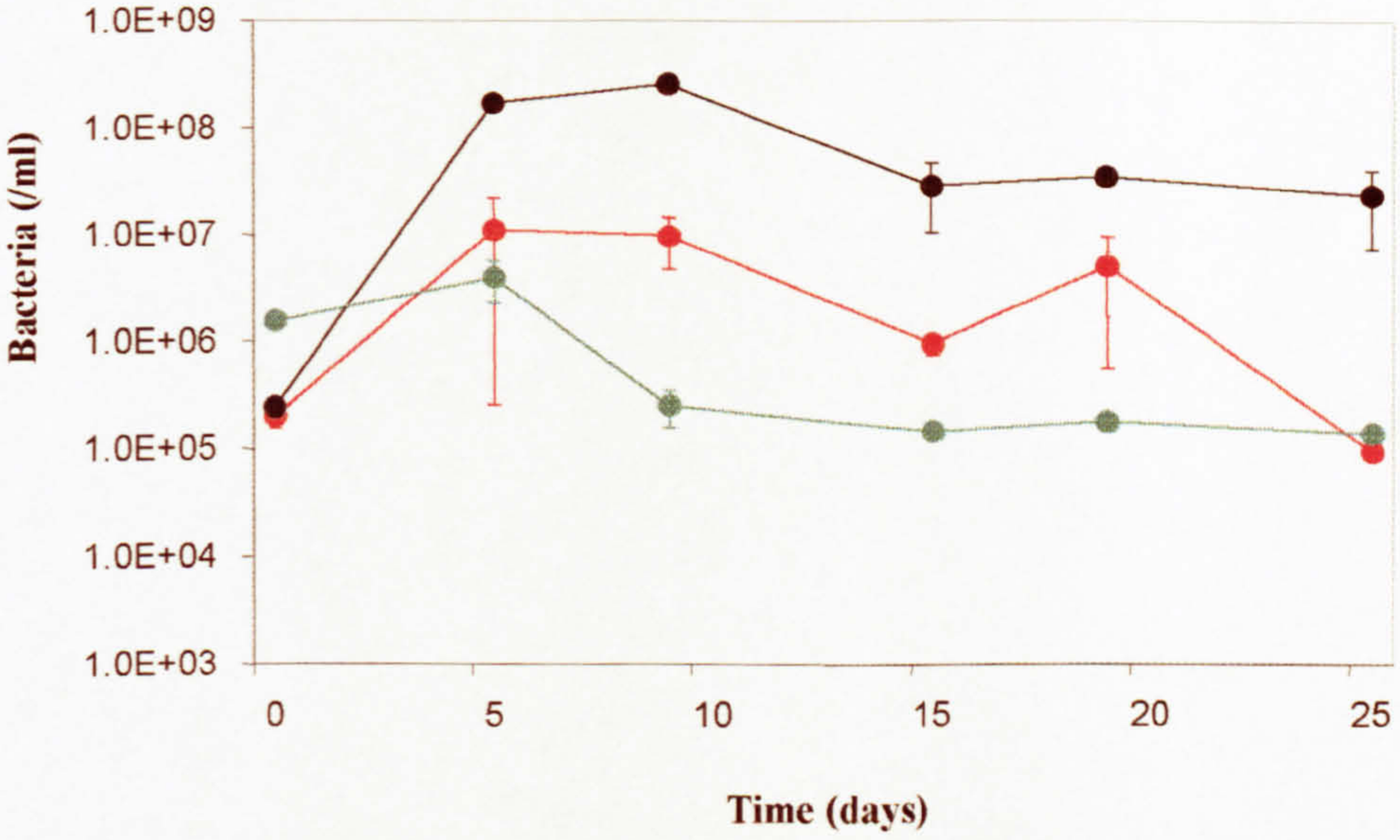


Figure 4.4: Changes in bacterial populations during the oxidation of Mintek pyrite concentrate by a consortium of four moderate thermophiles. Key: ●, *At. caldus* KU; ●, *Leptospirillum* MT6; ●, total numbers of isolate GSM and *Sulfobacillus* NC. (Evaporation of water was not taken into account.)

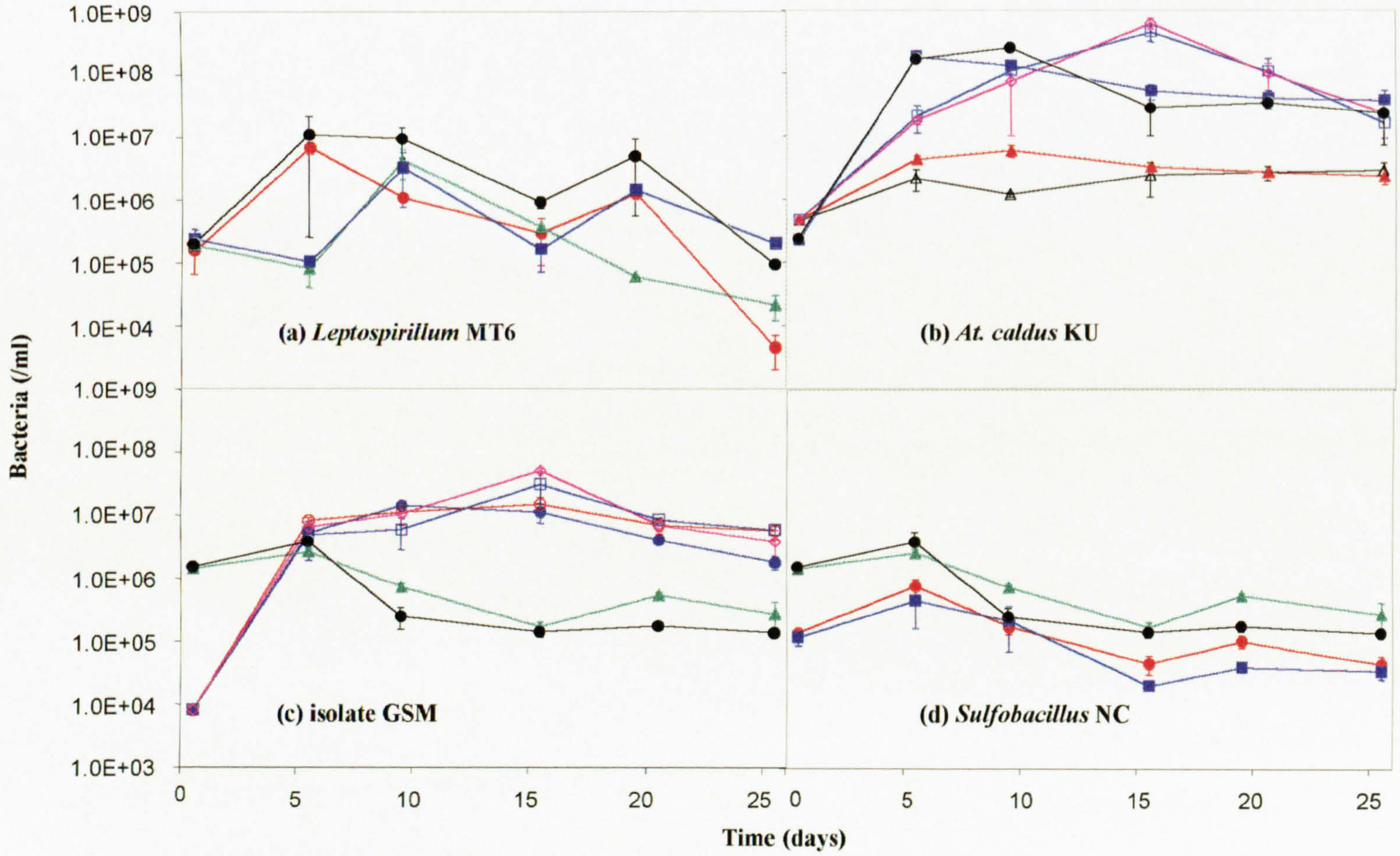


Figure 4.5: Changes in numbers of *Leptospirillum* MT6 (a), *At. caldus* KU (b), isolate GSM (c) and *Sulfobacillus* NC (d) in cultures containing *Leptospirillum* MT6+*Sulfobacillus* NC (●); *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM (▲); *Leptospirillum* MT6+*Sulfobacillus* NC+*At. caldus* KU (■); *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM+*At. caldus* KU (●); *At. caldus* KU (△); *At. caldus* KU+*Leptospirillum* MT6 (▲); *At. caldus* KU+isolate GSM (□); *At. caldus* KU+*Leptospirillum* MT6+isolate GSM (◇); isolate GSM (●); *Leptospirillum* MT6+isolate GSM (○). (Evaporation of water was not taken into account.)
Note: ▲ and ● in graph (c) and (d) are total numbers of isolate GSM and *Sulfobacillus* NC.

Measurements of DOC showed some interesting trends (Figures 4.6 and 4.7).

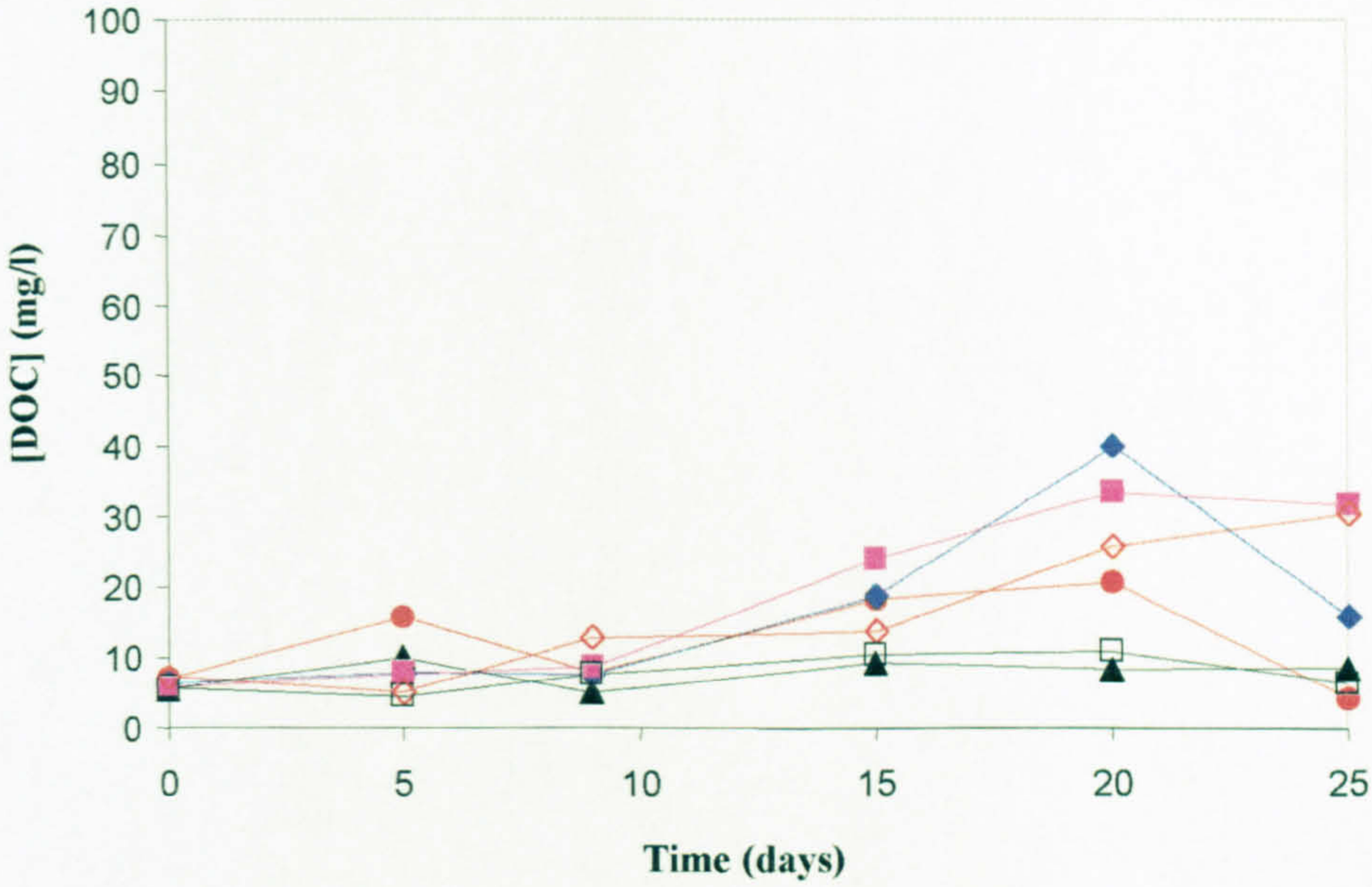


Figure 4.6: Changes in DOC concentrations during the oxidation of Mintek pyrite concentrate by pure cultures, and combinations of two moderate thermophiles. Key: ●, *Leptospirillum* MT6; ◆, isolate GSM; ▲, *At. caldus* KU; ■, *Leptospirillum* MT6+isolate GSM; □, *Leptospirillum* MT6+*At. caldus* KU, ◇, isolate GSM+*At. caldus* KU. (Evaporation of water was not taken into account).

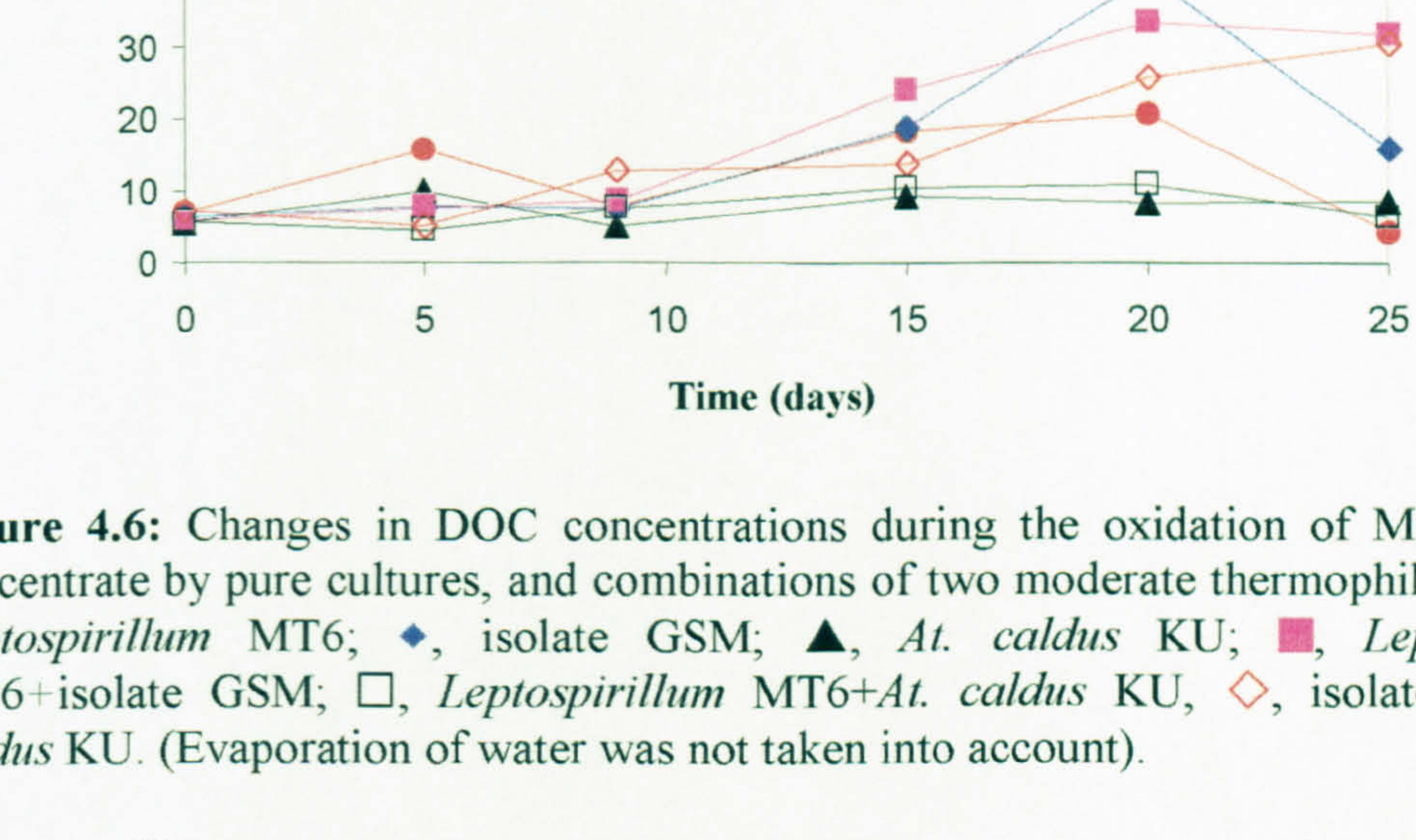


Figure 4.7: Changes in DOC concentrations during the oxidation of Mintek pyrite concentrate by combinations of three or four moderate thermophiles. Key: ◆, *Leptospirillum* MT6+isolate GSM+*At. caldus* KU; ○, *Leptospirillum* MT6+*Sulfobacillus* NC; △, *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM; △, *Leptospirillum* MT6+*Sulfobacillus* NC+*At. caldus* KU; ○, *Leptospirillum* MT6+*Sulfobacillus* NC+isolate GSM+*At. caldus* KU. (Evaporation of water was not taken into account.)

Concentrations of DOC did not show any marked increase in those cultures where pyrite oxidation was not effective (i.e. pure cultures of *At. caldus* and *Leptospirillum* MT6, and mixed cultures of *At. caldus* plus *Leptospirillum* MT6). DOC concentrations in pyrite-oxidising mixed cultures that excluded *Leptospirillum* MT6 and *Sulfobacillus* NC (total soluble iron <6,000 mg/l at day25) increased up to ~30 mgC/l. Pyrite-oxidising mixed cultures that included both *Leptospirillum* MT6 and *Sulfobacillus* NC (total soluble iron >10,000 mg/l) had higher DOC concentrations of 50-100 mgC/l. Among those mixed cultures that included both *Leptospirillum* MT6 and *Sulfobacillus* NC, cultures with *At. caldus* accumulated more DOC (70-100 mg/l) than those excluding *At. caldus* (~50 mg /l), probably due to contribution from the autotroph, *At. caldus*.

4.3 Oxidation of Mintek pyrite concentrate and Cae Coch rock pyrite by mixed cultures of *Leptospirillum* MT6 and other moderately thermophilic microorganisms.

4.3.1 Introduction

In earlier experiments (section 4.2), it was found that *Leptospirillum* MT6 was not able to oxidise the pyrite concentrate in pure cultures, but could do so in mixed cultures containing Gram-positive acidophiles. To test how widespread this phenomenon was, *Leptospirillum* MT6 was grown in mixed culture with a variety of other moderate thermophiles.

4.3.2 Methods

Pure cultures of moderate thermophiles (Table 4.1), and mixed cultures of *Leptospirillum* MT6 and one of these acidophiles, were prepared as inocula. One hundred millilitre flasks, each containing 50 ml of 2% pyrite medium (section 2.2.1.1.4) (2% Mintek pyrite concentrate, pH 2.0) were prepared and inoculated with the pre-grown cultures. Also, to investigate whether oxidation of rock pyrite was enhanced in mixed cultures of acidophiles (relative to pure *Leptospirillum* MT6), a parallel experiment using this pyrite was carried out. Samples were taken at regular intervals and tested for total soluble iron (section 2.4.3). Duplicate flasks were used for each set of organisms.

Table 4.1: List of moderate thermophiles used in shake flask pyrite oxidation experiments.

Leptospirillum MT6	
At. caldus KU (used only for rock pyrite oxidation)	
Sulfobacillus spp.	*Sb. thermosulfidooxidans*^{T}
	Sb. acidophilus ALV
	"Sb. yellowstonensis" YTF1
	Sulfobacillus NC
Isolate GSM	
Am. ferrooxidans TH3	
Alicyclobacillus YTH1	
Ferroplasma MT17	

4.3.3 Results

Oxidation of pyrite concentrate and rock pyrite by pure cultures, and by mixed cultures of *Leptospirillum* MT6 and various other moderate thermophiles (at day 20) are shown in Figures 4.8 and 4.9.

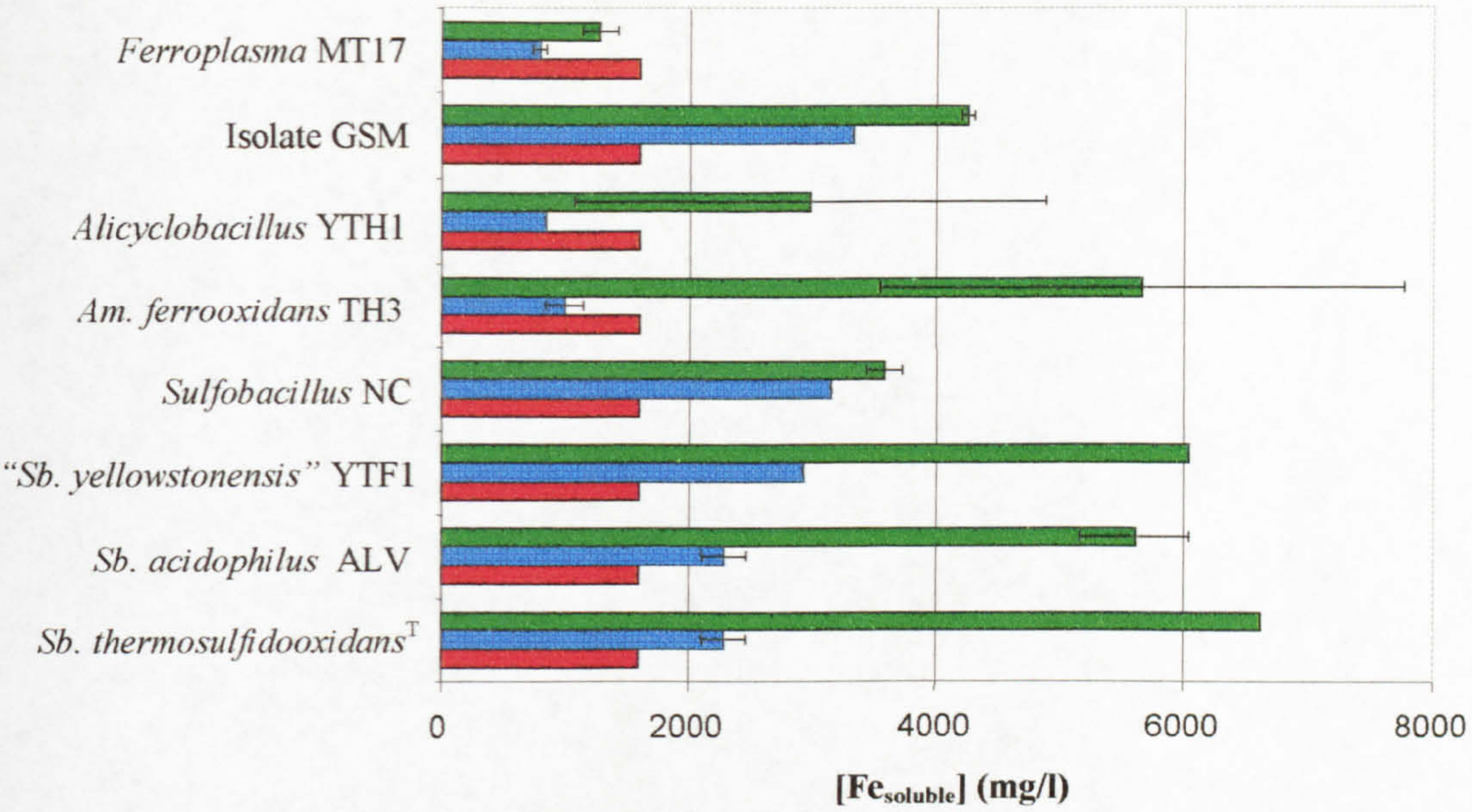


Figure 4.8: Oxidation of Mintek pyrite concentrate by pure cultures of moderate thermophiles and mixed cultures of *Leptospirillum* MT6 and other moderate thermophiles (examined as total soluble iron values after 20 days incubation). Key: ■, pure cultures of *Leptospirillum* MT6; ■, pure cultures of the other named moderate thermophile; ■, mixed cultures of the two acidophiles.

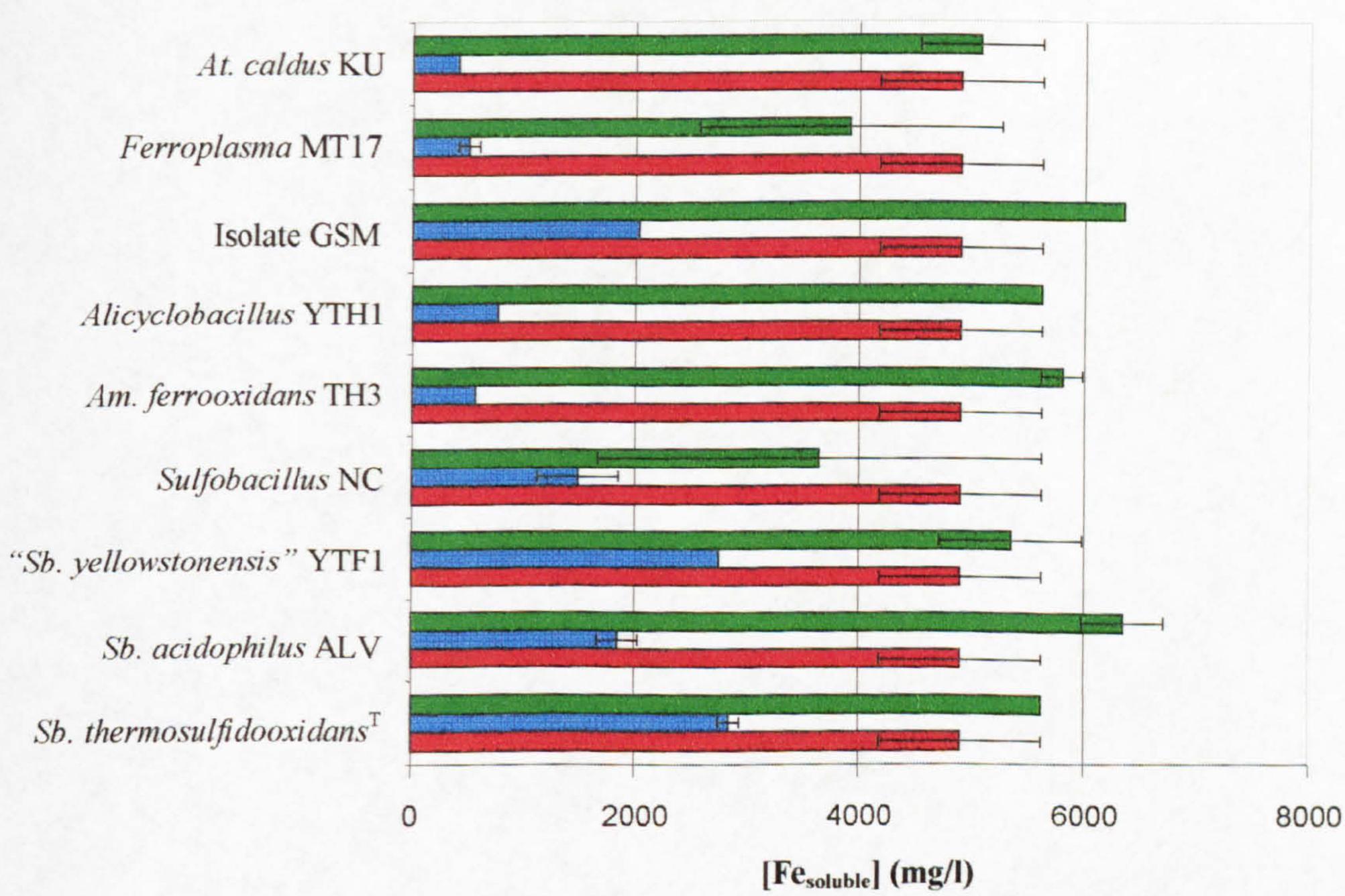


Figure 4.9: Oxidation of Cae Coch rock pyrite by pure cultures of moderate thermophiles and mixed cultures of *Leptospirillum* MT6 and other moderate thermophiles (examined as total soluble iron values after 20 days incubation). Key: ■, pure cultures of *Leptospirillum* MT6; ■, pure cultures of the other named moderate thermophile; ■, mixed culture of the two acidophiles.

In contrast to the earlier experiment using pyrite concentrate, there was some (limited) oxidation of this material by *Leptospirillum* MT6 in this experiment (as may be seen by comparison with pure cultures of *Ferroplasma* MT17, *Alicyclobacillus* YTH1 or *Am. ferrooxidans* TH3; Figure 4.8). For all of the mixed cultures tested (except *Leptospirillum* MT6 + *Ferroplasma* MT17), oxidation of pyrite concentrate was greater than by the corresponding pure culture, though this was marginal in the case of *Alicyclobacillus* YTH1 due to the large standard errors (Figure 4.8). Also, with the mixed cultures of *Leptospirillum* MT6 and either isolate GSM or *Sulfobacillus* NC, the extents to which oxidation of pyrite concentrate was enhanced relative to pure cultures of the Gram-positive acidophiles were relatively small (Figure 4.8).

In the case of rock pyrite, pure cultures of *Ferroplasma* MT17, *Alicyclobacillus* YTH1 *At. caldus* KU and *Am. ferrooxidans* TH3 were, again, non-effective. Oxidation of pyrite by pure cultures of *Leptospirillum* MT6 was greater (by day 20) than by pure cultures of all the other acidophiles tested. Consequently, a degree to which pyrite oxidation was enhanced by mixed cultures was less apparent with rock pyrite than with pyrite concentrate (Figure 4.9). Mixed cultures of *Ferroplasma* MT17 and *Leptospirillum* MT6 displayed marginally less oxidation of both types of pyrite than pure cultures, at day 20 (Figures 4.8 and 4.9). Somewhat surprisingly, in view of the results with the pyrite concentrate (section 4.2), inclusion of *Sulfobacillus* NC appeared to retard the oxidation of rock pyrite by *Leptospirillum* MT6 (Figure 4.9).

4.4 Effect of *At. caldus* on oxidation of rock pyrite by *Leptospirillum* MT6.

4.4.1 Introduction

Previous reports (Norris, 1990; Dopson and Lindström, 1999) have suggested that mixed cultures of moderate thermophiles containing *At. caldus* are more efficient mineral oxidising systems than pure cultures of iron-oxidisers. However, some of the current data appeared to contradict this (section 4.2). In an attempt to clarify the situation, the oxidation of rock pyrite by pure cultures and mixed cultures of *Leptospirillum* MT6 and *At. caldus* were examined further.

4.4.2 Methods

Leptospirillum MT6 and *At. caldus* KU were pre-grown in 2% pyrite medium (Cae Coch rock pyrite, pH 2.0; section 2.2.1.1.4). One hundred millilitre flasks (in duplicate), each containing 50 ml of the same medium, were prepared and inoculated either with pure cultures or the mixed culture of the two acidophiles. The flasks were incubated at 45°C, shaken, and samples were removed for determination of total soluble iron (section 2.4.3) and pH (section 2.4.1). In addition, cultures were tested (at day 77) for dissolved organic carbon (DOC) concentrations (section 2.4.7).

4.4.3 Results

The effect of *At. caldus* on the oxidation of rock pyrite by *Leptospirillum* MT6 is shown in Figure 4.10. As expected, no biological pyrite oxidation was observed in pure cultures of *At. caldus*. In contrast to the results obtained with pyrite concentrate (section 4.2), mixed cultures of *At. caldus* and *Leptospirillum* MT6 appeared to be superior in oxidising rock pyrite than pure cultures of the iron-oxidiser, although this effect was marginal, and only apparent when cultures were incubated for protracted periods (>35

days). In contrast, the pH of mixed cultures of *Leptospirillum* MT6 and *At. caldus* were much lower than those of pure cultures of *Leptospirillum* MT6 throughout incubation. DOC concentrations of the cultures at day 77 are shown in Figure 4.11; these appeared to correlate with pyrite oxidation.

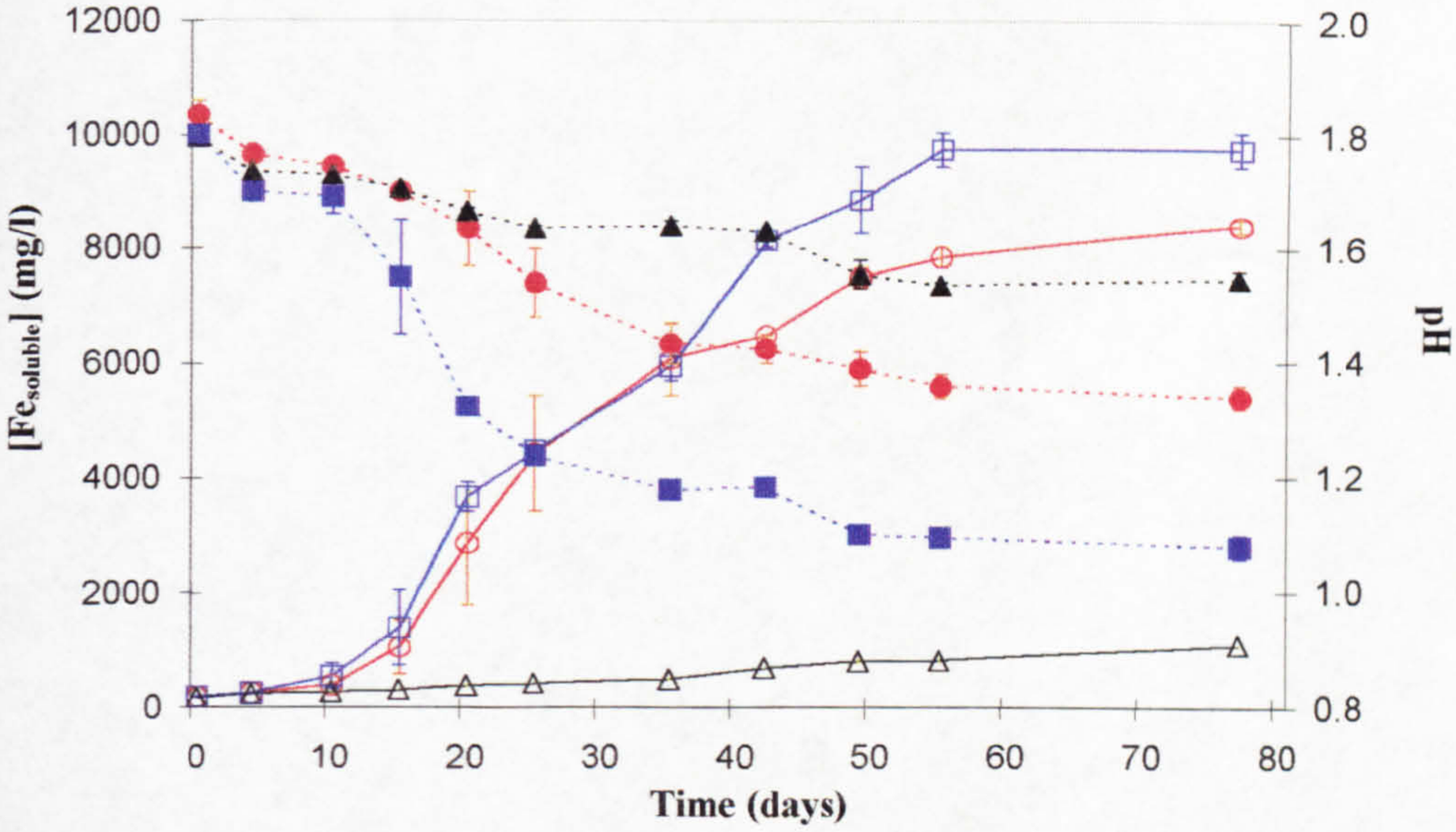


Figure 4.10: Oxidation of Cae Coch rock pyrite by pure and mixed cultures of *Leptospirillum* MT6 and *At. caldus* KU (solid lines) and pH changes in these cultures (broken lines). Key: ○●, *Leptospirillum* MT6 pure cultures; △▲, *At. caldus* KU pure cultures; ■□, mixed cultures of the two acidophiles.

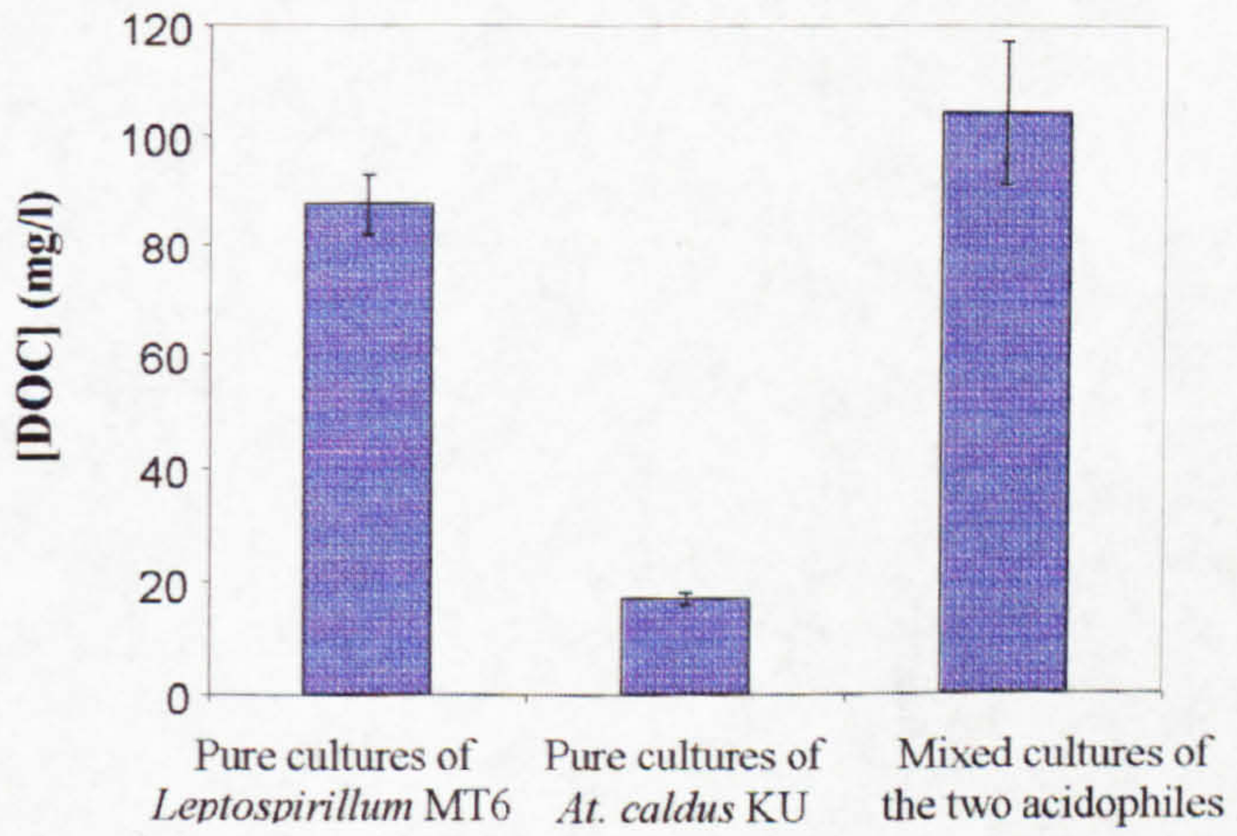


Figure 4.11: DOC concentrations in pure and mixed cultures of *Leptospirillum* MT6 and *At. caldus* KU at day 77.

4.5 Oxidation of pyrite by pure cultures of *Am. ferrooxidans* and mixed cultures of *Am. ferrooxidans*, *Leptospirillum* MT6 and *At. caldus*.

4.5.1 Introduction

Results from earlier experiments (section 4.3) suggested that pyrite oxidation by mixed cultures of *Leptospirillum* MT6 and many Gram-positive acidophiles were superior to pure cultures of these acidophiles. In this study, one of the Gram-positive acidophiles, *Am. ferrooxidans*, was examined further.

4.5.2 Methods

Shake flasks (100 ml), each containing 50 ml of 2% pyrite medium (Cae Coch or Mintek pyrite, pH 2.0; section 2.2.1.1.4) were autoclaved and inoculated (2%, v/v) with various combinations of pure and mixed populations of moderately thermophilic acidophiles, pre-grown in 1% Cae Coch pyrite media (pH 2.0). Duplicate flasks were incubated at 45°C, shaken (130 rpm), and samples removed aseptically at regular intervals for analysis of total soluble iron (section 2.4.3).

4.5.3 Results

Oxidation of rock pyrite and pyrite concentrate by pure and mixed cultures of these moderate thermophiles are shown in Figures 4.12 and 4.13. *Am. ferrooxidans* ICP and TH3 displayed different trends. In pure cultures, strain ICP oxidised pyrite concentrate more effectively than rock pyrite (Figure 4.12) whereas strain TH3 exhibited similar oxidation rates with both types of pyrite (Figure 4.13). Pyrite oxidation by mixed cultures of strain ICP and either (or both) of the two autotrophs (*Leptospirillum* MT6 and *At. caldus*) was more effective than pure cultures of strain ICP. All of the mixed

cultures were again more effective than pure cultures of strain TH3, though pyrite oxidation by mixed cultures of strain TH3 and *At. caldus* were not as effective as those that also included *Leptospirillum* MT6. It was also noted that in this experiment the oxidation of rock pyrite and pyrite concentrate by pure cultures of *Am. ferrooxidans* TH3 was superior to that observed in the previous experiment (section 4.3).

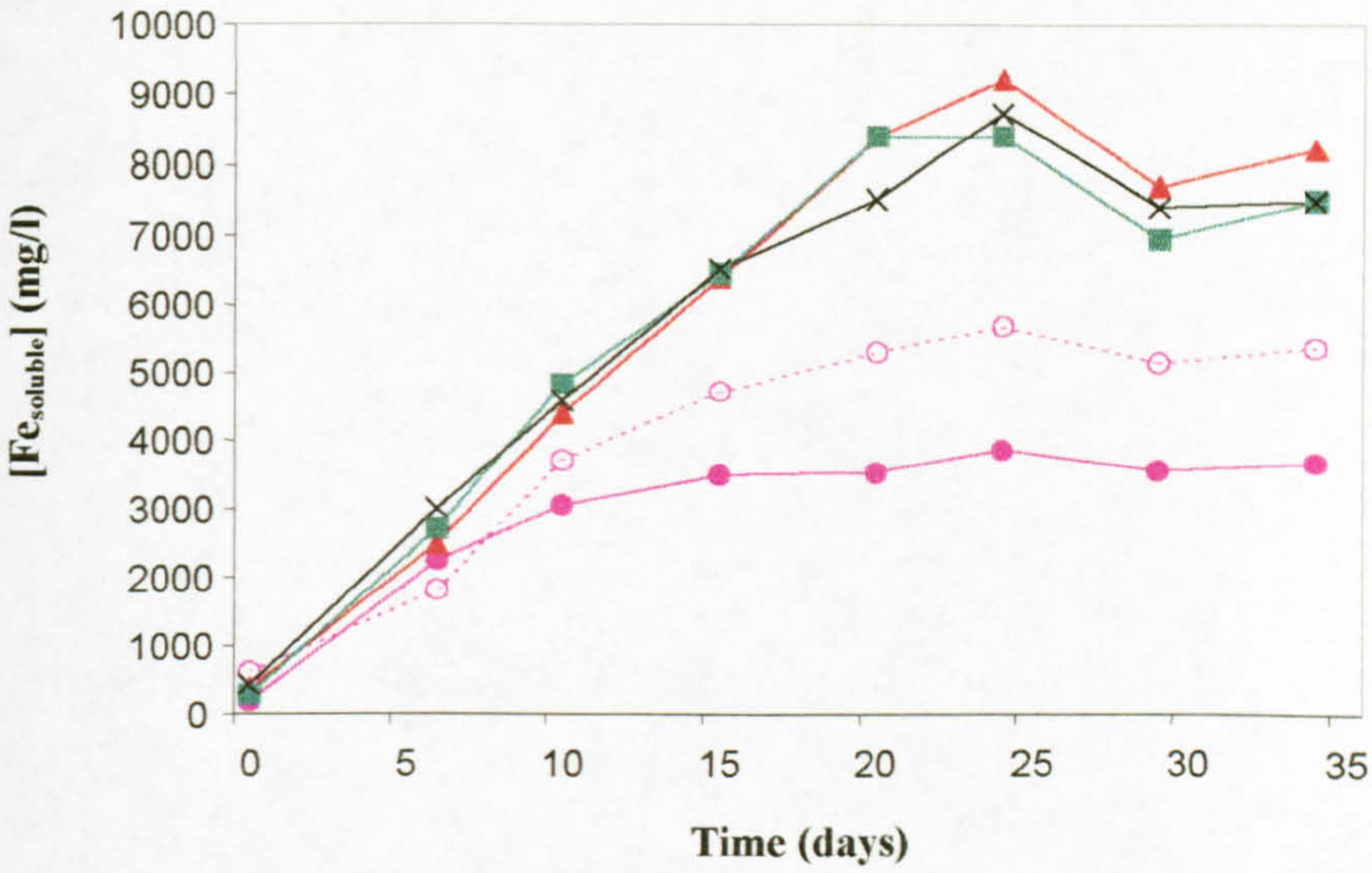


Figure 4.12: Oxidation of Cae Coch rock pyrite (solid lines) and Mintek pyrite concentrate (broken line) by pure cultures of *Am. ferrooxidans* ICP and mixed cultures of *Am. ferrooxidans* ICP, *Leptospirillum* MT6 and *At. caldus* KU. Key: ● ○, *Am ferrooxidans* ICP; ▲, *Am. ferrooxidans* ICP+*Leptospirillum* MT6; ■, *Am. ferrooxidans* ICP+*At. caldus* KU; ×, *Am. ferrooxidans* ICP+*Leptospirillum* MT6+*At. caldus* KU.

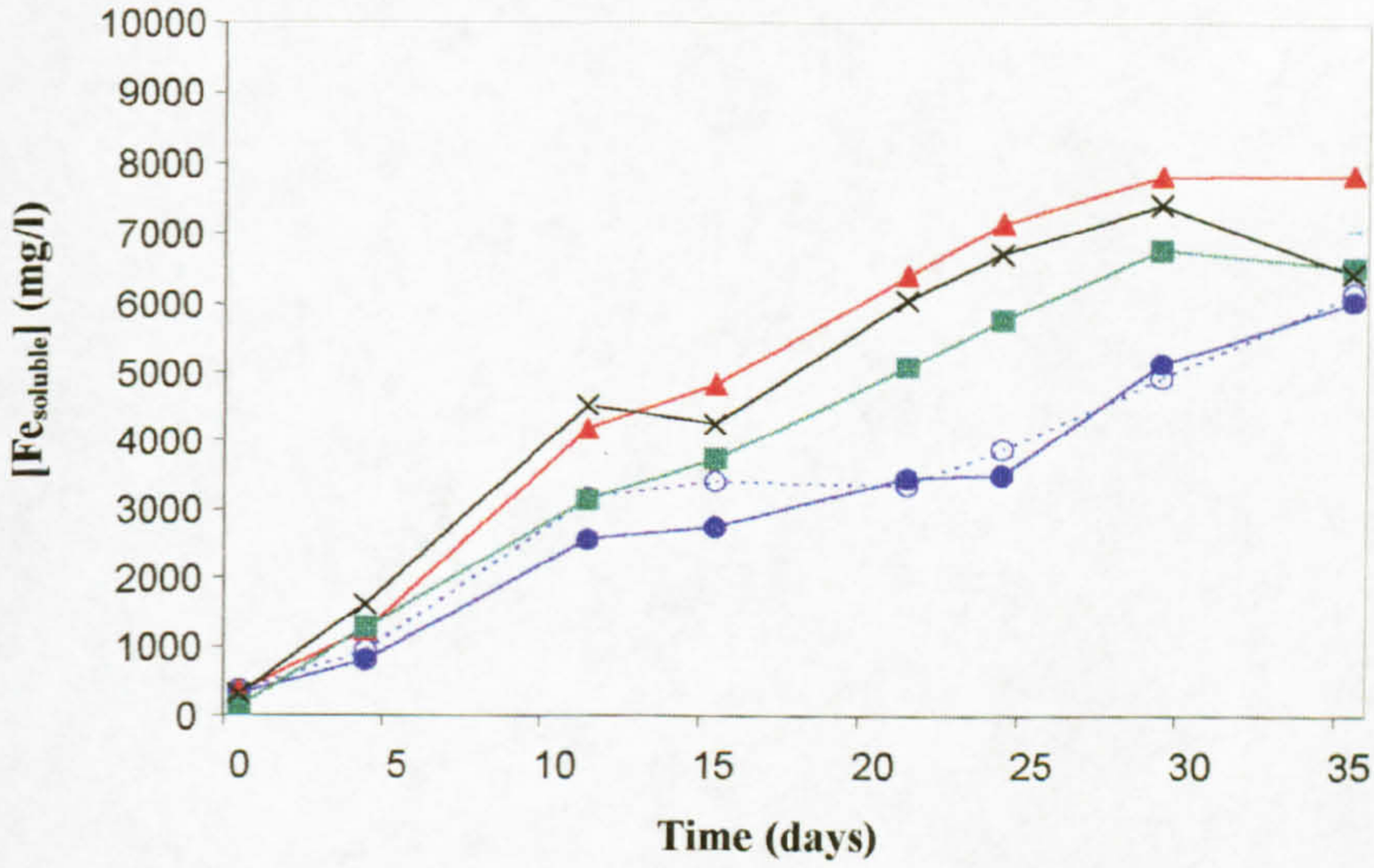


Figure 4.13: Oxidation of Cae Coch rock pyrite (solid lines) and Mintek pyrite concentrate (broken line) by pure cultures of *Am. ferrooxidans* TH3 and mixed cultures of *Am. ferrooxidans* TH3, *Leptospirillum* MT6 and *At. caldus* KU. Key: ● ○, *Am. ferrooxidans* TH3; ▲, *Am. ferrooxidans* TH3+*Leptospirillum* MT6; ■, *Am. ferrooxidans* TH3+*At. caldus* KU; ×, *Am. ferrooxidans* TH3+*Leptospirillum* MT6+*At. caldus* KU.

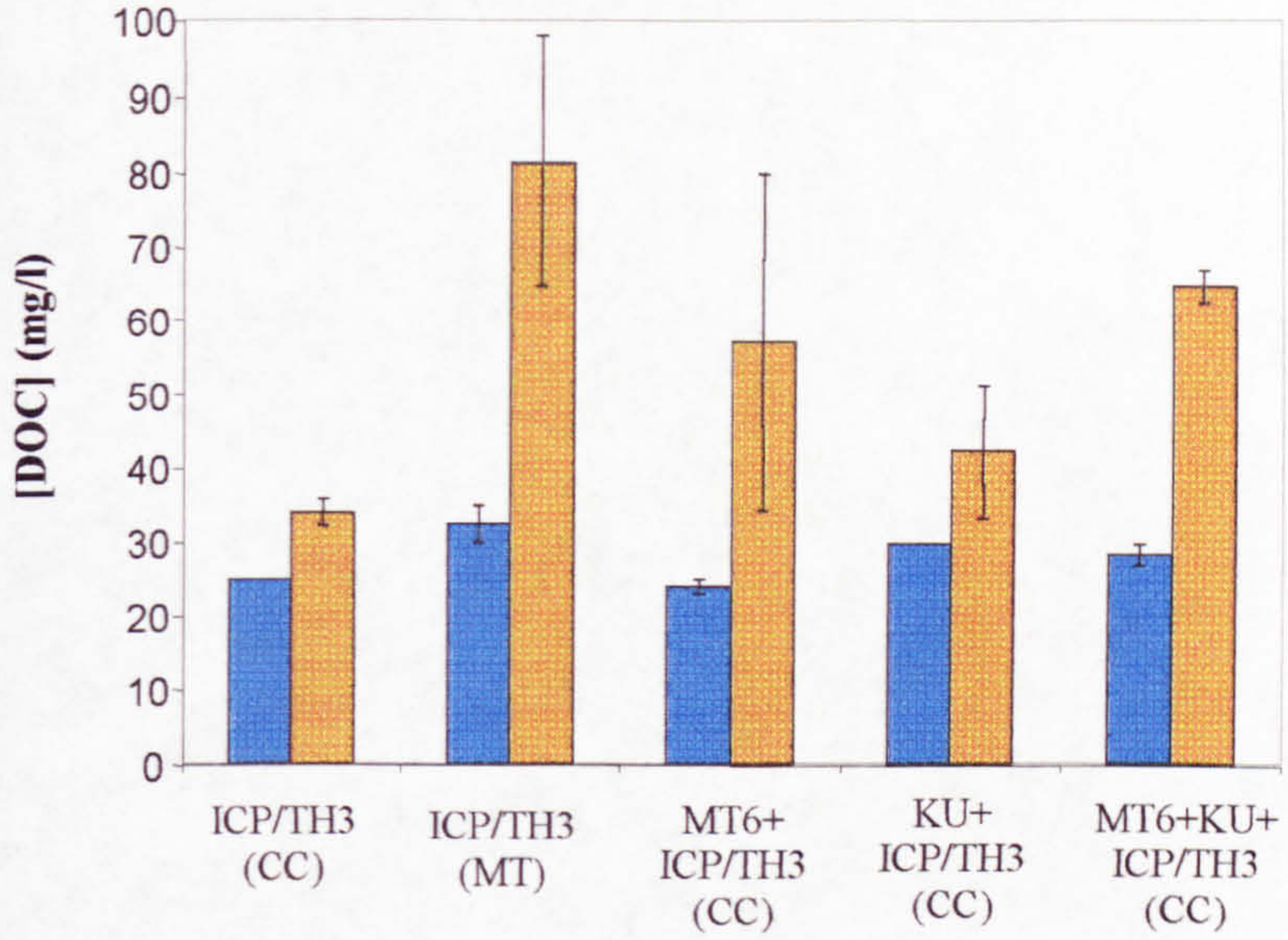


Figure 4.14: DOC concentrations at day 34 (*Am. ferrooxidans* ICP cultures) and at day 35 (*Am. ferrooxidans* TH3 cultures). Key: ■, *Am. ferrooxidans* ICP cultures; ■, *Am. ferrooxidans* TH3 cultures. "CC" and "MT" indicate Cae Coch rock pyrite and Mintek pyrite concentrate, respectively.

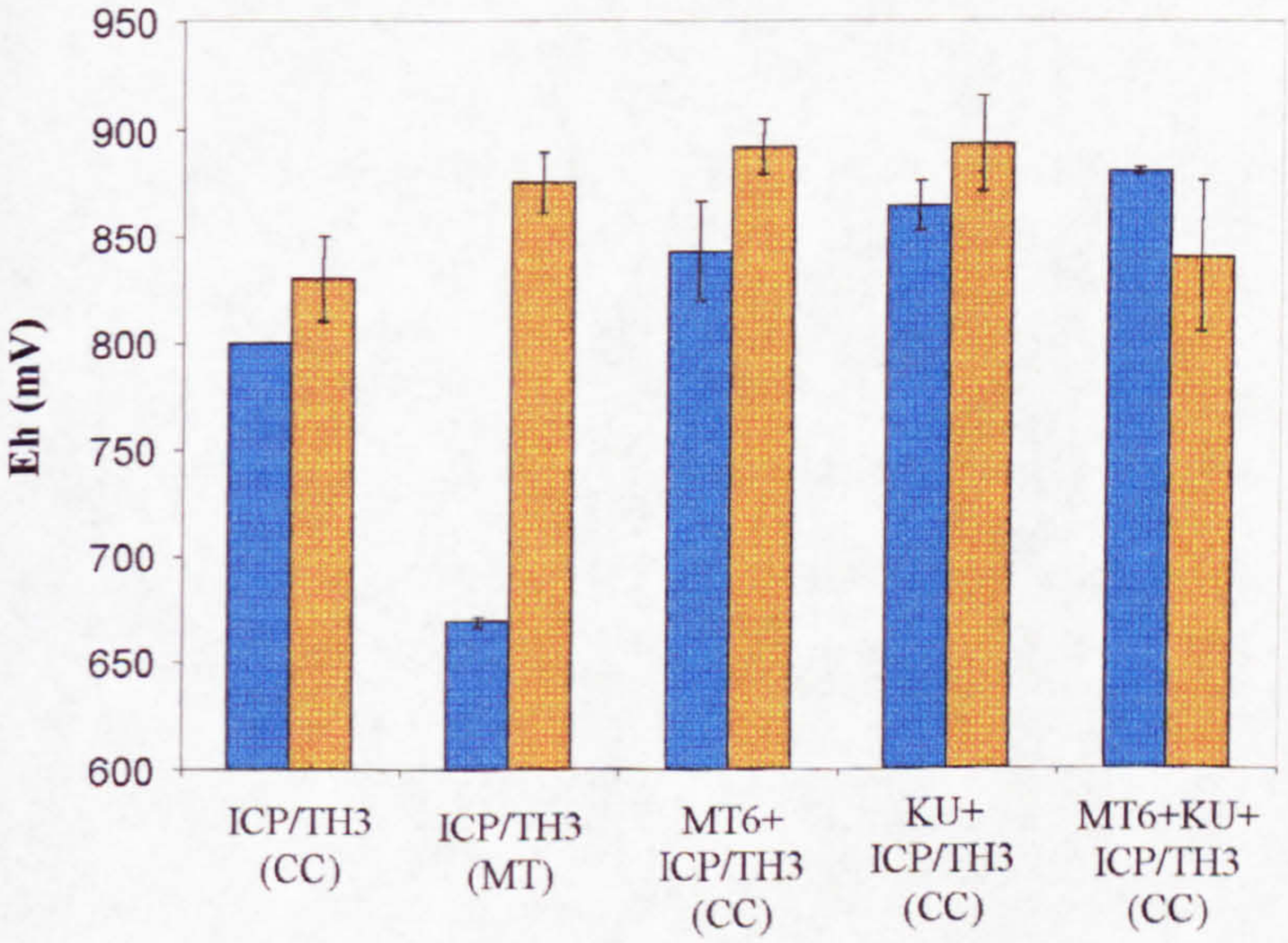


Figure 4.15: Redox potentials at day 34 (*Am. ferrooxidans* ICP cultures) and at day 35 (*Am. ferrooxidans* TH3 cultures). Key: ■, *Am. ferrooxidans* ICP cultures; ■, *Am. ferrooxidans* TH3 cultures. "CC" and "MT" indicate Cae Coch rock pyrite and Mintek pyrite concentrate, respectively.

DOC concentrations and redox potentials measured at day 34 (*Am. ferrooxidans* ICP) and day 35 (*Am. ferrooxidans* TH3) are shown in Figures 4.14 and 4.15, respectively. DOC concentrations in *Am. ferrooxidans* ICP cultures tended to be smaller than in *Am. ferrooxidans* TH3 cultures, and did not differ much between the different cultures. In contrast, DOC concentrations in *Am. ferrooxidans* TH3 cultures displayed wide variations, with those in pure cultures of *Am. ferrooxidans* TH3 (with pyrite concentrate) having the highest values (approx. 80 mg C/l). Redox potentials of mixed cultures (for both ICP and TH3) were slightly higher than those of pure cultures with rock pyrite (reflecting higher Fe^{3+}/Fe^{2+} ratios in the more effective mixed cultures). Significantly lower redox potentials of pure cultures of *Am. ferrooxidans* ICP were observed with pyrite concentrate than with rock pyrite though pyrite oxidation was greater with pyrite concentrate than with rock pyrite; the reason for this was not clear.

4.6 Discussion

The pure and mixed populations of moderately thermophiles used in these experiments oxidised rock pyrite and pyrite concentrate at different rates and to varying extents.

It should be noted that in experiments in section 4.2, about 60 % of water appeared to be evaporated at day 25, and growth of acidophiles might have been affected (water evaporation was taken into account for all later experiments).

With the initial experiments using pyrite concentrate and four species of moderate thermophiles (section 4.2), the most efficient bacterial systems contained both *Leptospirillum* MT6 and *Sulfobacillus* NC. Pure cultures were either non-effective (*Leptospirillum* MT6 or *At. caldus*) or relatively poor (*Sulfobacillus* NC or isolate GSM) at oxidising the pyrite concentrate, though pure cultures of *Leptospirillum* MT6 were able to oxidise the rock pyrite effectively. Although *At. caldus* was not able to

oxidise pyrite in pure culture, it was numerically the dominant bacterium in all mixed cultures, presumably because it oxidises the various reduced sulfur compounds formed from ferric iron attack on sulfidic minerals, which yield greater energy than ferrous iron oxidation (Sand *et al.*, 1995). Results showing that pure cultures of *Leptospirillum* MT6 were able to oxidise rock pyrite but not pyrite concentrate suggested that some compound(s) in the latter inhibited the growth of this bacterium (this is examined further in chapter 6). When grown in mixed culture with *Sulfobacillus* NC, the apparent inhibition of *Leptospirillum* MT6 was removed, resulting in the most efficient pyrite concentrate oxidation observed. This was not the case with either isolate GSM or *At. caldus*, where rates of mineral dissolution by the mixed cultures of these and *Leptospirillum* MT6 were similar to those of pure cultures of these acidophiles. In cultures containing *Sulfobacillus* NC, *Leptospirillum* MT6 was the most numerically dominant iron-oxidiser, suggesting that where the inhibition was removed, this iron-oxidiser was the most significant pyrite-oxidising bacterium of those investigated.

In theory, there could be three means by which *At. caldus* (and other sulfur-oxidising isolates *Sulfobacillus* NC and GSM) might affect pyrite oxidation; first, by decreasing culture pH due to their oxidation of sulfur and RISCs, forming sulfuric acid (thus preventing, at least in part, the formation of ferric precipitates); second, by removing sulfur deposits from the pyrite surface (thus facilitating ferric iron attack of the pyrite surface); third, by providing organic materials (lysates and exudates) for heterotrophic or mixotrophic iron-oxidisers (e.g. *Am. ferrooxidans*). However, in the current experiments, there was no evidence that *At. caldus* (and isolate GSM) had a positive effect on mineral oxidation by the first two means, since inclusion of either (or both) of these bacteria in cultures containing both *Leptospirillum* MT6 and *Sulfobacillus* NC resulted in a somewhat lower rates of mineral oxidation than in those containing only

Leptospirillum MT6 and *Sulfobacillus* NC. The lowest pH values (pH 0.9) were recorded in those mixed cultures that included *At. caldus* (higher values were found in mixed cultures containing isolate GSM) and such pH values were well below the optimum pH (1.5) for *Leptospirillum* MT6. However, these low pH values, probably did not inhibit *Leptospirillum* MT6 significantly, since mixed cultures with isolate GSM (which did not cause lower pH) rather than *At. caldus* also displayed similar lower rates of pyrite concentrate oxidation. The reason why inclusion of either (or both) isolate GSM and *At. caldus* to cultures containing both *Leptospirillum* MT6 and *Sulfobacillus* NC resulted in a somewhat lower rate of mineral oxidation might be that exudates and/or lysates from these two bacteria resulted in a build up of soluble carbon materials (such as some organic acids which are toxic to some autotrophs) that had a negative impact on the primary iron-oxidiser in the system, *Leptospirillum* MT6.

The hypothesis that *At. caldus* may not, in all situations, have a positive effect on pyrite oxidation by *Leptospirillum* MT6 was supported by data from another experiment using rock pyrite (section 4.4). Despite the fact that pH declined more rapidly from the start of the experiment in mixed cultures with *At. caldus*, there was no difference in pyrite oxidation by pure cultures of *Leptospirillum* MT6 and by mixed cultures containing the two bacteria, until day 35. Only after prolonged incubation was pyrite oxidation by mixed cultures found to be superior, quite possibly because, at this time, some ferric precipitates formed in pure cultures of *Leptospirillum* MT6 (due to the slightly higher pH) but not in the mixed cultures with *At. caldus*.

In subsequent experiments (section 4.3), it was shown that mixed cultures of *Leptospirillum* MT6 and a range of Gram-positive bacteria could, apparently, enhance oxidation of the pyrite concentrate, relative to pure cultures. Either these Gram-positive bacteria were also able to remove inhibitory compound(s) present in the pyrite

concentrate, thereby allowing *Leptospirillum* MT6 to emerge as the primary iron-oxidiser, or mineral oxidation by the (generally mixotrophic) Gram-positive moderate thermophiles was stimulated by addition of the autotroph as a source of organic carbon. In the case of mixed cultures of *Leptospirillum* MT6 and *Alicyclobacillus* YTH1, however, the second explanation is not tenable as *Alicyclobacillus* YTH1 is unable to oxidise ferrous iron or pyrite. The reason why mixed cultures with *Leptospirillum* MT6 and *Sulfobacillus* NC were not as effective in later (section 4.3) as in earlier experiments (section 4.2) is not clear. The only difference was that, in the first experiment (section 4.2), *Sulfobacillus* NC was present as a contaminant of a supposedly pure culture of *Leptospirillum* MT6, and this mixed culture had been sub-cultured at least twice on pyrite concentrate prior to inoculation. In contrast, in the later experiment (section 4.3), the two bacteria were inoculated separately from pure cultures. It is possibly that, for the optimum synergy between these two moderate thermophiles that resulted in such a considerably strong enhancement of pyrite concentrate oxidation observed in the first experiment, the mixed culture community has to be reasonably well established.

In the case of rock pyrite, enhanced pyrite oxidation was again observed by some mixed cultures of *Leptospirillum* MT6 and Gram-positive acidophiles, relative to pure cultures. However, such effects were marginal, due to the ability of pure cultures of *Leptospirillum* MT6 to oxidise rock pyrite effectively. Inclusion of *Sulfobacillus* NC in mixed cultures resulted in no enhancement (or a slightly negative effect) of rock pyrite oxidation. Therefore, it was concluded that enhanced oxidation of pyrite by *Leptospirillum* MT6 may not always be achieved by co-culturing with *Sulfobacillus* NC.

Ferroplasma MT17 showed negative effects on the oxidation of both rock pyrite and pyrite concentrate by *Leptospirillum* MT6. This might be because of soluble carbon materials released from this archaeon that inhibit the growth of the bacterium, though the exact reason for this effect was not established.

In the earlier experiment, the iron-oxidising moderate thermophile, *Am. ferrooxidans* (strain TH3) was found to be relatively ineffective at oxidising rock pyrite/pyrite concentrate in pure culture, but did enhance rock pyrite and pyrite concentrate oxidation when grown in mixed culture with *Leptospirillum* MT6. Subsequently, two strains of *Am. ferrooxidans* (strain TH3 and ICP) were examined further. In experiments described in section 4.5, strain TH3 was found to oxidise both rock pyrite and pyrite concentrate in pure culture (~3000 mg/l at day 20 and ~6000 mg/l at day 35), in contrast to earlier results (section 4.3). Strain ICP also oxidised both rock pyrite and pyrite concentrate, though the oxidation of pyrite concentrate was greater than that of rock pyrite. Although *Am. ferrooxidans* is capable of autotrophic growth on ferrous iron and heterotrophic growth on yeast extract (Clark and Norris, 1996a), it was reported in an earlier paper that autotrophic growth of strain TH3 was somewhat inconsistent (Norris and Barr, 1985). This may explain the variable autotrophic pyrite oxidation by strain TH3 found in the present study. Strain ICP was not studied in section 4.3; however, this strain appeared to grow more readily as an autotroph than strain TH3.

Pyrite oxidation by mixed cultures of *Am. ferrooxidans* and either (or both) of the obligate autotrophs (*Leptospirillum* MT6 and *At. caldus*) was always greater than by pure cultures of *Am. ferrooxidans.* Very similar pyrite oxidation rates of all mixed cultures containing strain ICP suggest that this strain of *Am. ferrooxidans* may benefit from *Leptospirillum* MT6 and *At. caldus* primarily by obtaining carbon from the

autotrophs. Similarly, all mixed cultures including strain TH3 were superior at oxidising pyrite than were pure cultures of this acidophile. Mixed cultures of strain TH3 and *At. caldus* were displayed inferior pyrite oxidation than mixed cultures of strain TH3 and *Leptospirillum* MT6, suggesting that the primary mineral oxidiser in the latter was the Gram-negative acidophile. However, since no pure cultures of *Leptospirillum* MT6 were used in this experiment, it is not possible to comment on how these compared with mixed cultures containing *Am. ferrooxidans*. Also, since bacterial populations were not determined, it was not known whether *Leptospirillum* MT6 or *Am. ferrooxidans* was the dominant iron-oxidiser in mixed cultures. This issue was, however, addressed later in bioreactor experiments (chapter 5).

Culture DOC concentrations often appeared to correlate with pyrite oxidation. There were some exceptions: DOC concentrations (~100 mg/l) were greater in mixed cultures of *Leptospirillum* MT6 + *Sulfobacillus* NC + *At. caldus* than in mixed cultures of *Leptospirillum* MT6 + *Sulfobacillus* NC alone (~50 mg/l) though oxidation of pyrite was superior in the latter cultures (Figure 4.16). Greater DOC was probably due to the large numbers of autotrophic *At. caldus* in the former cultures, even though this sulfur-oxidiser did not appear to contribute to net pyrite oxidation.

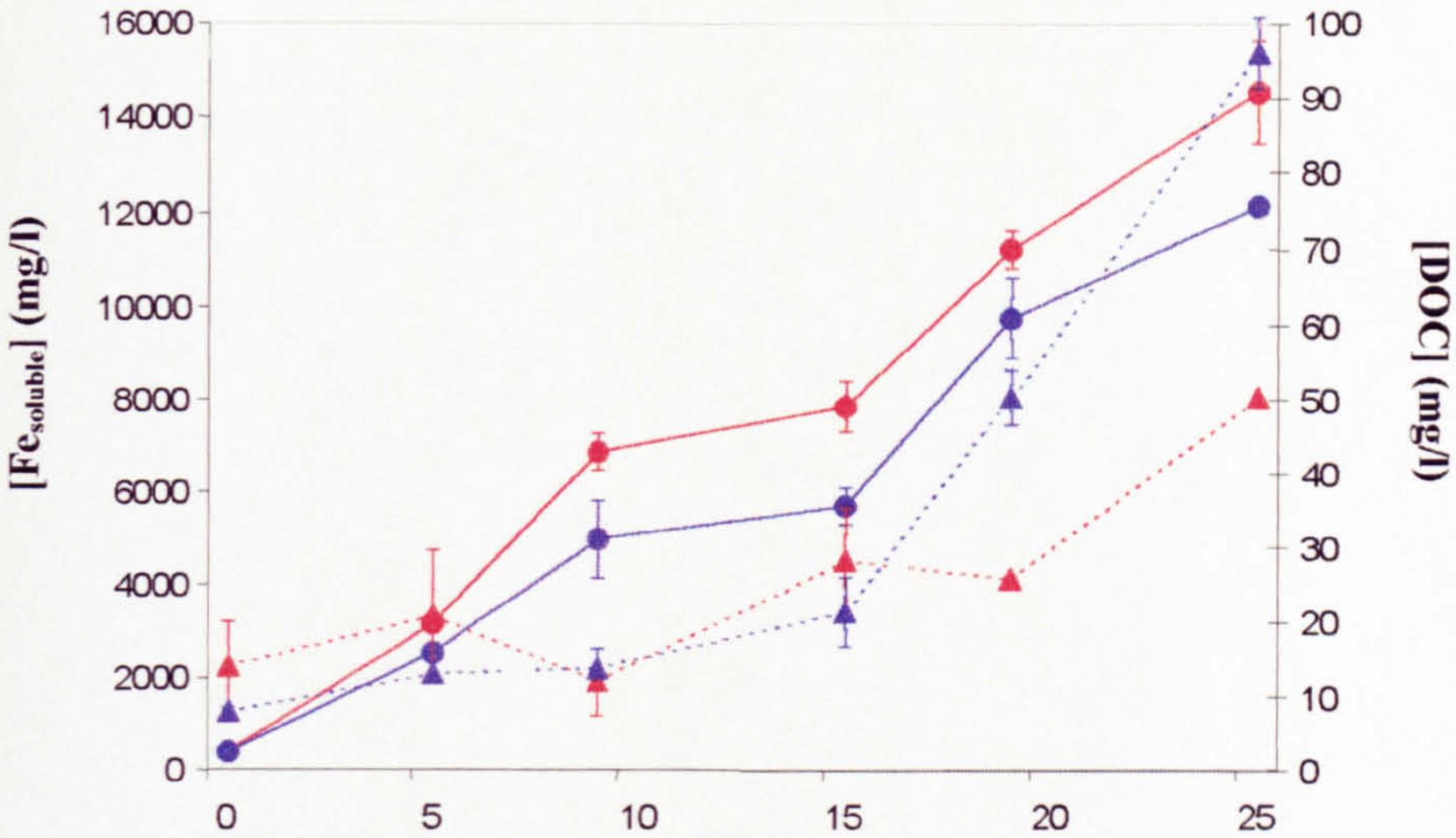


Figure 4.16: Oxidation of pyrite concentrate (solid lines) and DOC concentrations (broken lines) in mixed cultures of *Leptospirillum* MT6 and *Sulfobacillus* NC (▲●) and in mixed cultures of *Leptospirillum* MT6, *Sulfobacillus* NC and *At. caldus* (▲●). (Evaporation of water was not taken into account.)

The DOC that accumulated in some of these cultures were surprisingly large for chemoautotrophic systems. Some idea of the scale of biomass required for the DOC concentrations recorded can be gained from the following. Since a typical bacterium weighs ~10^{-12} g and ~50% of bacterial dry weight (10% of the wet weight) is carbon, a typical bacterium contains 10^{-13} g carbon. Therefore, a DOC concentration of 100 mg/l (=10^{-4} g/ml) is equivalent to 10^{9} bacteria/ml. Soluble organic materials originate as exudates from active cells, as well as lysates from dead and dying cells (not all of which would be soluble), and about 10% of the carbon fixed by *At. ferrooxidans* has been estimated to be present as small molecular weight exudates in iron-grown cultures (Schnaitman and Lundgren, 1965).

There was some evidence of utilisation of soluble carbon materials by mixotrophic iron-oxidisers. Mixed cultures containing *Leptospirillum* MT6, *Sulfobacillus* NC, *At. caldus*

and isolate GSM had lower DOC concentrations than mixed cultures that excluded isolate GSM. This might have resulted from isolate GSM consuming organic materials in mixed cultures. Similarly, although mixed cultures of *Am. ferrooxidans* ICP and an autotrophic *At. caldus* and/or *Leptospirillum* MT6 showed greater pyrite oxidation than pure cultures of *Am. ferrooxidans*, DOC concentrations were almost the same in all cultures. This might have resulted from strain ICP consuming carbon materials originating from the autotroph(s), though this was not the case with strain TH3. Removal of organic carbon might also explain why mixed cultures containing *Am. ferrooxidans* ICP were superior at oxidising rock pyrite and pyrite concentrate than those containing strain TH3. The reason why DOC concentrations were markedly greater in pure cultures of *Am. ferrooxidans* TH3 when oxidising pyrite concentrate than when oxidising rock pyrite is not clear.

Redox potentials (reflecting ratios of Fe^{3+}/Fe^{2+}) also correlated often with pyrite oxidation, though this trend was not consistent when different types of pyrite were compared (redox potential of *Am. ferrooxidans* ICP pure cultures were much lower with pyrite concentrate than with rock pyrite, despite pyrite oxidation being greater with the concentrate). Again, the reason for this is not clear.

Clearly, with increasing awareness of the biodiversity of moderately thermophilic and thermotolerant prokaryotes, the use of defined mixed populations of these microorganisms to maximise the oxidation of sulfidic minerals merits further study. The results from the experiments described in this chapter indicate that defined mixed cultures of these prokaryotes may accentuate or diminish the rates and extent of sulfide mineral oxidation, relative to pure cultures. Pyrite oxidation by mixed cultures of

moderate thermophiles was investigated further using pH-controlled bioreactors in chapter 5.

Chapter 5

Biooxidation of Pyrite by Defined Mixed Cultures of Moderately Thermophilic Acidophiles: pH-Controlled Bioreactors

5.1 Introduction

In shake flask experiments (chapter 4), it was found that some mixed cultures of moderate thermophiles were superior to pure cultures in oxidising pyrite. In this chapter, using rock pyrite (from the Cae Coch mine) as the test sulfide mineral, selected combinations of moderate thermophiles were tested for mineral oxidation in pH-controlled bioreactors. The first biooxidation system tested used various combinations of *Leptospirillum* MT6, *Am. ferrooxidans* ICP, *At. caldus* KU and a non-iron/sulfur-oxidising heterotrophic isolate, *Alicyclobacillus* Y004. A later system assessed pyrite oxidation by mixed cultures of *Leptospirillum* MT6 and *Ferroplasma* MT17, in the presence and absence of *At. caldus* KU. *Ferroplasma*-like acidophiles had been found to become gradually dominant in pilot plant aerated stirred tanks as mineral oxidation progressed (section 3.2). Therefore, *Ferroplasma* MT17 was chosen to be included in this system to examine the role of this novel isolate in longer-term mineral oxidation in bioreactors.

5.2 Methods

The iron-oxidising acidophiles used were pre-grown in 2% pyrite medium (Cae Coch rock pyrite, pH 1.5; section 2.2.1.1.4). Yeast extract was added (0.02%, w/v) to the pyrite medium when growing *Ferroplasma* MT17. *At. caldus* and *Alicyclobacillus* Y004 were pre-grown in tetrathionate medium (section 2.2.1.1.2) and heterotrophic medium (section 2.2.1.1.3), respectively. Bacteria and archaea were enumerated using a Thoma counting chamber (section 2.2.2.2.1) and bioreactors (section 2.2.1.3) containing 1.5 L of 5% pyrite medium (Cae Coch rock pyrite, pH 1.5) were inoculated with 2.0×10^9 cells of each acidophile (resulting in 1.3×10^6/ml of each acidophile at the start of the experiments).

Bioreactors were run as follows:

System 1.
Leptospirillum MT6 + *Am. ferrooxidans* ± *At. caldus*
(run in parallel for 46 days)

System 2.
Leptospirillum MT6 + *At. caldus* ± *Am. ferrooxidans*
(run in parallel for 43 days)

System 3.
Leptospirillum MT6 ± *Alicyclobacillus* Y004
(run for 44 days)

System 4.
Am. ferrooxidans ± *At. caldus*
(run for 44 days)

System 5.
Leptospirillum MT6 + *Ferroplasma* MT17 ± *At. caldus*
(run in parallel for 44 days)

Culture pH was controlled automatically by addition of 2 M H_2SO_4 or 2M NaOH to the bioreactors. The initial pH was 1.50 (with lower/upper limits of 1.45/1.55) and, at day 32 or 33, pH control was removed for about 3 days and pH changes were monitored. After that, the pH was set at 1.20 (with lower/upper limits of 1.15/1.25) and then to 1.0 (with lower/upper limits of 0.95/1.05). Bioreactors were aerated at 0.2 L of atmospheric air/min, and stirred at 100 rpm. Acid and alkali inputs were monitored regularly. Prior to sampling, bioreactors were stirred vigorously (300 rpm) for approximately 5 minutes to distribute the pyrite equally in the cultures. Samples were removed for analysis of total soluble iron (section 2.4.3), ferrous iron (section 2.4.2.2), sulfate (section 2.4.5), dissolved organic carbon (DOC) (section 2.4.7) and microbial populations. The latter involved plating serially-diluted samples onto ferrous iron and ferrous iron/tetrathionate overlay plates (sections 2.2.1.2.1.1 and 2.2.1.2.1.2), and using FISH (section 2.5.12). Redox potentials were calculated from measured ferrous iron and ferric iron concentrations using the Nernst equation (equation 5.1):

$$E = E^{\circ} + RT/nF.\log^{[Fe(III)]}/_{[Fe(II)]} \qquad [5.1]$$

Where E is the redox potential, E° the standard redox potential (+770 mV for the ferrous/ferric couple at pH 2.0), R is the gas constant, T is temperature (°K), n is the number of electrons involved in the half-cell couple (1 for ferrous/ferric), and F is the Faraday constant. At 20°C, for the ferrous/ferric couple, this simplifies as:

$$\text{Redox potential (mV)} = 770 + 59.2 \log ([Fe^{2+}]/[Fe^{3+}]) \qquad [5.2]$$

To analyse relative numbers of microbes using FISH, fixed cells were hybridised with a Cy3-labelled probe that targeted a specific acidophile, and simultaneously with a fluorescein-labelled eubacterial probe that targeted all eubacterial cells. Relative numbers of a specific acidophile were compared to total numbers of eubacterial cells targeted by a general eubacterial probe, and also to total numbers of microorganisms

stained with DAPI in the same field of view, to work out the abundance of a particular acidophile. To target *Leptospirillum* MT6, *Am. ferrooxidans* ICP, *At. caldus* KU and *Ferroplasma* MT17, LF655, ACM995, THC642 and FER656 probes (all Cy3-labelled) were used (section 2.5.12, Table 2.5). The abundance of *Alicyclobacillus* Y004 in the reactor containing *Leptospirillum* MT6 and *Alicyclobacillus* Y004 was calculated by subtracting numbers of *Leptospirillum* MT6 from those of total eubacterial cells. Since *Ferroplasma* MT17 was the only archaeon used in these experiments, when this acidophile was included in mixed cultures, total cell numbers were calculated by adding numbers of eubacterial cells (targeted by EUB338Fl probe) to those of *Ferroplasma* MT17 cells (using the FER656 probe) in the same field of view.

5.3 Results

5.3.1 Total soluble iron and sulfate concentrations and pyrite oxidation rates

Total soluble iron and sulfate concentrations in bioreactors containing pure or mixed cultures of *Leptospirillum* MT6, *At. caldus*, *Am. ferrooxidans* and *Alicyclobacillus* Y004 are shown in Figure 5.1-I. Bioreactors containing mixed cultures of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* were run twice (Figure 5.1-Ia and b), and displayed slightly different extent of pyrite oxidation, possibly caused by different lag phases. Data from experiments using *Ferroplasma* MT17 are shown in Figure 5.1-II.

A number of trends are apparent in Figures 5.1-I & -II. Firstly, there were more pronounced lag periods before pyrite oxidation commenced when *Leptospirillum* MT6 than when *Am. ferrooxidans* was the iron-oxidiser present. In mixed cultures containing both these acidophiles, lag periods were again less than with *Leptospirillum* MT6 alone. Secondly, there was a marked "flattening off" of pyrite oxidation in some of these experiments. This was not due to the pyrite being limiting; even in cultures which displayed the greatest extents of pyrite oxidation, the iron solubilised represented only about 50% of that present in the pyrite (5%, w/v, of ground rock pyrite, which contains ~80% FeS_2, would give a theoretical maximum concentration of 18,567 mg soluble iron/l). Thirdly, there were clear differences, in many cases, between rates and extents of pyrite oxidation in bioreactor cultures run in parallel. The most dramatic of these were the far greater pyrite oxidation observed by mixed culture of *Am. ferrooxidans*+*At. caldus* than by the pure culture of *Am. ferrooxidans* (Figure 5.1-Id), and by the mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* than by the *Leptospirillum* MT6+*Ferroplasma* MT17 mixed culture (Figure 5.1-II). The mixed culture containing *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* was superior both

to the mixed culture containing *Leptospirillum* MT6+*At. caldus* (Figure 5.1-Ia) and to that containing *Leptospirillum* MT6+*Am. ferrooxidans* (Figure 5.1-Ib). Differences between the mixed culture containing *Leptospirillum* MT6+*Alicyclobacillus* Y004 and the pure culture of *Leptospirillum* MT6 were marginal. The most notable difference was the smaller lag period with the mixed culture, though this have been due, at least in part, to these two cultures being slightly out of synchrony (the pure culture was commissioned later than the mixed culture, and the *Leptospirillum* inoculum was possibly less active in the former case).

When comparing pyrite oxidation by pure and mixed cultures that were run at different times, factors such as that mentioned above, need to be borne in mind. However, there were interesting trends in both the rates at which pyrite was oxidised and the extents of pyrite oxidation (estimated as maximum concentrations of soluble iron and sulfate-sulfur: Table 5.1). The least effective pyrite oxidising system of those tested (in terms of rates and yields) was the pure culture of *Am. ferrooxidans*, while the mixed culture of this iron-oxidiser and the sulfur-oxidiser *At. caldus* was one of the most effective cultures used. Interestingly, although total pyrite solubilised was similar in the *Am. ferrooxidans*+*At.caldus*+*Leptospirillum* MT6 culture, the rates of pyrite oxidation in both culture runs were less than that in the *Am. ferrooxidans*+*At. caldus* system. One of the highest rates of pyrite oxidation occurred with the pure *Leptospirillum* MT6 culture, though maximum soluble iron in this culture was about 25% lower than in the *Am. ferrooxidans* mixed cultures. Data in Table 5.1 also indicate that, whilst *At. caldus* had a positive impact on pyrite oxidation by *Am. ferrooxidans*, it had a negative impact on *Leptospirillum* MT6.

The "flattening off" of pyrite oxidation was most obvious in cultures where the only iron-oxidiser (and therefore primary mineral-oxidiser) was *Am. ferrooxidans*, and also

in the *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* mixed culture. In most mixed cultures that included *Leptospirillum* MT6, pyrite oxidation continued even when the pH was lowered to pH 1.2 (Figure 5.1-I). This suggests that, given more prolonged incubation, final yields of iron solubilised would have been greater in cultures containing the Gram-negative iron-oxidiser. The exceptions to this were cultures which included both *Ferroplasma* MT17 and *Leptospirillum* MT6 (Figure 5.1-II). Interestingly, although *Ferroplasma* MT17 appeared to retard pyrite oxidation by *Leptospirillum* MT6 (as observed in shake flask experiments; chapter 4) the mixed culture of these two iron-oxidisers plus *At. caldus* was the most effective of those tested, both in terms of rate and extent of pyrite oxidised (Table 5.1).

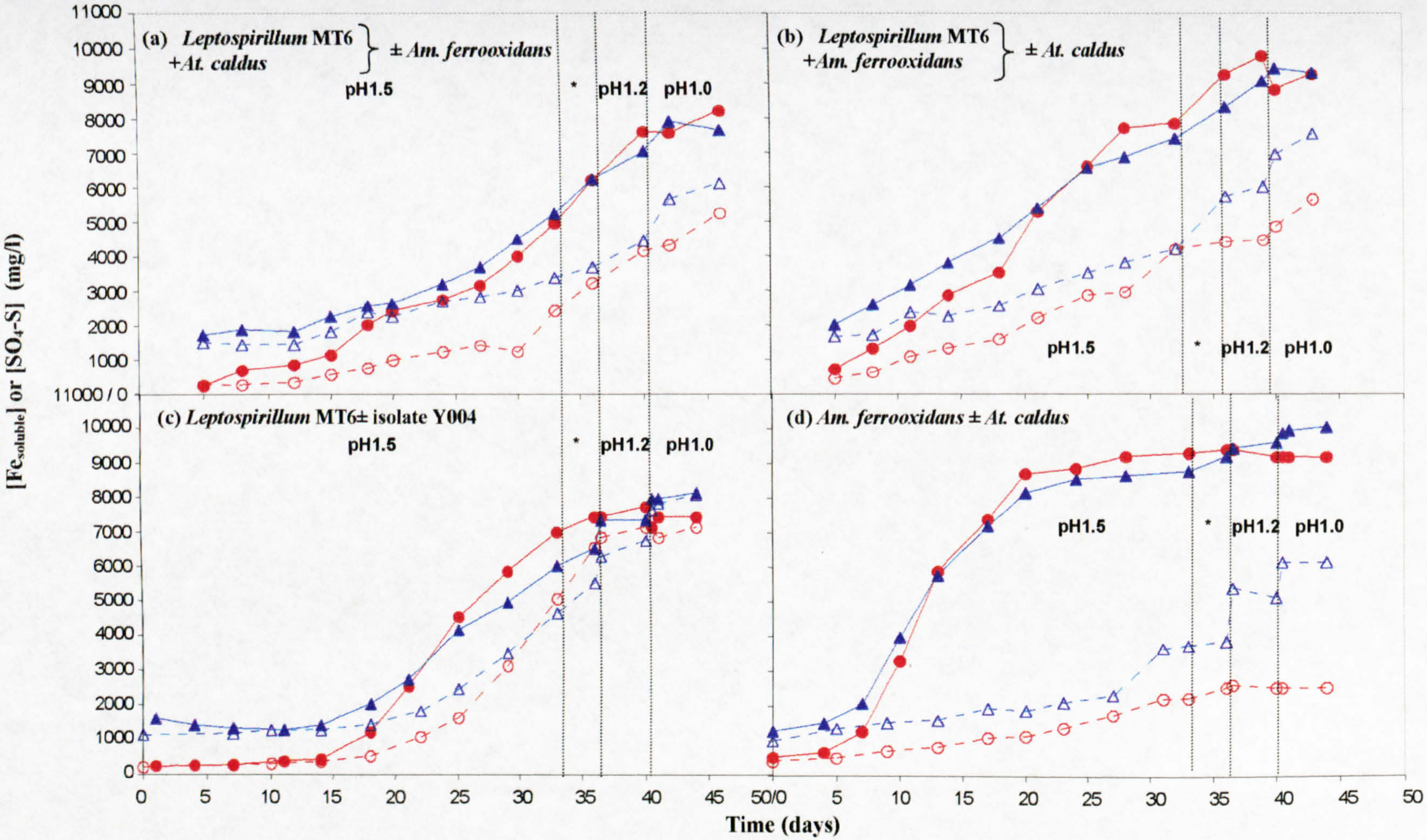


Figure 5.1-I: Total soluble iron (● ○) and sulfate concentrations (▲ △) in pyrite-oxidising bioreactors. (a) *Leptospirillum* MT6+*At. caldus*+*Am. ferrooxidans* (solid lines, ● ▲) and *Leptospirillum* MT6+*At. caldus* (broken lines, ○ △); (b) *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* (solid lines, ● ▲) and *Leptospirillum* MT6+*Am. ferrooxidans* (broken lines, ○ △); (c) *Leptospirillum* MT6+*Alicyclobacillus* Y004 (solid lines, ● ▲) and *Leptospirillum* MT6 (broken lines, ○ △); (d) *Am. ferrooxidans*+*At. caldus* (solid lines, ● ▲) and *Am. ferrooxidans* (broken lines, ○ △). * indicate when pH control was removed.

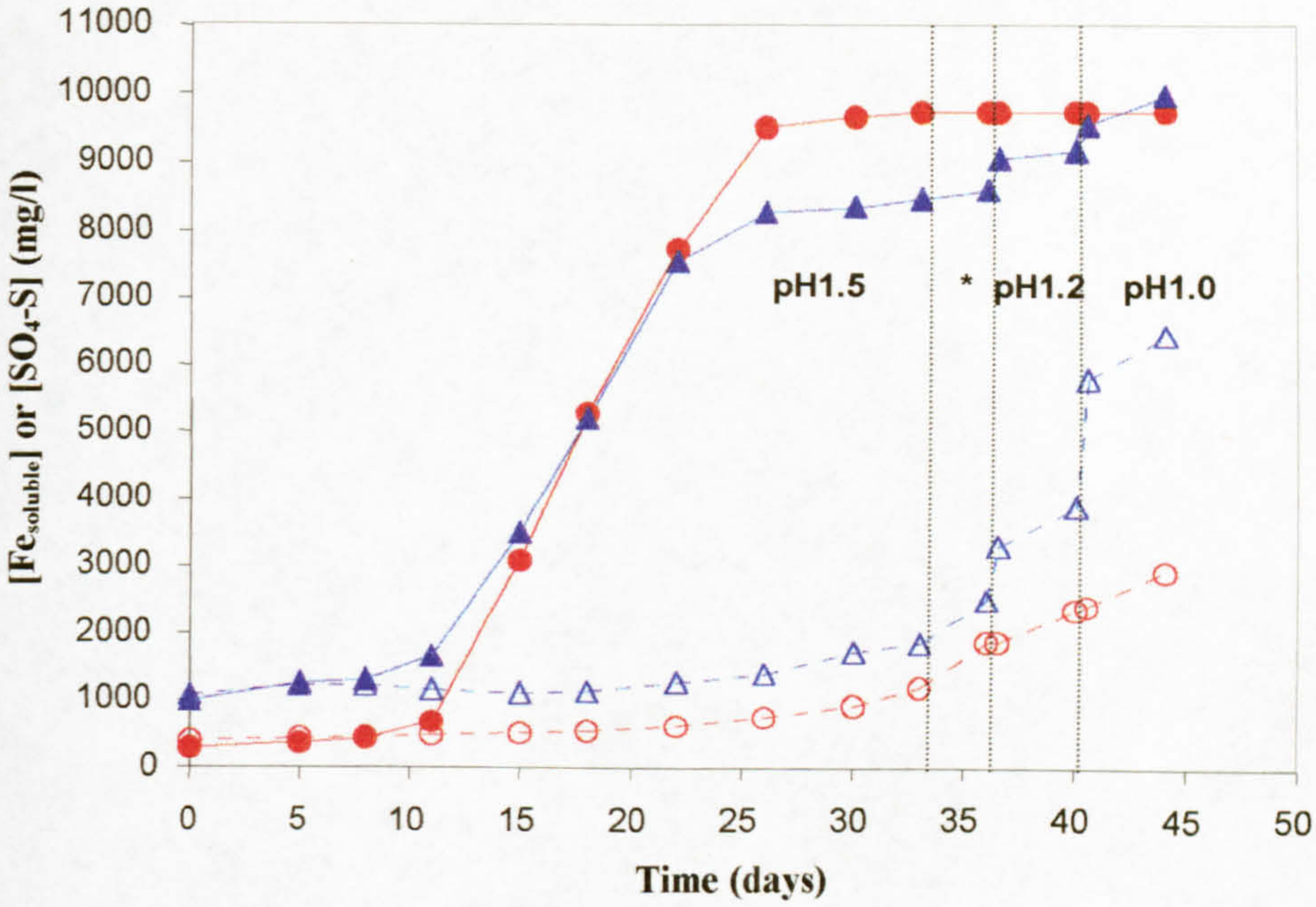


Figure 5.1-II: Total soluble iron (● ○) and sulfate concentrations (▲ △) in pyrite-oxidising bioreactors containing *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* (● ▲) and *Leptospirillum* MT6+*Ferroplasma* MT17 (○ △). * indicates when pH control was removed.

Table 5.1: Maximum total soluble iron and sulfate-S (corrected for sulfate present at day 0) concentrations, and pyrite oxidation rates in pure and mixed cultures of acidophilic microorganisms.

	Maximum [$Fe_{soluble}$] (mg/l)	Maximum [sulfate-S] (mg/l) *	Maximum Pyrite oxidation rates (mg Fe/day)
Leptospirillum MT6+*At. caldus* +*Am. ferrooxidans* **	8200 & 9700	5100 & 7400	290 & 320
Leptospirillum MT6+*At. caldus*	5200	2900	150
Leptospirillum MT6+*Am. ferrooxidans*	5600	4500	150
Leptospirillum MT6+*Alicyclobacillus* Y004	7400	5600	390
Leptospirillum MT6	7100	5400	570
Am. ferrooxidans+*At. caldus*	9200	8300	550
Am. ferrooxidans	2500	1100	83
Leptospirillum MT6+*Ferroplasma* MT17 +*At. caldus*	9700	8200	610
Leptospirillum MT6+*Ferroplasma* MT17	3000	2700	120

* corrected for sulfate-S present in the medium; ** data from two separate experimental runs.

When pH control of the cultures was temporarily suspended (at about day 33, when culture pH was 1.45-1.50) the pH of all cultures fell somewhat, but to different extents (Table 5.2). The extent to which pH declined over these 3-day periods appeared to correlate with pyrite oxidation rates during the time that pH control was suspended, and was greatest in mixed cultures of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus*. Subsequently, when pH control of cultures was re-established (at lower pH values) there were increases in sulfate concentrations, due to the addition of sulfuric acid (Figure 5.1).

Table 5.2: pH changes over 3-day periods, in pure and mixed cultures following suspension of pH control.

	pH change
Leptospirillum MT6+*At. caldus*+*Am. ferrooxidans*	1.45→1.32 (Figure 5.1-Ia) 1.45→1.31 (Figure 5.1-Ib)
Leptospirillum MT6+*At. caldus*	1.46→1.44
Leptospirillum MT6+*Am. ferrooxidans*	1.46→1.40
Leptospirillum MT6+*Alicyclobacillus* Y004	1.47→1.46
Leptospirillum MT6	1.45→1.39
Am. ferrooxidans+*At. caldus*	1.50→1.43
Am. ferrooxidans	1.50→1.47
Leptospirillum MT6+*Ferroplasma* MT17+*At. caldus*	1.50→1.41
Leptospirillum MT6+*Ferroplasma* MT17	1.48→1.43

5.3.2 Stoichiometry of pyrite oxidation

When pyrite is fully oxidised, two moles of sulfate are generated for each mole of soluble iron released, assuming the mineralogical formula FeS_2 for the mineral In Figure 5.2, sulfate concentrations in bioreactors (that resulting from pyrite oxidation, and excluding sulfate that was initially present in medium) were compared to "theoretical sulfate concentrations", calculated from multiplying soluble iron concentrations (mM) by two. Sulfate concentrations at the first sampling point were adjusted to 0 mM, except for the mixed culture of *Leptospirillum* MT6+*At. caldus* where sulfate concentrations at day 15 were adjusted to 0 mM, due to acid input to this culture up to day 15 (Figure 5.3).

Data, shown in Figure 5.2, show that in some cultures there was a close fit between theoretical and actual sulfate concentrations, though in other cultures the two were significantly different, with actual sulfate concentrations being much lower than those predicted from concentrations of soluble iron. Greatest divergence was found in cultures which exhibited more extensive pyrite oxidation.

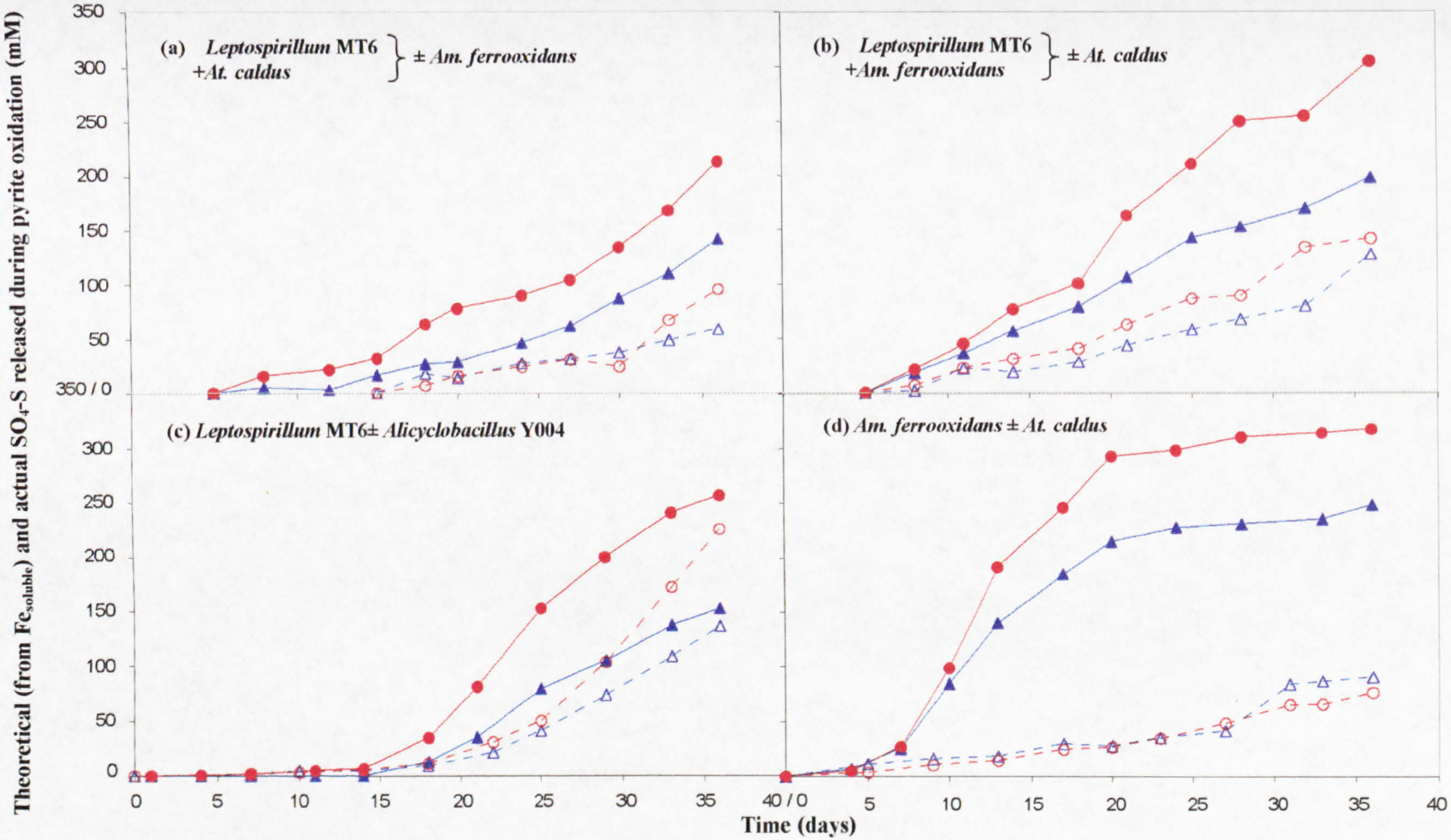


Figure 5.2-I: Theoretical sulfate-S concentrations achieved by complete oxidation of pyrite (● ○) and actual sulfate concentrations in bioreactors (▲ △). (a) *Leptospirillum* MT6+*At. caldus*+*Am. ferrooxidans* (solid lines, ● ▲) and *Leptospirillum* MT6+*At. caldus* (broken lines, ○ △); (b) *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* (solid lines, ● ▲) and *Leptospirillum* MT6+*Am. ferrooxidans* (broken lines, ○ △); (c) *Leptospirillum* MT6+*Alicyclobacillus* Y004 (solid lines, ● ▲) and *Leptospirillum* MT6 (broken lines, ○ △); (d) *Am. ferrooxidans*+*At. caldus* (solid lines, ● ▲) and *Am. ferrooxidans* (broken lines, ○ △).

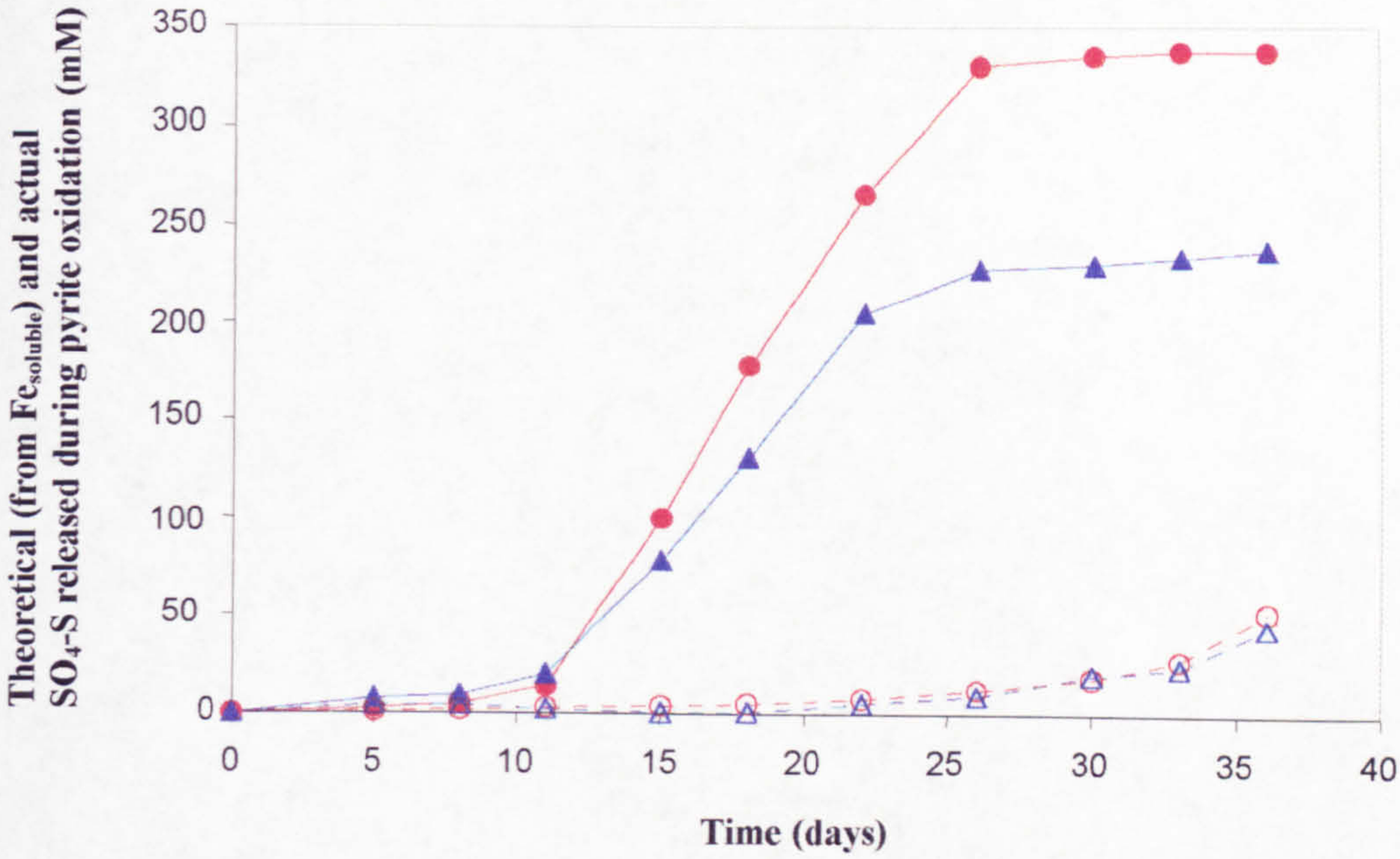


Figure 5.2-II: Theoretical sulfate-S concentrations achieved by complete oxidation of pyrite (● ○) and actual sulfate concentrations (▲ △) in bioreactors containing *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* (solid lines, ● ▲) and *Leptospirillum* MT6+*Ferroplasma* MT17 (broken lines, ○ △).

5.3.3 pH control and acid and alkali input

The amount of alkali (added as 2M NaOH) used to control pH in the bioreactor cultures is shown in Figure 5.3. Alkali addition was required in the most active cultures since pyrite oxidation is an acid-generating reaction. Apart from that which was required to drop culture pH to either 1.2 or 1.0, no acid was required to control pH in the bioreactors, with the single exception of the mixed culture of *Leptospirillum* MT6+*At. caldus*. With this culture, acid was pumped in to maintain pH 1.5 until day 15 (data not shown). Between days 0 and 15, there was no detectable increase of soluble iron concentrations in this culture and the initial ferrous iron was almost completely oxidised (Figure 5.4-Ia), resulting in proton consumption in the culture.

There was an apparent correlation between the extent of pyrite oxidation and the amount of alkali required to maintain the pH at 1.5 during the first phase of each of the bioreactor experiments. Also, in those experiments where cultures including *At. caldus* were compared with those not containing this sulfur-oxidiser, there was a very marked increase in alkali consumption in the former (Figures 5.3-Ib and 5.3-II). At least some of this may be accounted for by the more extensive pyrite oxidation in the mixed cultures that included *At. caldus*; however, whilst *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* oxidised about twice as much pyrite from day 0-32 than the corresponding culture not containing *At. caldus*, alkali consumption was about 4-fold greater in the former culture.

During the "free fall" periods (with a lower pre-set limit of pH 1.2), since culture pH did not go less than 1.2 in all bioreactors, there was no alkali input. At pH 1.2, there was still some alkali input in mixed cultures containing *Leptospirillum* MT6+*At. caldus*+*Am. ferrooxidans* (Figures 5.3-Ia and b). Although concentrations of total soluble iron still increased to some extent at pH 1.0 in these mixed cultures, this did not result in additional alkali input.

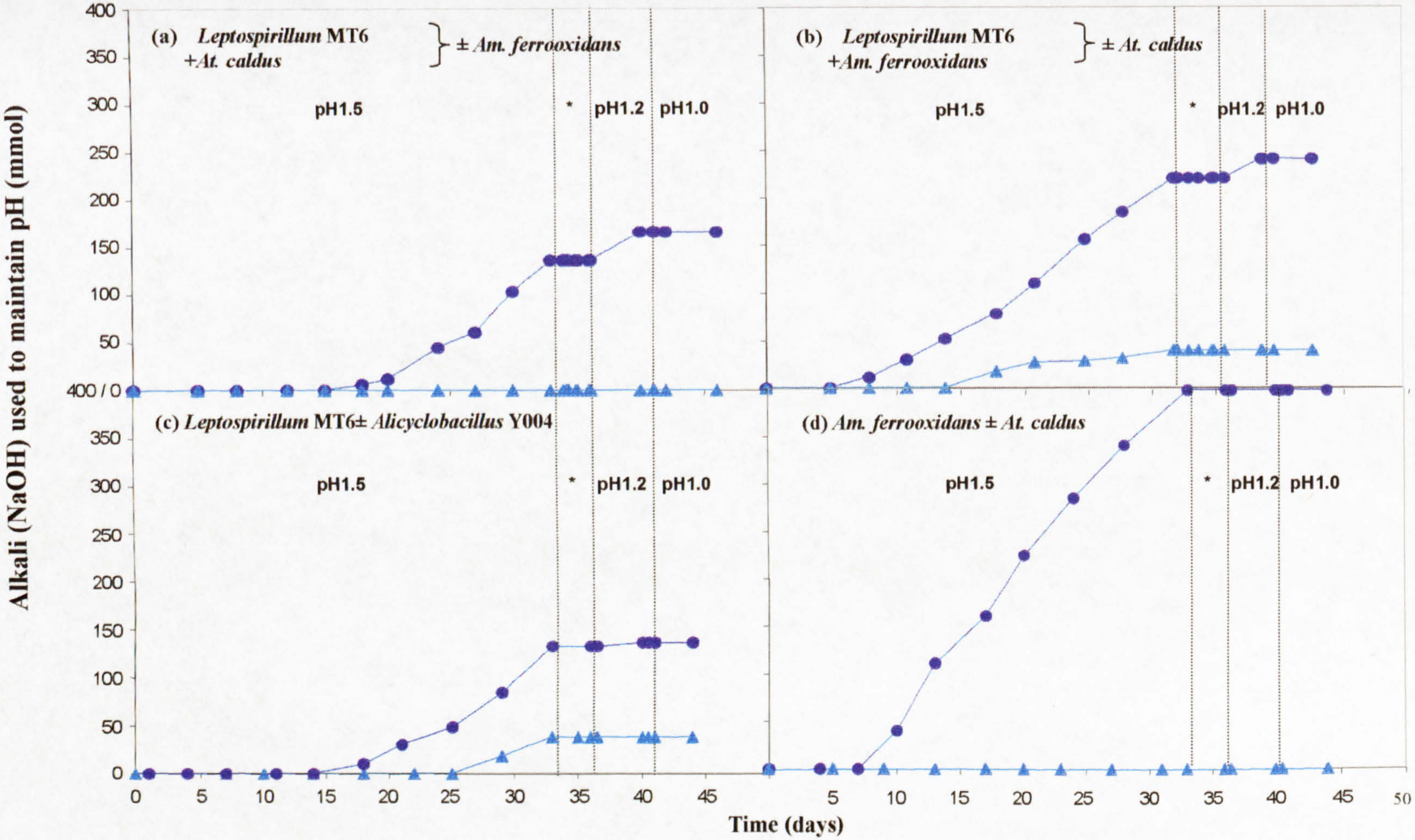


Figure 5.3-I: Alkali added to bioreactors to maintain pH. (a) *Leptospirillum* MT6+*At. caldus*+*A. ferrooxidans* (●) and *Leptospirillum* MT6+*At. caldus* (▲); (b) *Leptospirillum* MT6+*A. ferrooxidans*+*At. caldus* (●) and *Leptospirillum* MT6+*A. ferrooxidans* (▲); (c) *Leptospirillum* MT6+*Alicyclobacillus*Y004 (●) and *Leptospirillum* MT6 (▲); (d) *A. ferrooxidans*+*At. caldus* (●) and *Am. ferrooxidans* (▲). * indicate when pH control was removed.

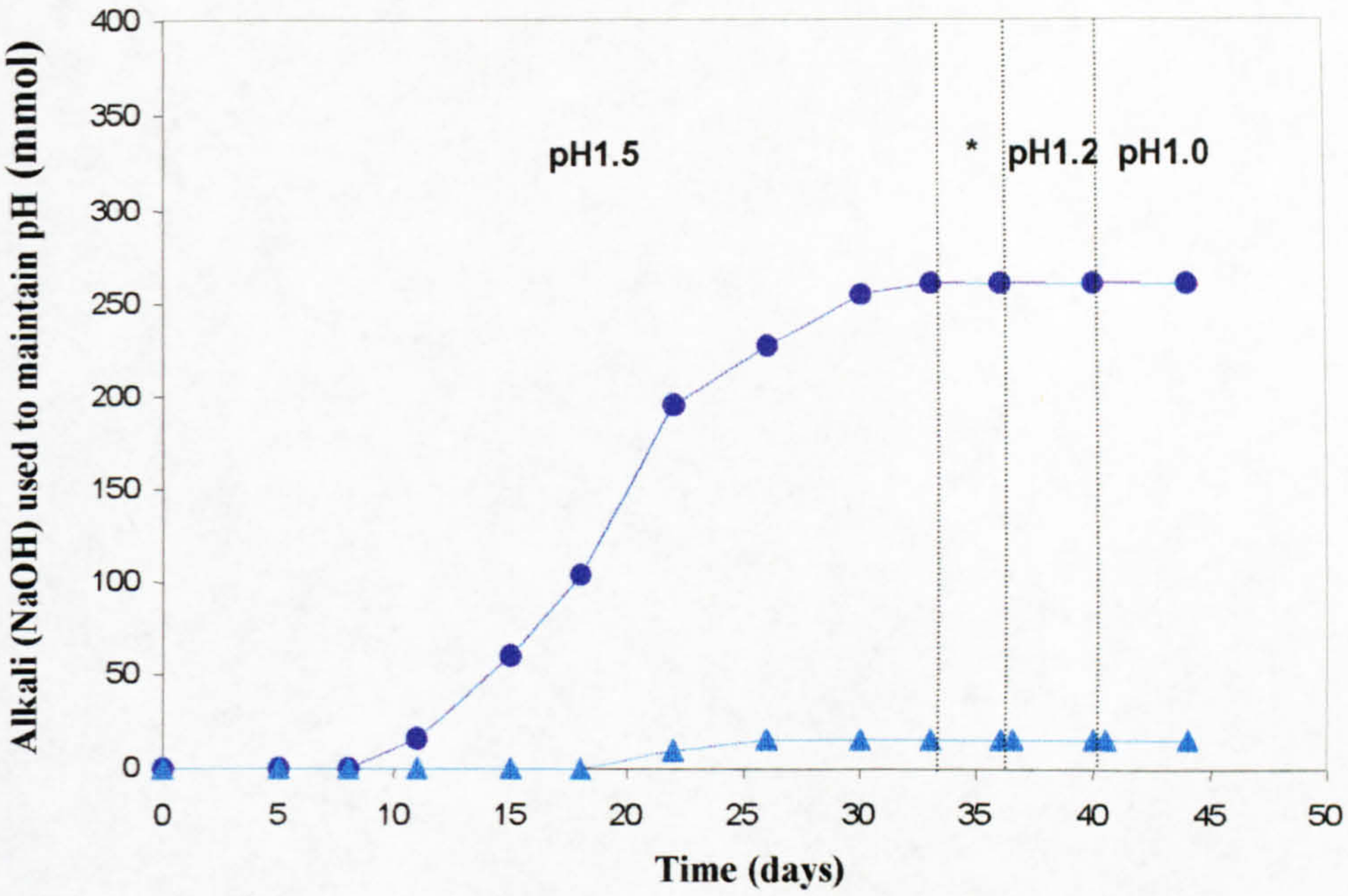


Figure 5.3-II: The amount of alkali used to maintain pH in bioreactors containing *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* (●) and *Leptospirillum* MT6+*Ferroplasma* MT17 (▲). * indicates when pH control was removed.

5.3.4 Ferrous iron concentrations and redox potentials

Ferrous iron concentrations and calculated redox potentials in bioreactors are shown in Figure 5.4. Although not determined in all cases, there was ~200-500 mg/l of ferrous iron present in the media at the start of the experiments, presumably resulting from the autoclaving of pyrite. In most cases, this ferrous iron was generally oxidised rapidly, particularly in mixed cultures that included *Am. ferrooxidans*. Thereafter, in most cases, concentrations of ferrous iron remained low (<250 mg/l) and redox potentials relatively high (+850-900 mV) throughout the experimental periods. The importance of continued aeration in maintaining high redox potentials is illustrated in Figure 5.4-Ia, where problems with the aeration supply at days 18, 24 and 27 caused a rapid increase in ferrous iron concentrations and consequent decreases in redox potentials. In the pure culture of *Am. ferrooxidans*, after some of the ferrous iron was oxidised by this bacterium by day 5, concentrations of ferrous iron increased throughout the experiment, resulting in redox potentials of <+770 mV, due to ferrous iron concentrations being always equal to or greater than those of ferric iron.

Contrasting redox potentials were also evident in cultures of *Leptospirillum* MT6+*Ferroplasma* MT17, with and without *At. caldus* (Figure 5.4-II). Higher redox potentials in the presence of the sulfur-oxidiser corresponded to the very different rates of pyrite oxidation observed with these cultures (Figure 5.1-II).

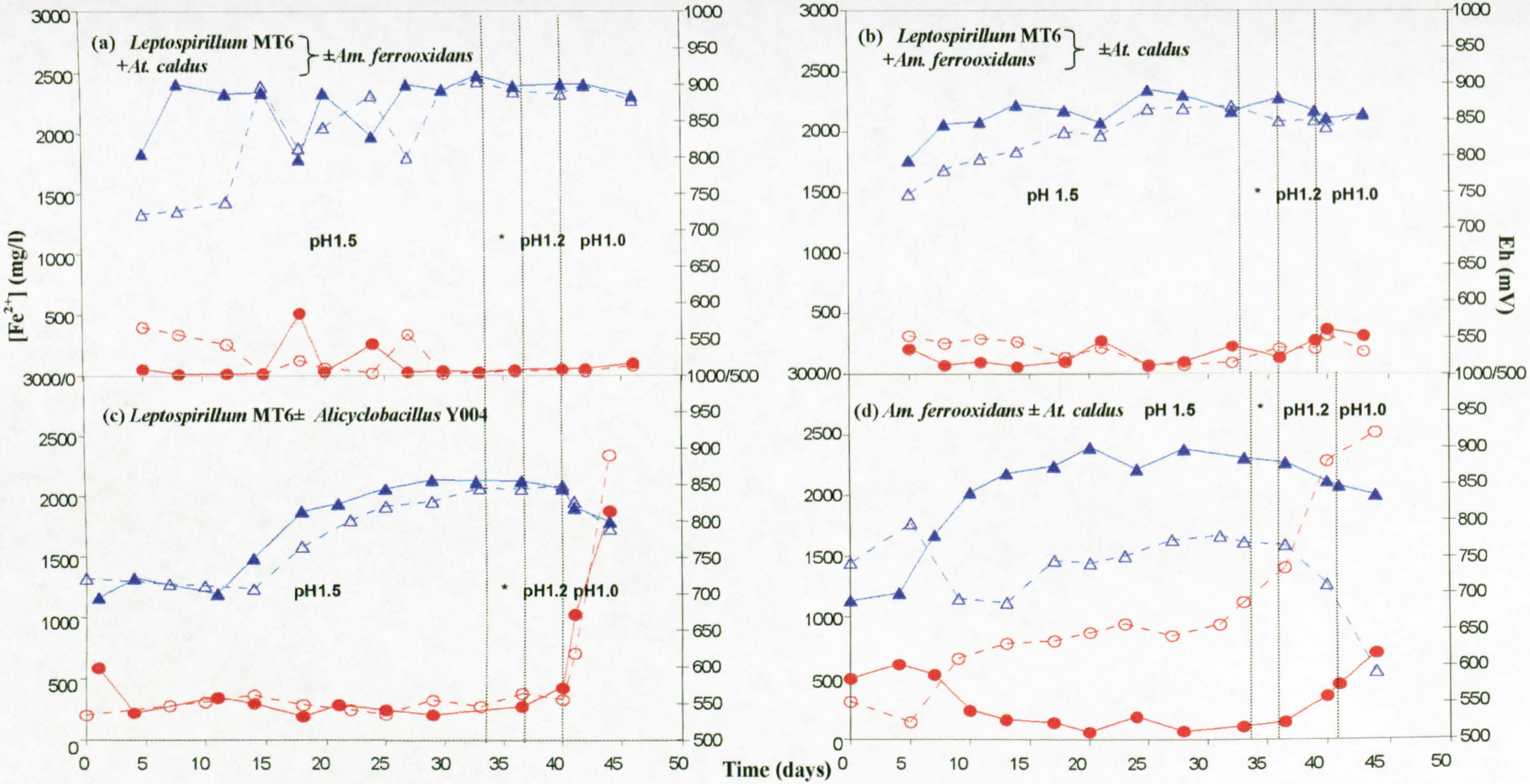


Figure 5.4-I: Ferrous iron concentrations (● ○) and redox potentials (▲ △) in bioreactors. (a) *Leptospirillum* MT6+*At. caldus*+*Am. ferrooxidans* (● ▲, solid lines) and *Leptospirillum* MT6+*At. caldus* (○ △, broken lines); (b) *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* (● ▲, solid lines) and *Leptospirillum* MT6+*Am. ferrooxidans* (○ △, broken lines); (c) *Leptospirillum* MT6+isolate Y004 (● ▲, solid lines) and *Leptospirillum* MT6 (○ △, broken lines); (d) *Am. ferrooxidans*+*At. caldus* (● ▲, solid lines) and *Am. ferrooxidans* (○ △, broken lines). * indicate when pH control was removed.

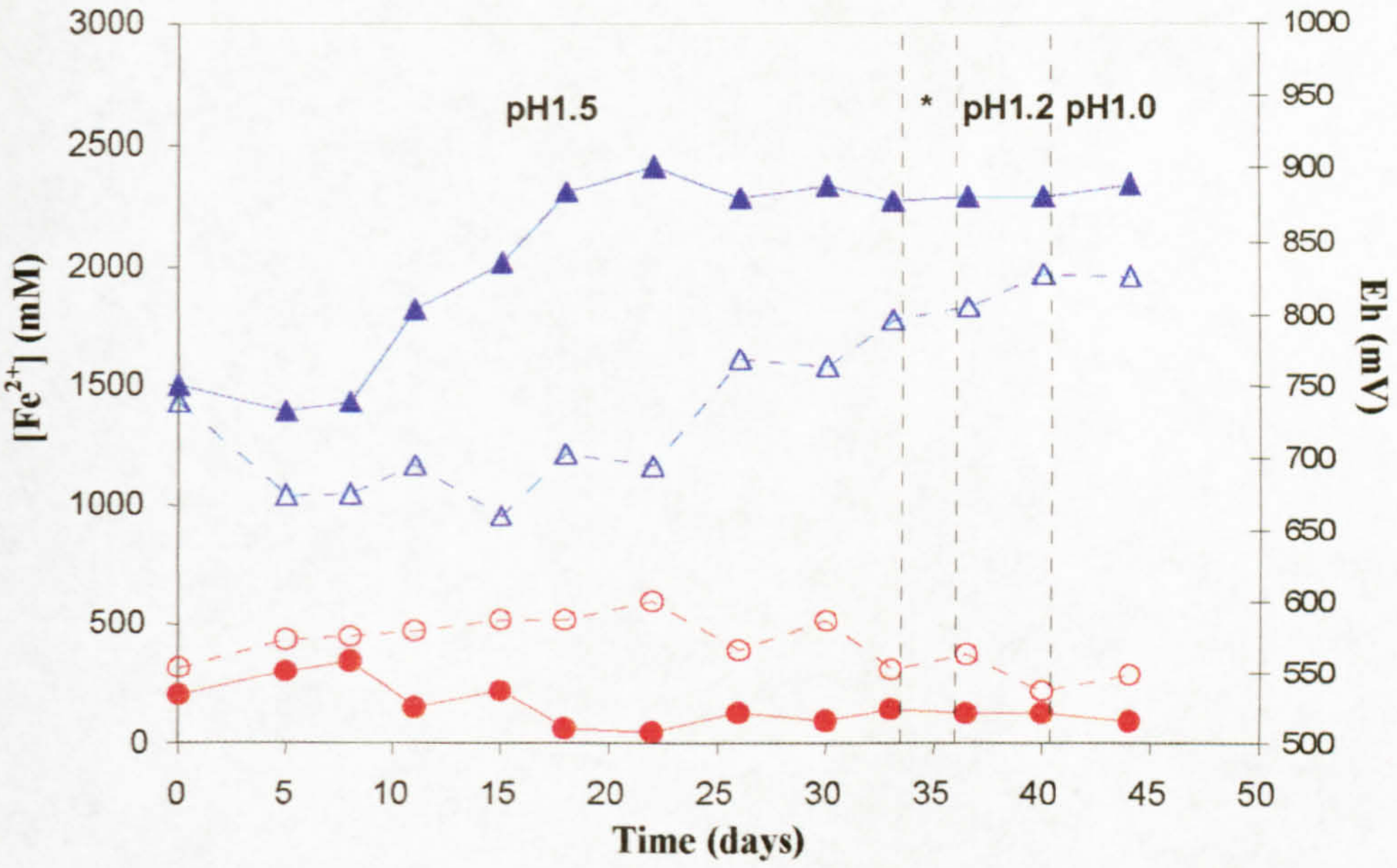


Figure 5.4-II: Ferrous iron concentrations (● ○) and redox potentials (▲ △) in bioreactors containing *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* (● ▲, solid lines) and *Leptospirillum* MT6+*Ferroplasma* MT17 (○ △, broken lines). * indicates when pH control was removed.

5.3.5 Microbial population changes and DOC concentrations

Microbial population changes in bioreactor cultures determined by plate counts and by FISH are shown in Figures 5.5-5.9. Total bacterial numbers determined by DAPI staining were inconsistent and did not appear to reflect actual bacterial numbers in bioreactors (Figure 5.10). Although cell preparation from pyrite media always followed the same procedure, DAPI counts showed wide variations. Therefore, FISH results shown here are relative numbers of bacteria, as percentages in pie graphs. With the FISH technique, all cells stained by DAPI were also detected with the EUB338-flu probe (except the archaeon, *Ferroplasma* MT17). Representative micrographs are shown in Figures 5.11 and 5.12.

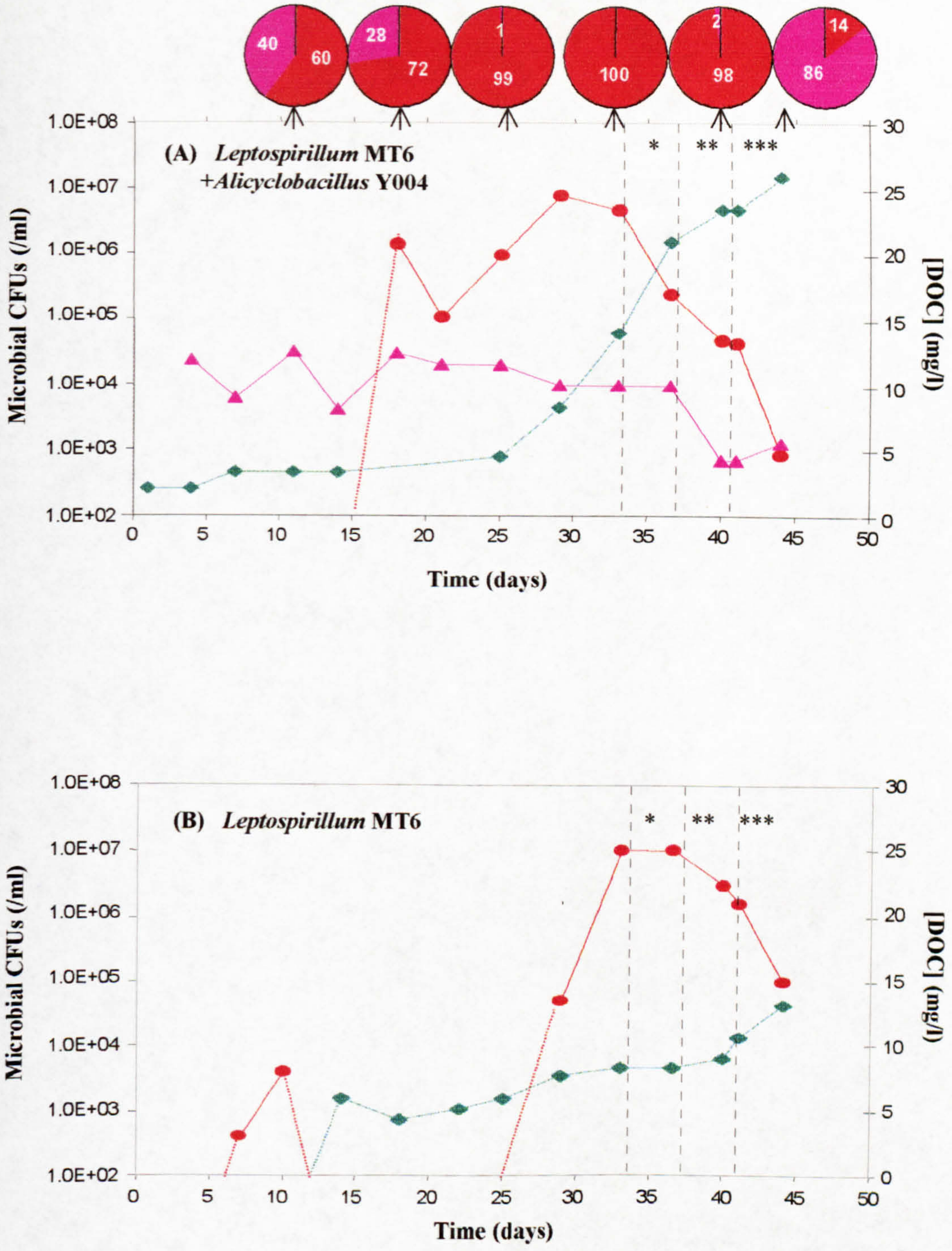


Figure 5.5: Microbial populations (from plate counts (CFUs) and FISH) and DOC concentrations in mixed culture of *Leptospirillum* MT6+*Alicyclobacillus* Y004 (A) and pure culture of *Leptospirillum* MT6 (B). Key: ●, *Leptospirillum* MT6; ▲, *Alicyclobacillus* Y004 (all plate counts); ◆, DOC concentrations. Pie charts show relative microbial counts in the bioreactors determined *via* FISH$^{(+)}$ (%). The colours in pie charts correspond to those in line charts. $^{(+)}$Arrows indicate times of FISH analysis. *, ** and *** indicate when pH control was removed, pH 1.2 and pH 1.0, respectively.

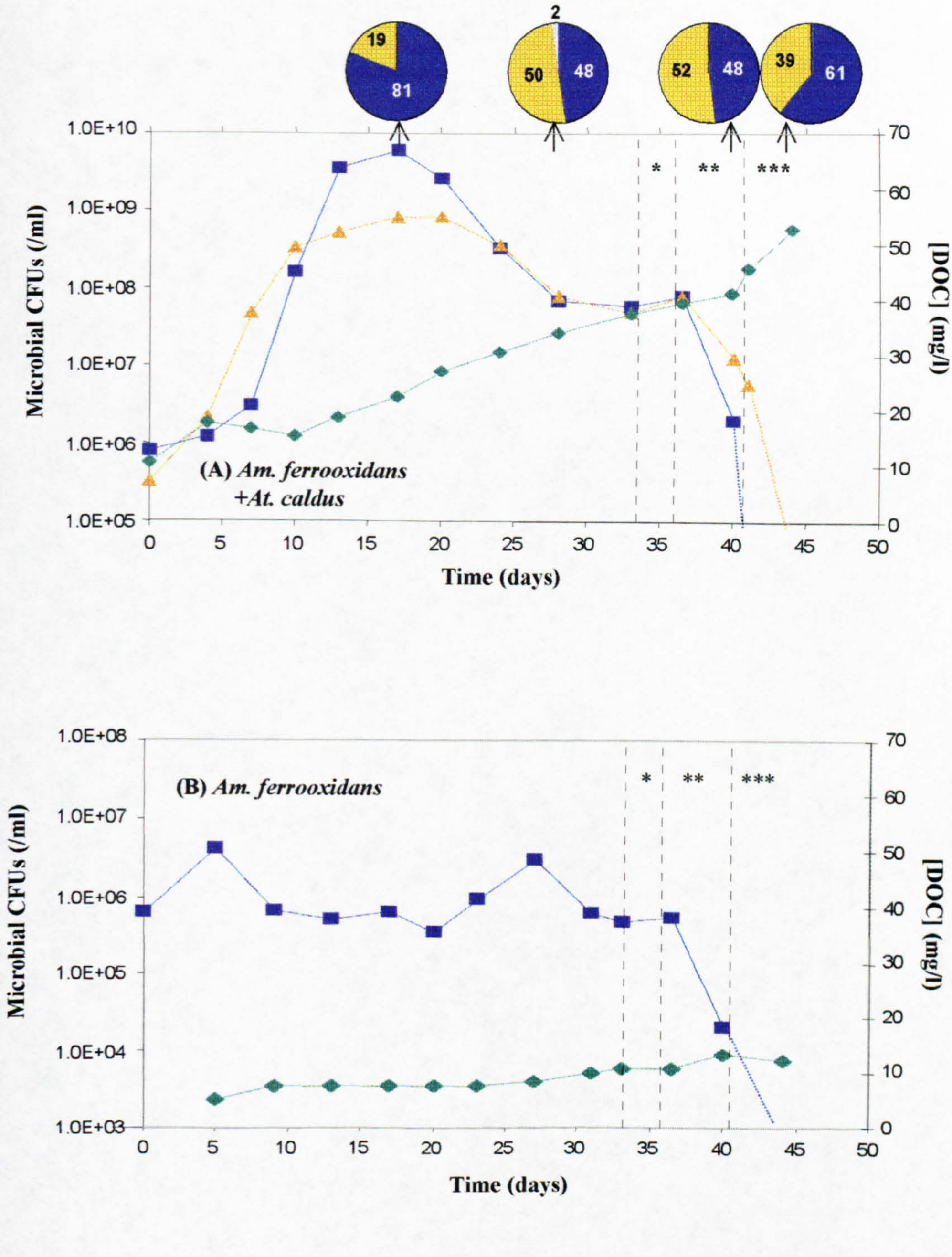


Figure 5.6: Microbial populations (from plate counts (CFUs) and FISH) and DOC concentrations in mixed culture of *Am. ferrooxidans*+*At. caldus* (A) and pure culture of *Am. ferrooxidans* (B). Key: ■, *Am. ferrooxidans*; ▲, *At. caldus* (all plate counts); ◆, DOC concentrations. Pie charts show relative microbial numbers in the bioreactors determined *via* FISH$^{(+)}$ (%). The colours in pie charts correspond to those in line charts (grey colour in pie charts represents unidentified microorganisms). $^{(+)}$Arrows indicate times of FISH analysis. *, ** and *** indicate when pH control was removed, pH 1.2 and pH 1.0, respectively.

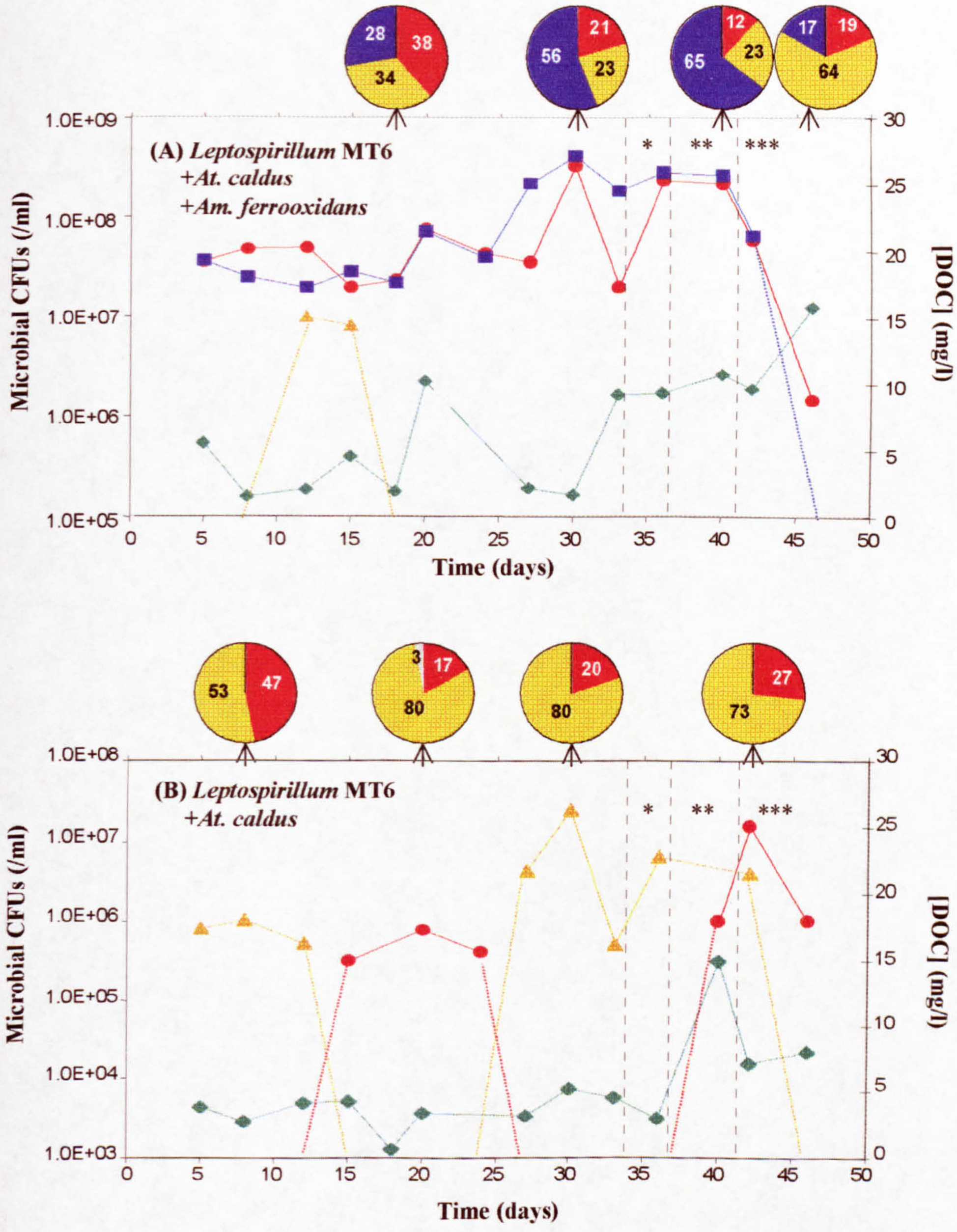


Figure 5.7: Microbial populations (from plate counts (CFUs) and FISH) and DOC concentrations in mixed culture of *Leptospirillum* MT6+*At. caldus*+*Am. ferrooxidans* (A) and *Leptospirillum* MT6+*At. caldus* (B). Key: ●, *Leptospirillum* MT6; ▲, *At. caldus* KU; ■, *Am. ferrooxidans* ICP (all plate counts); ◆, DOC concentrations. Pie charts show relative microbial numbers in the bioreactors determined *via* FISH$^{(+)}$ (%). The colours in pie charts correspond to those in line charts (grey colour in pie charts represents unidentified microorganisms). $^{(+)}$Arrows indicate times of FISH analysis. *, ** and *** indicate when pH control was removed, pH 1.2 and pH 1.0, respectively.

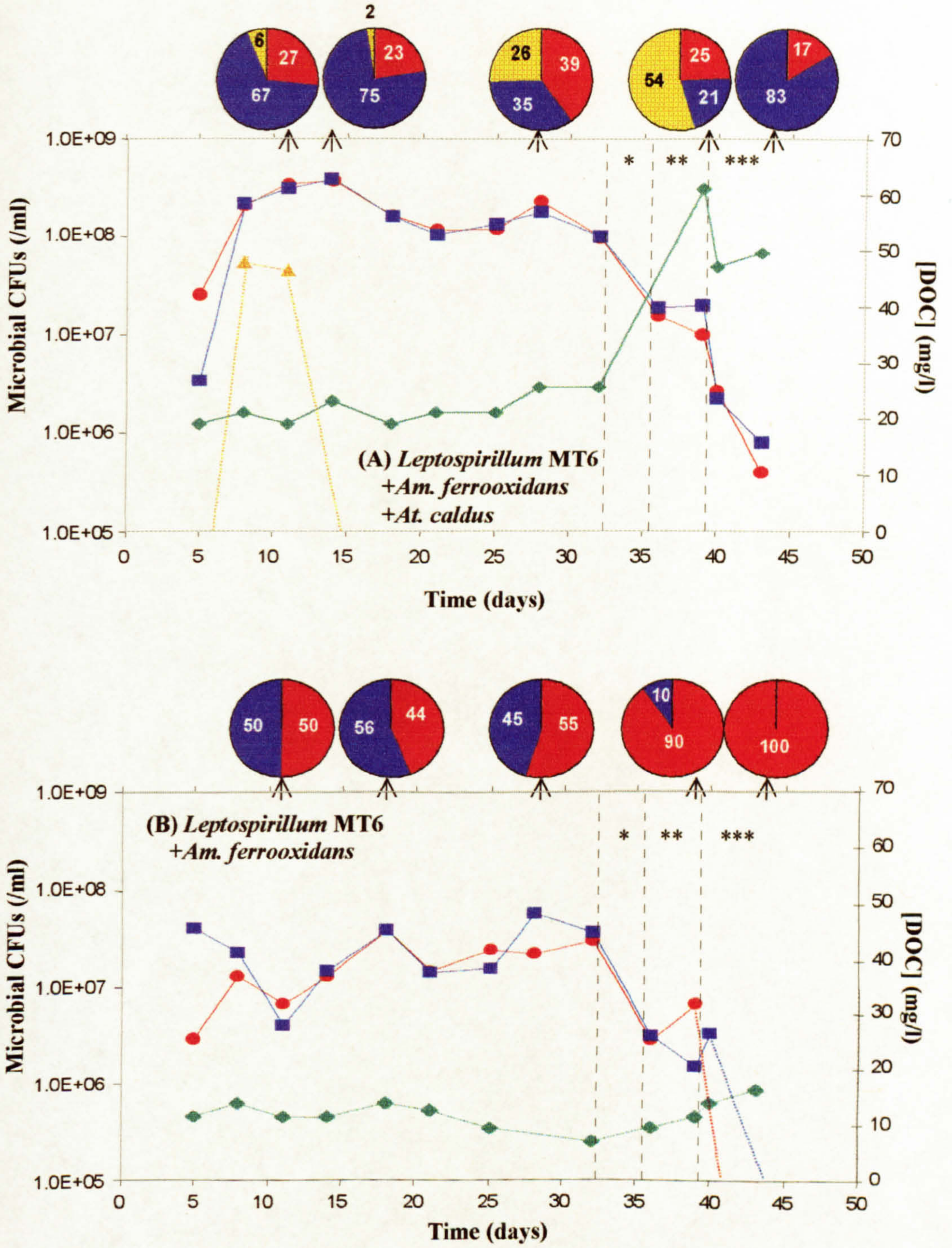


Figure 5.8: Microbial populations (from plate counts (CFUs) and FISH) and DOC concentrations in mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* (A) and *Leptospirillum* MT6+*Am. ferrooxidans* (B). Key: ●, *Leptospirillum* MT6; ■, *Am. ferrooxidans* ICP; ▲, *At. caldus* KU (all plate counts); ◆, DOC concentrations. Pie charts show relative microbial numbers in the bioreactors determined *via* FISH$^{(+)}$ (%). The colours in pie charts correspond to those in line charts. $^{(+)}$Arrows indicate times of FISH analysis. *, ** and *** indicate when pH control was removed, pH 1.2 and pH 1.0, respectively.

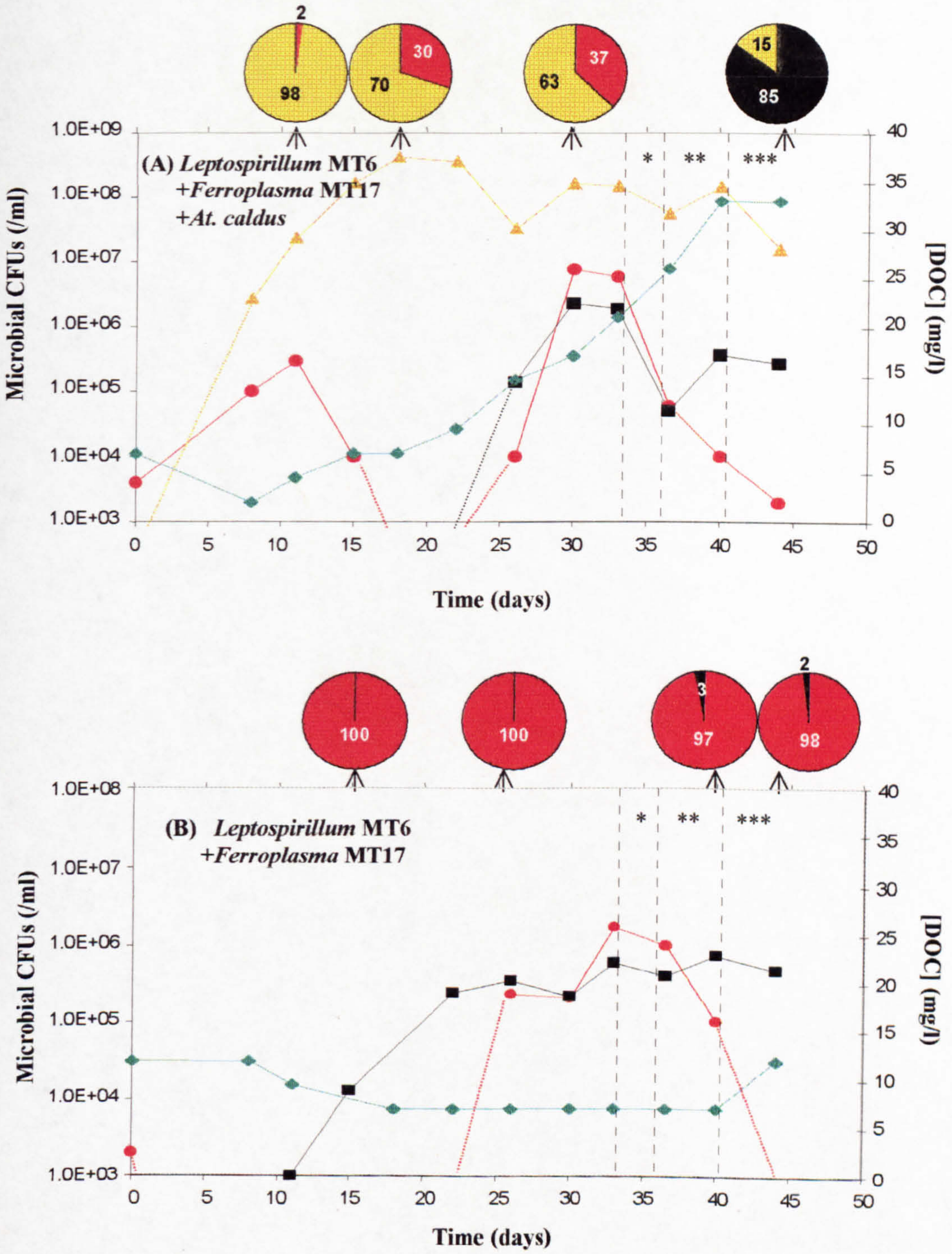


Figure 5.9: Microbial populations (from plate counting (CFUs) and FISH) and DOC concentrations in mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* (A) and *Leptospirillum* MT6+*Ferroplasma* MT17 (B). Key: ●, *Leptospirillum* MT6; ■, *Ferroplasma* MT17; ▲, *At. caldus* (all plate counts); ◆, DOC concentrations. Pie charts show relative microbial numbers in the bioreactors determined *via* FISH$^{(+)}$ (%). The colours in pie charts correspond to those in line charts. $^{(+)}$Arrows indicate times of FISH analysis. *, ** and *** indicate when pH control was removed, pH 1.2 and pH 1.0, respectively.

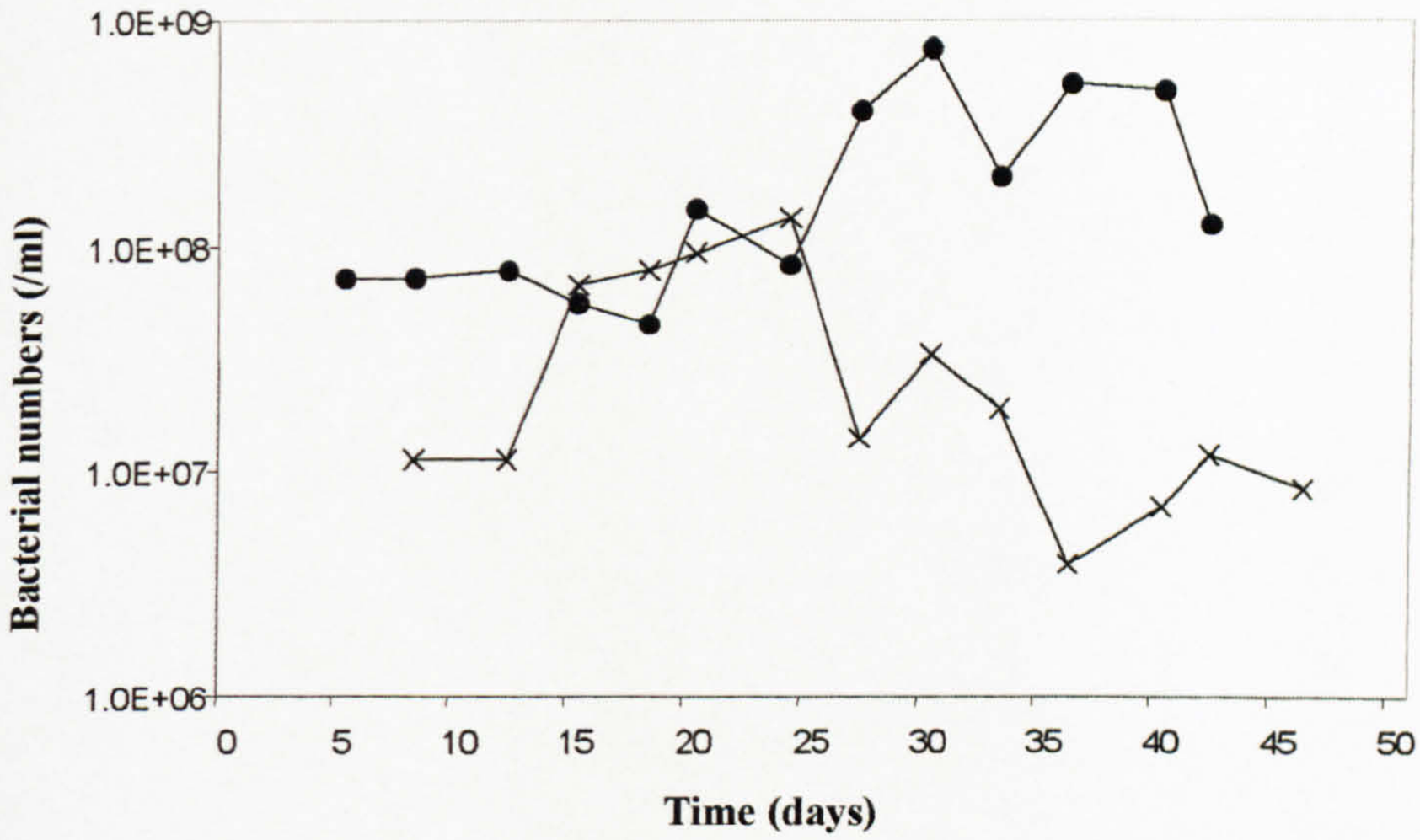
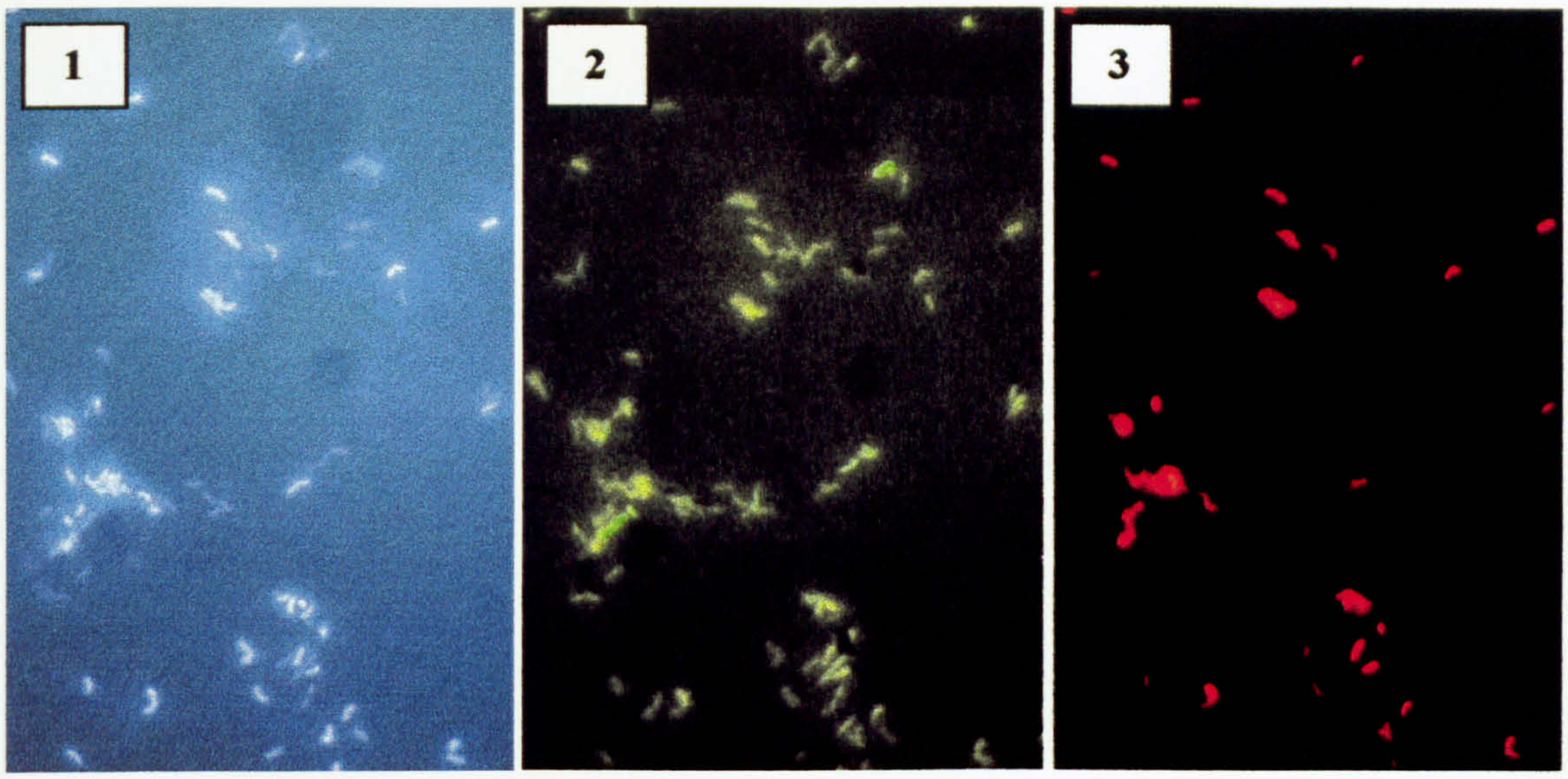
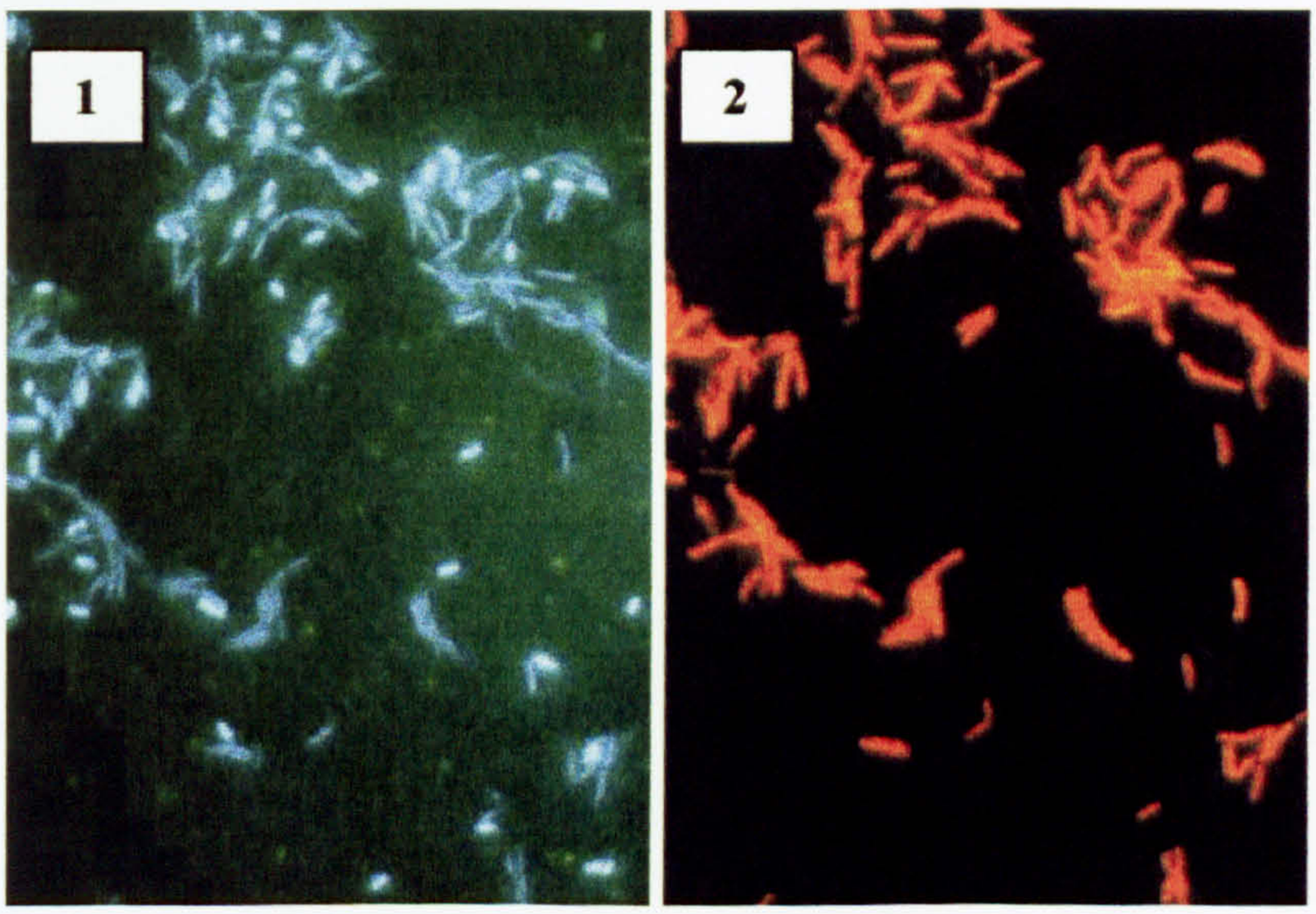


Figure 5.10: Total bacterial populations in mixed culture of *Leptospirillum* MT6+*At. caldus*+*Am. ferrooxidans* determined by plate counts (●) and direct counts (DAPI-staining) (×).

(A)



(B)



(C)

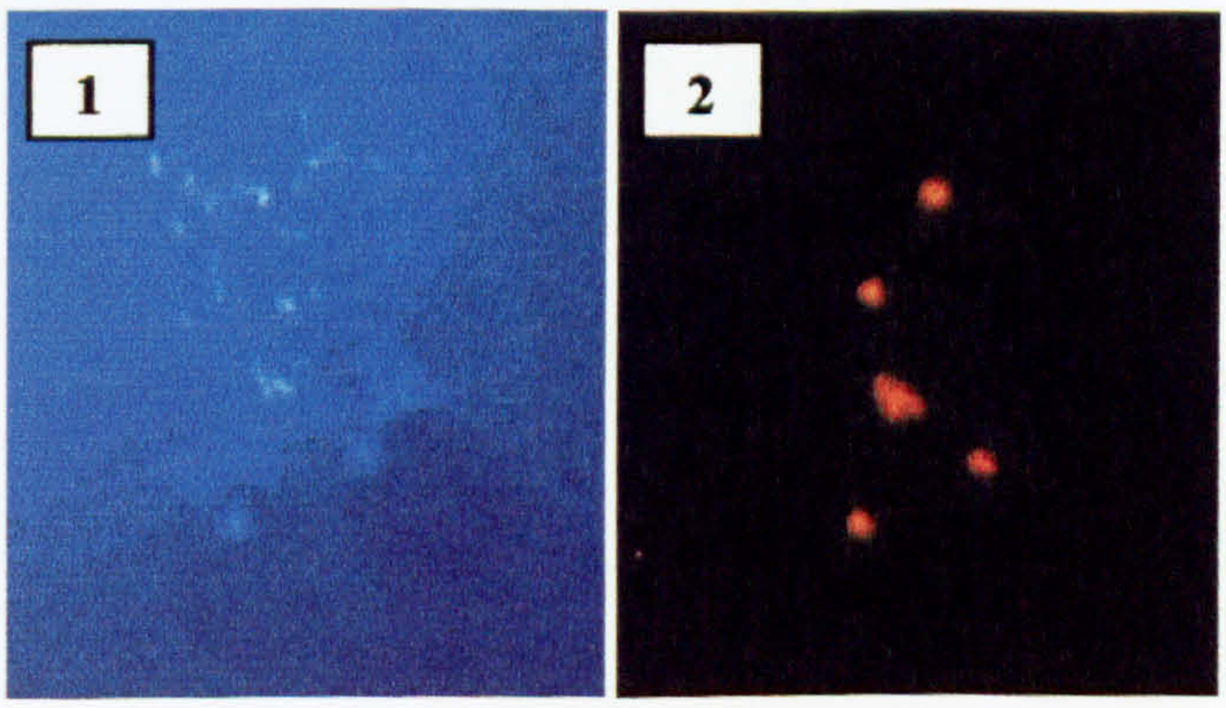


Figure 5.11: Micrographs from: (A) Mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* stained with DAPI (1), hybridised with EUB388Fl (2) and hybridised with LF655Cy3 (3). (B) Mixed culture of *Am. ferrooxidans*+*At. caldus* stained with DAPI (1) and hybridised with ACM995Cy3 (2). (C) Mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* stained with DAPI (1) and hybridised with THC642Cy3 (2).

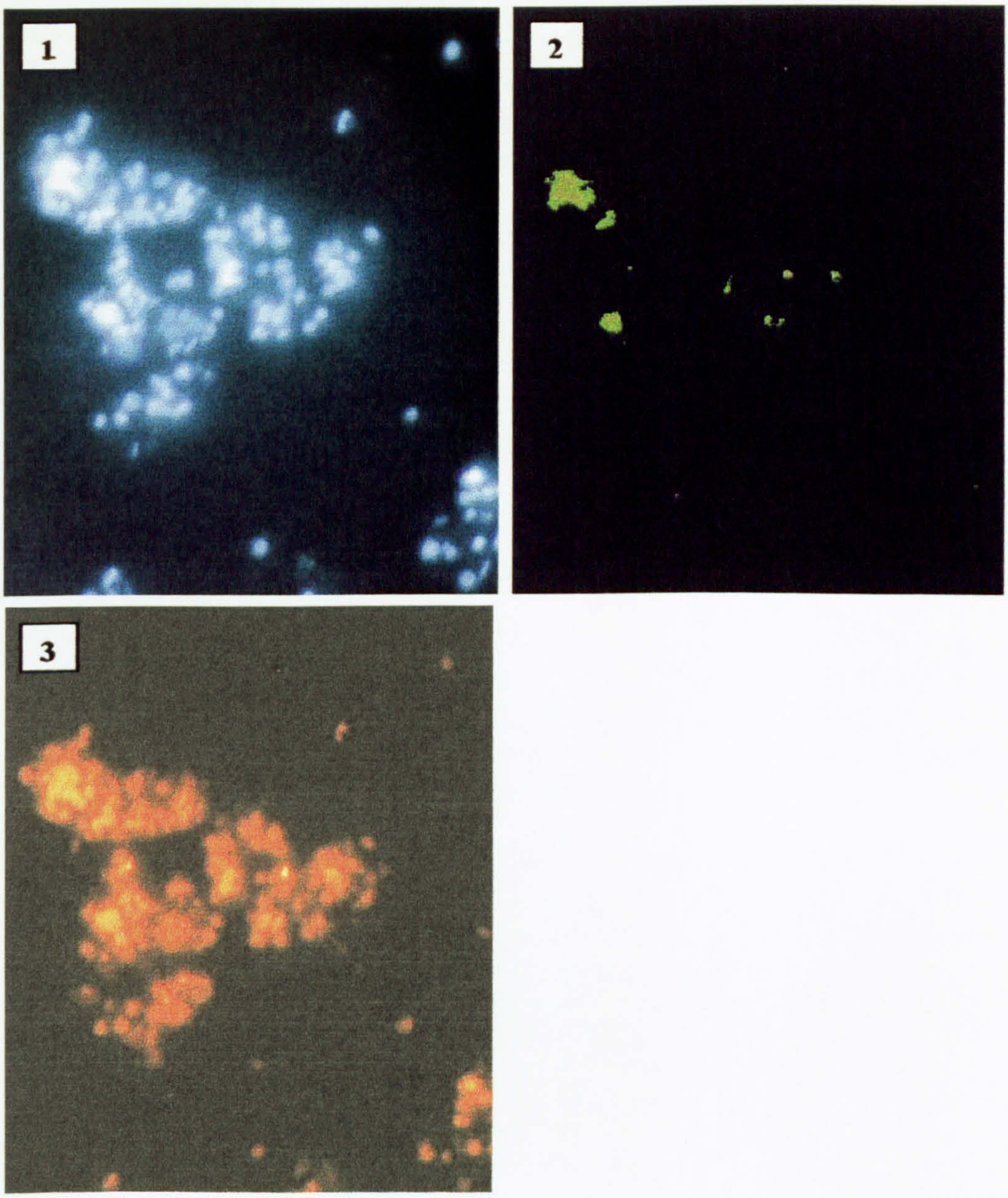


Figure 5.12: Micrographs from mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* stained with DAPI (1), hybridised with EUB388Fl (2) and hybridised with FER656Cy3 (3).

It was found that plate counts of some of the acidophiles tended to be spasmodic. This was most pronounced with *At. caldus* and *Leptospirillum* MT6, where sometimes no colonies were detected with the dilutions used whilst at other times colony forming units (CFUs) of these bacteria were readily obtained. The FISH analysis gave relative, rather than absolute, numbers of bacteria and archaea; these data were useful in assessing whether the fluctuating trends with plate counts truly reflected the microbial populations in the bioreactors.

With the pure culture of *Leptospirillum* MT6, numbers of CFUs decreased to $<10^2$/ml after day 10, and did not increase again until after day 25 (Figure 5.5b). In the corresponding mixed culture with *Alicyclobacillus* Y004, numbers of *Leptospirillum* MT6 increased earlier, in line with the observed oxidation of pyrite (Figure 5.1-Ic). Interestingly, CFUs of *Alicyclobacillus* Y004 were detected throughout, albeit at relatively low numbers ($\sim10^4$/ml). Both organisms went into sharp decline when the culture pH fell. FISH data indicated that *Leptospirillum* MT6 was always the numerically dominant bacterium in the mixed culture, except at the last sampling (at pH 1.0) where *Alicyclobacillus* Y004 accounted for 86% of stained cells (plate counts also showed slightly greater numbers of the Gram-positive acidophile at this time). DOC increased to greater concentrations in the mixed culture, particularly at the time that the culture pH declined.

The pure culture of *Am. ferrooxidans*, which had been noted to be very ineffective in oxidising pyrite, gave CFUs of about 10^6/ml throughout the first (pH 1.5) phase, and these declined markedly when the culture fell to $\leq$1.2 (Figure 5.6b). In contrast, in the mixed culture with *At. caldus*, CFUs of *Am. ferrooxidans* increased to 6×10^9/ml, before declining somewhat. Plate counts of *At. caldus* mirrored (but never exceeded) those of *Am. ferrooxidans*. Again, plate counts of both bacteria declined rapidly when

the culture pH was adjusted to pH 1.2. Interestingly, there appeared to be a good correlation between microbial populations in this mixed culture whether assessed by plate counts or by FISH. Again, DOC was observed to increase more rapidly and to a greater extent in the more effective (in terms of pyrite oxidation) mixed culture, particularly at pH 1.0.

Comparative data from the mixed culture of *Leptospirillum* MT6+*At. caldus*±*Am. ferrooxidans*, are shown in Figure 5.7. In both cases, CFUs of *At. caldus* were absent on solid media for at least part of the experimental run. Colonies of *Leptospirillum* MT6 were also only seen sporadically with inocula from the *Leptospirillum* MT6+*At. caldus* culture, but were obtained more consistently from the culture containing all three acidophiles. Colonies of *Am. ferrooxidans* were also recovered from the latter cultures throughout the experiment, in numbers similar to those of *Leptospirillum* MT6. FISH data from the *Leptospirillum* MT6+*At. caldus* culture indicated that both bacteria were present throughout the experiment, and that *At. caldus* was numerically dominant (accounting for 53-80% of cells). With the other system, FISH analysis detected all three bacteria, at similar abundance in the early stage, before becoming increasingly dominated by *Am. ferrooxidans* (until the pH was adjusted to 1.0, when *At. caldus* accounted for 64% of stained cells).

The other permutation on this leaching consortium, in which the bacterium omitted was *At. caldus* rather than *Am. ferrooxidans*, showed some interesting trends (Figure 5.8). The mixed culture with the two iron-oxidisers contained similar numbers of *Leptospirillum* MT6 and *Am. ferrooxidans* (both plate counts and FISH) during the time that the culture was maintained at 1.5. When the culture pH fell, CFUs of both bacteria declined severely, and FISH data indicated that the impact of acidification was far greater for *Am. ferrooxidans* than for *Leptospirillum* MT6, which accounted for 90-

100% of stained cells at pH 1.2 and 1.0, respectively. In the corresponding mixed culture containing *At. caldus*, recovery of the latter bacterium on solid media was, again, very sporadic. Plate counts of both iron-oxidising bacteria were about an order of magnitude greater than those from the mixed culture that did not contain *At. caldus.* All three bacteria were detected using FISH, with *At. caldus* becoming increasingly abundant (relative to other bacteria) as leaching progressed. However, in contrast to the earlier mixed culture (Figure 5.7a), most cells detected by FISH when the culture pH was adjusted to 1.0 were *Am. ferrooxidans*, rather than *At. caldus.* DOC concentrations were also notably greater in the later mixed culture of the three bacteria than in the earlier culture (Figures 5.8a and 5.7a) and also greater than in the mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans* (Figure 5.8b).

Data from the last of the mixed leaching cultures to be examined, which included the archaeon *Ferroplasma* MT17, showed some contrasting microbiological trends (Figure 5.9). With the mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17, although CFUs of the archaeon were often similar or even greater than those of the bacterium, *Ferroplasma* MT17 was only detected by FISH when the culture pH was lowered to 1.2 and 1.0, and even then it only accounted for 2-3% of stained cells. In the corresponding mixed culture containing *At. caldus*, CFU recovery of the sulfur-oxidiser of 10^8-10^9/ml were obtained for much of the period that the culture was held at pH 1.5. FISH analysis confirmed that *At. caldus* was the dominant organism in the culture during this time, and that *Leptospirillum* MT6 was the more numerous iron oxidiser. A very different picture emerged when the culture was sampled at day 40, when the pH was 1.0. At that time, *Ferroplasma* MT17 accounted for 85% of cells in FISH analysis, with the remaining 15% being *At. caldus.* Again, DOC concentrations were found to increase to

much higher levels in the more efficient leaching culture (i.e. *Leptospirillum* MT6+ *Ferroplasma* MT17+*At. caldus*).

5.4 Discussion

The objectives of this part of the research project were to compare the microbial oxidation of pyrite by defined pure cultures and consortia of moderately thermophilic acidophiles, and to examine changes in microbial populations in these cultures using a combination of cultivation and molecular techniques. Because of time constraints imposed by running parallel cultures in bioreactors for up to 44 days, the number of consortia permutations had necessarily to be limited. One moderately thermophilic acidophile that was omitted from these experiments was the iron/sulfur-oxidiser, *Sulfobacillus*. Work with the Mintek stirred tank samples (section 3.2) had shown that, although a "*Sb. yellowstonensis*"-like bacterium was present in these cultures, these accounted for, at most, <6% of CFUs, and was therefore considered not be a very significant microorganism in that consortium. Similarly, shake flask leaching of Cae Coch rock pyrite (chapter 4) had indicated that *Sulfobacillus*-like bacteria had a slightly negative impact on pyrite oxidation by *Leptospirillum* MT6. In contrast, although no *Am. ferrooxidans*-like bacteria were detected in the Mintek cultures, data from shake flask experiments had indicated that inclusion of this acidophile in mixed cultures might enhance pyrite oxidation, relative to pure cultures.

In the pyrite-oxidising experiments using pH-controlled bioreactors, cultures containing different microbial consortia displayed different trends. The least effective pyrite oxidising system of those tested (in terms of rates and yields) was the pure culture of *Am. ferrooxidans*, where redox potentials were <+770 mV throughout (whereas all the

other cultures developed redox potentials of +850-900 mV) and there were no marked increases in numbers of bacteria. However, the mixed culture of *Am. ferrooxidans* and the sulfur-oxidiser, *At. caldus,* was one of the most effective cultures used. It was suggested from shake flask experiments that *Am. ferrooxidans* might benefit from both *Leptospirillum* MT6 and *At. caldus* by utilising organic carbon compounds originating from these autotrophs (chapter 4). However, in pH-controlled bioreactors, the mixed culture of *Am. ferrooxidans*+*Leptospirillum* MT6 was far less effective than that of *Am. ferrooxidans*+*At. caldus,* though both mixed cultures were more effective than the pure culture of *Am. ferrooxidans*. This, at least in part, might have been due to the greater numbers of *At. caldus* (~10^2-fold) than *Leptospirillum* MT6 present in mixed cultures with *Am. ferrooxidans*, and, consequently, provision of more organic carbon for *Am. ferrooxidans*. This hypothesis was supported by higher DOC concentrations found in the mixed culture of *Am. ferrooxidans*+*At. caldus* (though presumably not all of the organic carbon originating from *At. caldus* would have been metabolised by *Am. ferrooxidans*). In addition, *At. caldus* might have contributed to more effective pyrite oxidation by *Am. ferrooxidans* by removing sulfur deposits from mineral surfaces. It would be interesting to test whether pyrite oxidation by *Am. ferrooxidans* in the presence of added organic materials (e.g. yeast extract) would be as effective as in the presence of *At. caldus* (thereby eliminating any possible enhancement due to sulfur oxidation). In this mixed culture (and also the mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus*) there was a marked "flattening off" of pyrite oxidation. One possible reason for this might have been limiting concentrations of one or more inorganic nutrients, though the exact cause was not determined.

Interestingly, although *At. caldus* had a positive impact on pyrite oxidation by *Am. ferrooxidans*, it had a negative impact on *Leptospirillum* MT6 (as noted also in shake

flask experiments; chapter 4). One reason for this might be that these two obligate autotrophs compete for inorganic carbon, though the numbers of *Leptospirillum* MT6 in mixed culture with *At. caldus* were quite similar to those in the pure culture. It was also found that both *Am. ferrooxidans* and *Ferroplasma* MT17 also had a negative impact on pyrite oxidation by *Leptospirillum* MT6. *Leptospirillum* MT6 and *Am. ferrooxidans* would be expected to compete for their common energy source (ferrous iron) but, again, plate counts indicated that numbers of *Leptospirillum* MT6 in the pure culture and the mixed culture with *Am. ferrooxidans* were quite similar. Pyrite oxidation was markedly less efficient in the mixed culture of *Leptospirillum* MT6 and *Ferroplasma* MT17 than in mixed cultures of *Leptospirillum* MT6 with either *At. caldus* or *Am. ferrooxidans*. This suggests the possibility that an antimicrobial agent, which is active against *Leptospirillum* spp., is produced by this archaeon, though this hypothesis was not tested. In contrast to the negative impact on pyrite oxidation by *Leptospirillum* MT6 caused either by *Ferroplasma* MT17 or (to a lesser extent) by *At. caldus*, the mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* was particularly adept at oxidising pyrite, both in terms of rate and extent of mineral oxidation. *Ferroplasma* MT17 appeared to be an obligately heterotrophic iron-oxidiser (chapter 3) and, earlier shake flask experiments indicated that *Ferroplasma* MT17 was not able to oxidise pyrite in mixed cultures with *At. caldus* (chapter 3), though it is possible that the efficient pyrite oxidation observed in the bioreactor containing *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* resulted from mutualistic interactions involving all three acidophiles, including utilisation of organic exudates and lysates from *At. caldus* (which were present in large numbers) by the heterotrophic *Ferroplasma*. A similar scenario has been described for pyrite oxidation by the mesophiles *"Ferrimicrobium acidiphilum"* (a

heterotrophic iron-oxidiser) and *At. thiooxidans* (an autotrophic sulfur-oxidiser; Bacelar-Nicolau and Johnson, 1999)

Differences between the mixed culture of *Leptospirillum* MT6+*Alicyclobacillus* Y004 and the pure culture of *Leptospirillum* MT6 were marginal. Pyrite oxidation by the pure culture of *Leptospirillum* MT6 was clearly more effective than by the pure culture of *Am. ferrooxidans*, but less effective than by *Am. ferrooxidans* mixed cultures. *Alicyclobacillus* Y004, as a non-iron/sulfur-oxidising heterotroph, did not show any noticeable impact on pyrite oxidation by *Leptospirillum* MT6, though it did persist in relatively low numbers (~10^4/ml) throughout incubation.

The mixed culture containing *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* was superior both to the mixed culture containing *Leptospirillum* MT6+*At. caldus* and to that containing *Leptospirillum* MT6+*Am. ferrooxidans*. Since numbers of the two iron-oxidisers in this mixed culture were similar, it is not possible to conclude which one of them was the primary iron-oxidising bacterium in the system. The major differences observed with mixed cultures of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* and *Am. ferrooxidasns*+*At. caldus* was that, in cultures that included *Leptospirillum* MT6, the pyrite oxidation rate was slower and pyrite oxidation continued even when the pH was lowered to pH 1.2 (no "flattening off" was observed during incubation), suggesting that, given more prolonged incubation, final yields of iron solubilised might have been greater with the mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* than with *Am. ferrooxidans*+*At. caldus*. Therefore, inclusion of *Leptospirillum* MT6 might be a way to avoid the "flattening off" phenomenon observed in a number of mixed cultures, thereby potentially achieving greater yields of pyrite oxidised. However, this hypothesis needs to be verified by testing long-term pyrite oxidation by mixed cultures containing and not containing *Leptospirillum* MT6.

There was a close fit between theoretical sulfate concentrations (calculated from total iron solubilised and assuming the mineralogical formula for pyrite to be FeS_2) and measured sulfate concentrations in some cultures, though the two were noticeably different in others. Although Schippers and Sand (1999) found different amounts of sulfate produced during pyrite oxidation by *L. ferrooxidans* and iron/sulfur-oxidising *At. ferrooxidans*, in the current experiments, inclusion of sulfur-oxidiser, *At. caldus* did not result in enhanced concentrations of soluble sulfate.

The initial attack on pyrite is generally acknowledged to be mediated by ferric iron (equation [5.3]).

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+ \quad [5.3]$$

The oxidation of thiosulfate may be catalysed either biologically (equation [5.4]), or chemically (equation [5.5]).

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \quad [5.4]$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \quad [5.5]$$

The closest fit between the theoretical and actual sulfate concentrations was observed in mixed cultures of *Leptospirillum* MT6+*Ferroplasma* MT17, *Leptospirillum* MT6+*At. caldus*, and the pure culture of *Am. ferrooxidans*, and also at the initial stages of pyrite oxidation in some other cultures. This might indicate that thiosulfate oxidation (either biological or chemical) tends to be more complete when rates of pyrite oxidation are low. In cultures where pyrite oxidation was extensive, actual sulfate concentrations were much lower than those predicted theoretically, implying that thiosulfate oxidation (either biological [5.4] or chemical [5.5]) does not progress as fast as iron solubilisation [5.3]. Another reason why there were differences between theoretical and actual sulfate concentrations is that the true chemical composition of the pyrite used not being FeS_2,

but being FeSx (x<2), which would mean that measured sulfate concentrations would be lower than those predicted from iron solubilisation.

In contrast to the sulfate data, it was found that more alkali was required to maintain pH in cultures that included *At. caldus* than in those that did not contain this sulfur-oxidiser. Whilst *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* oxidised about twice as much pyrite (resulting in twice as much sulfate produced) from day 0-32 than the corresponding culture not containing *At. caldus*, alkali consumption was about 4-fold greater in the former, indicating that the system including *At. caldus* produced more protons (presumably as sulfuric acid). This was also the case in mixed cultures of *Am. ferrooxidans*+/-*At. caldus* and *Leptospirillum* MT6+*Ferroplasma* MT17+/-*At. caldus.*

The FISH analysis was found to be a very useful tool to assess relative microbial abundance in mineral oxidation systems, especially in assessing whether the fluctuating trends with plate counts truly reflected the microbial populations in the bioreactors. The reason why plating periodically failed to detect some microbial populations may due to the physiological state of the acidophiles, in that cells might only have formed colonies on plates if they were metabolically active. Numbers of iron-oxidising bacteria in mixed cultures often appeared to correlate with pyrite oxidation. In the *Leptospirillum* MT6 systems, numbers of this iron-oxidiser were greatest in the most effective mixed cultures of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus* and *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus.* Also in the *Am. ferrooxidans* systems, the numbers of this iron-oxidiser were greatest in the most effective mixed culture (*Am. ferrooxidans*+*At. caldus*) and slightly lower in the mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans*+*At. caldus*. The numbers of *At. caldus* in these highly effective mixed cultures were also greater than in any other cultures. Interestingly, despite its

inability to oxidise pyrite, *At. caldus* was often present in similar or greater numbers than the primary iron-oxidiser(s) in mixed cultures, presumably reflecting its ability to utilise RISCs (which are more energy rich than ferrous iron) produced during pyrite oxidation (Sand *et al.*, 1995). Interesting microbial population trends were observed in mixed cultures of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus.* Despite the fact that *Ferroplasma* MT17 was not detected either by plating or by FISH, inclusion of this archaeon appeared to have a positive impact on pyrite oxidation. Both rates of pyrite oxidation and the numbers of *At. caldus* were much greater in this culture than in the corresponding mixed culture of *Leptospirillum* MT6+*At. caldus.* Even at pH 1.2-1.0, *Ferroplasma* MT17 accounted only for 2-3% of the total population in the mixed culture without *At. caldus,* while in the culture containing *At. caldus,* this archaeon (at pH 1.0) accounted for 85% of cells in FISH analysis. Population changes in the mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus* analysed by FISH were similar to those observed in the Mintek pilot plant aerated tank operation described in section 3.2, where the numbers of *Leptospirillum* and (more latterly) of *At. caldus* decreased as mineral pyrite oxidation progressed, while those of the *Ferroplasma* isolates increased.

In the majority of cases, *Am. ferrooxidans* was found to be most acid-sensitive of the microorganisms used in these leaching studies, and plate counts of this iron-oxidiser decreased significantly when the culture was lowered to 1.2 and then to 1.0, though numbers of all the other bacteria also decreased when cultures were acidified. However, it was found that *Ferroplasma* MT17 was far less acid-sensitive, and dominated the microbial population at pH 1.2 to 1.0 in the mixed culture with *Leptospirillum* MT6 and *At. caldus.* A further possible reason for the success of this archaeon at low pH is that

death of the bacteria present would have resulted in enhanced levels of DOC from lysed cells that would have been utilised by the heterotroph.

In commercial mineral processing, it would be advantageous to achieve enhanced mineral oxidation rates and yields with minimum costs. In light of the data presented in the current work, it was found that these objectives may be achieved, at least in part, by selecting particular mixed populations of bioleaching microorganisms. The most effective mixed cultures included heterotrophic iron-oxidisers (*Am. ferrooxidans* and/or *Ferroplasma* MT17), and the requirement of these heterotrophic iron-oxidisers for organic carbon could be met by that originating from autotrophic acidophiles, which would minimise the cost of adding extraneous organic matter. Since some of the more highly effective mixed cultures (which showed shorter lag phases, and faster rates of pyrite oxidation) tended to display a "flattening off" of pyrite oxidation, further studies on circumventing incomplete mineral oxidation are necessary to optimise the bioleaching process.

Chapter 6

Effect of Flotation Chemicals on Some Moderately Thermophilic Acidophilic Prokaryotes

6.1 Introduction

The results described in chapter 4 suggested that growth of the autotrophic iron-oxidiser, *Leptospirillum* MT6, might have been inhibited in cultures containing Mintek pyrite concentrate due to the presence of residual flotation chemicals. Depending on the operational conditions and choice of chemicals, residual flotation reagents may remain in the ore slurry and be carried over to the leaching reactors. Inhibition of *At. ferrooxidans* by flotation reagents has been reported (Loon and Madgwick, 1995; Valdivia and Chaves, 2001; Tuovinen, 1978). Although *At. ferrooxidans* had been considered to be the most important acidophile in bioleaching systems for many years, the greater importance of acidophiles such as *Leptospirillum* spp. and some thermotolerant acidophiles in bioleaching system is now increasingly recognised (chapter 1).

In this chapter, the effect of flotation chemicals on some mesophilic and moderately thermophilic acidophiles is described. The flotation chemicals studied here were those used in commercial mineral processing processes, and were provided by BHP Billiton (Randberg, South Africa). The "sensitivity" of *Leptospirillum* MT6 to pyrite concentrate was compared to the type strains of mesophilic *L. ferrooxidans* and *At. ferrooxidans*. Five species of moderate thermophiles were tested for their sensitivity to a variety of floatation chemicals. Also, the potential use of *Sulfobacillus* NC for detoxification of pyrite concentrate, thereby facilitating growth of *Leptospirillum* MT6, was investigated.

Flotation reagents

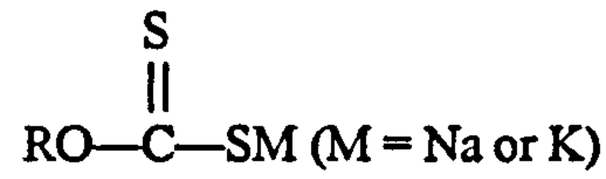
In a metal ore, the valuable minerals are present as part of a coherent mixture of intergrown mineral crystals, each having a definite chemical composition. To liberate the individual mineral particles, it is necessary to grind the ore to very fine pulps. To separate the desired mineral in a concentrate from the accompanying unwanted gangue minerals, the finely ground ores are treated with flotation reagents.

Collectors

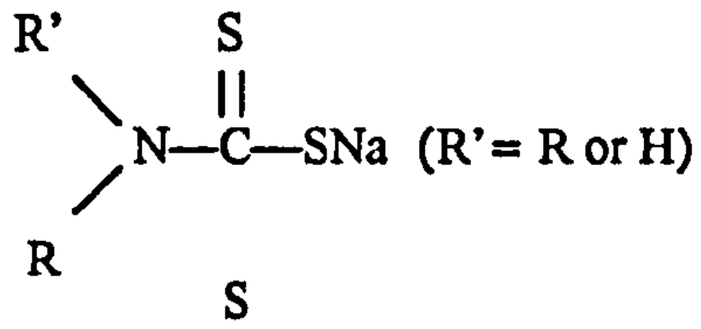
In the flotation process, the physical separation of minerals is achieved by the use of chemical reagents known as collectors. The collectors impart a hydrophobic coating to the mineral particle to be floated and a hydrophilic character to unwanted gangue minerals, when the mineral-containing ore is conditioned with them. Once the mineral surface has obtained a hydrophobic coating due to the action of a collector, it can adhere to an air bubble and thus will "float" to the surface. A collector consists of two moieties: firstly, a part of the molecule that becomes attached to an air bubble, i.e. the non-polar part of the molecule, and secondly a part that reacts with the mineral surface.

One of the most widely used groups is sulphydric collectors. All sulfidic minerals can be floated to varying degrees of success by any of the sulphydric collectors. This group includes the following, where R and R' are carbon chains of varying lengths.

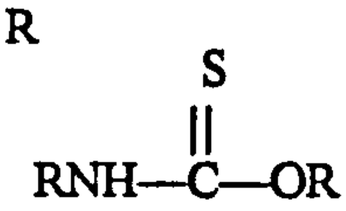
Xanthates



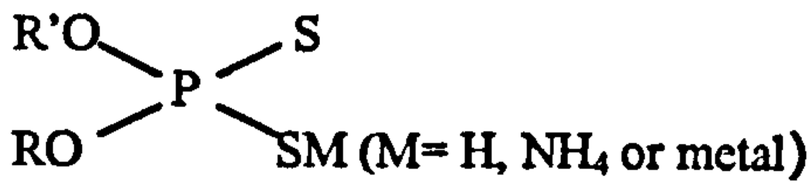
Dithiocarbamates



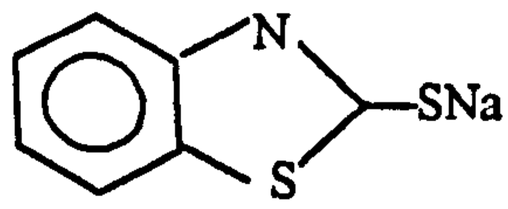
Thionocarbamates



Dithiophosphates



Mercaptobenzothiazoles



Frothers

Frothers are surface-active, usually non-ionic, molecules whose function in the flotation system is to provide a large air-water interface of sufficient stability to ensure that floated particles will not fall back into the flotation pulp before they can be removed. This froth can be skimmed off to yield a concentrate in which the desired mineral is present in a much higher concentration than in the original ore.

6.2 Effect of different concentrations of Mintek pyrite concentrate on pyrite and iron oxidation by *Leptospirillum* spp.

6.2.1 Introduction

In earlier experiments (chapter 4), it was found that *Leptospirillum* MT6 was not able to oxidise Mintek pyrite concentrate in pure culture. This was considered to be possibly due to some inhibitory compound(s) present in the pyrite concentrate. In this study, to examine whether or not this iron-oxidiser was able to oxidise the pyrite concentrate when supplied at lower concentrations (and corresponding lower concentrations of the "inhibitory compound(s)") different concentrations of pyrite concentrate were tested for oxidation by *Leptospirillum* MT6. The type strains of *L. ferrooxidans* and *At. ferrooxidans* were also tested for oxidation of pyrite concentrate as reference bacteria. Also, to test whether "inhibitory compound(s)" are present either (or both) in liquid or solid phase of the pyrite media after autoclaving, the liquid and solid phases of autoclaved pyrite cultures (containing 1 or 2 % pyrite concentrate) were separated prior to inoculation of *Leptospirillum* MT6.

6.2.2 Methods

For the pyrite concentrate oxidation experiment, replicate 100 ml flasks, each containing 50 ml of pyrite medium (Mintek pyrite concentrate, pH 2.0; section 2.2.1.1.4) and different concentrations of pyrite concentrate (0.1, 0.5, 1.0, 1.5 and 2.0%, w/v) were inoculated with *Leptospirillum* MT6, pre-grown in 1% Cae Coch rock pyrite medium. Also, flasks containing 1% or 2% Cae Coch rock pyrite medium were inoculated, as controls. The flasks were incubated for 25-30 days at 45°C, shaken, and samples removed every 5 days to determine total soluble iron concentrations (section

2.4.3). Cultures inoculated with the type strains of *L. ferrooxidans* and *At. ferrooxidans* were prepared similarly, and incubated at 30°C.

In the second series of experiments, replicate 100 ml flasks, each containing 40 ml of 1% or 2% pyrite medium (Mintek pyrite concentrate, pH 2.0; section 2.2.1.1.4) were autoclaved and the mineral-free liquors and the solid pyrites concentrate were separated by centrifugation. The mineral-free solutions were transferred into sterile 100 ml flasks; sterile ferrous sulfate solution (to 25 mM) was added to one of the replicate flasks in each case. The separated pyrite concentrates were also transferred to sterile 100 ml flasks, and 40 ml of fresh sterile basal salts solutions added. For positive controls, replicate 100 ml flasks, each containing 40 ml ferrous iron medium (25 mM ferrous sulfate, pH 2.0; section 2.2.1.1.1) or 2% Cae Coch rock pyrite medium (pH 2.0; section 2.2.1.1.4) were prepared. In addition, replicate 100 ml flasks, each containing 2% Mintek pyrite medium (pH 2.0; section 2.2.1.1.4) were prepared, as negative controls. The flasks were inoculated with *Leptospirillum* MT6 and incubated, shaken, at 45°C. Samples were removed to determine concentrations of ferrous iron (section 2.4.2.2) or total soluble iron (section 2.4.3).

6.2.3 Results

Oxidation of pyrite concentrate and rock pyrite by *Leptospirillum* MT6, *L. ferrooxidans* and *At. ferrooxidans* are shown in Figures 6.1-6.3. *Leptospirillum* MT6 and *L. ferrooxidans* were unable to oxidise the pyrite concentrate, irrespective of the initial concentrations of pyrite concentrate (even after prolonged incubation). In contrast, rock pyrite was oxidised by *Leptospirillum* MT6 after a 15-day lag-period (Figure 6.1). After 20 days lag-period, *L. ferrooxidans* also started to oxidise the rock pyrite (Figure 6.2).

In contrast, *At. ferrooxidans* was able to oxidise both the pyrite concentrate and the rock pyrite.

In the second series of experiments, mineral-free pyrite concentrate liquor media separated from 1% and 2% pyrite media following autoclaving contained ~4.0 mM and 7.6 mM ferrous iron, respectively. *Leptospirillum* MT6 readily oxidised all of the available ferrous iron in mineral-free pyrite concentrate liquors (both supplemented and initially present ferrous iron). Although *Leptospirillum* MT6 began to oxidise the rock pyrite (positive controls) by day 20, no pyrite oxidation was observed in cultures containing Mintek pyrite concentrate (both 1% and 2%) and in the negative control cultures (2% Mintek pyrite cultures; data not shown).

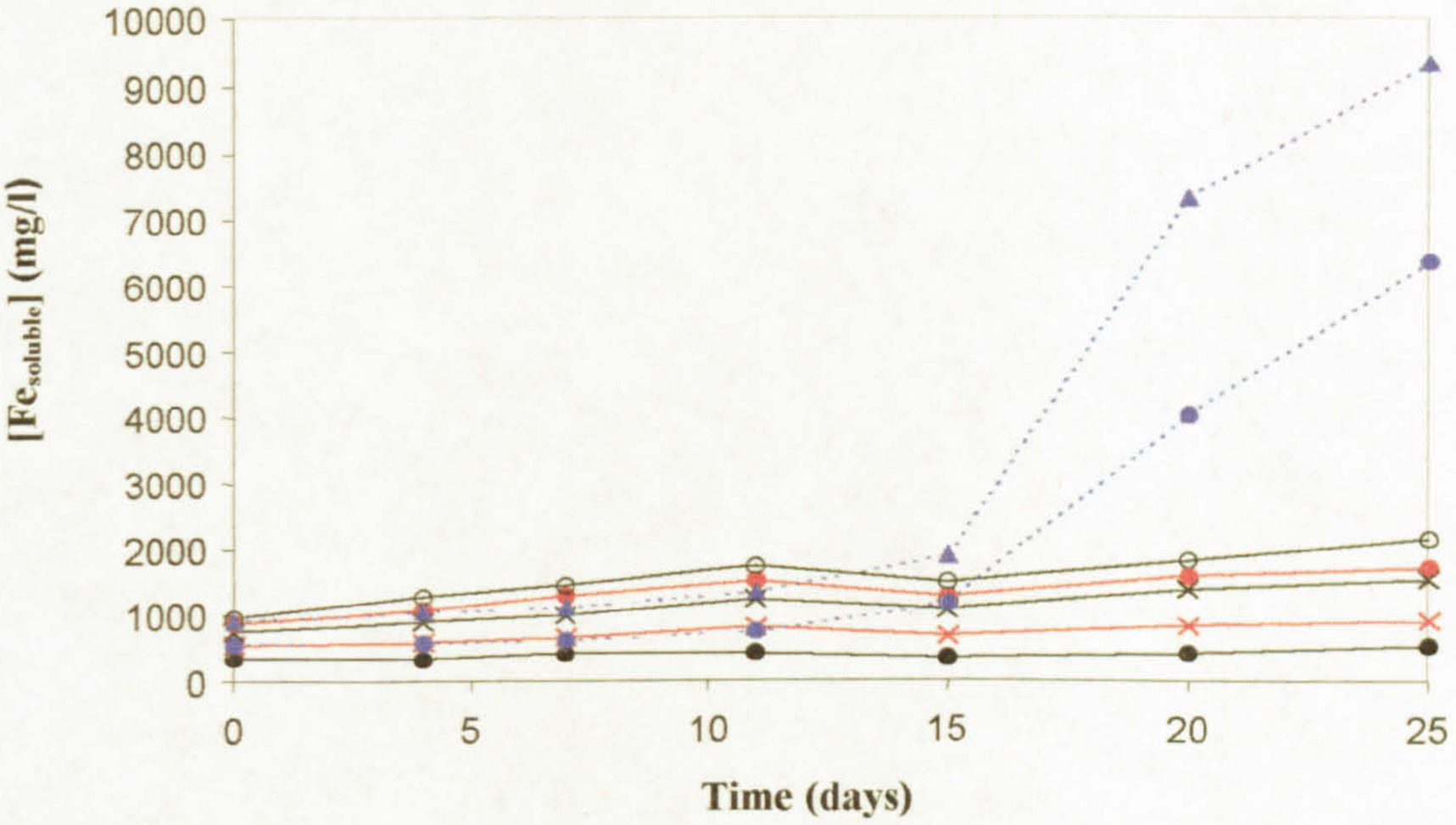


Figure 6.1: Oxidation of pyrite concentrate (solid lines) and rock pyrite (broken lines) by *Leptospirillum* MT6. Key: ●, 0.1% pyrite concentrate; ×, 0.5% pyrite concentrate; ×, 1.0% pyrite concentrate; ●, 1.5% pyrite concentrate; ○, 2.0% pyrite concentrate; ●, 1.0% rock pyrite; ▲, 2.0% rock pyrite.

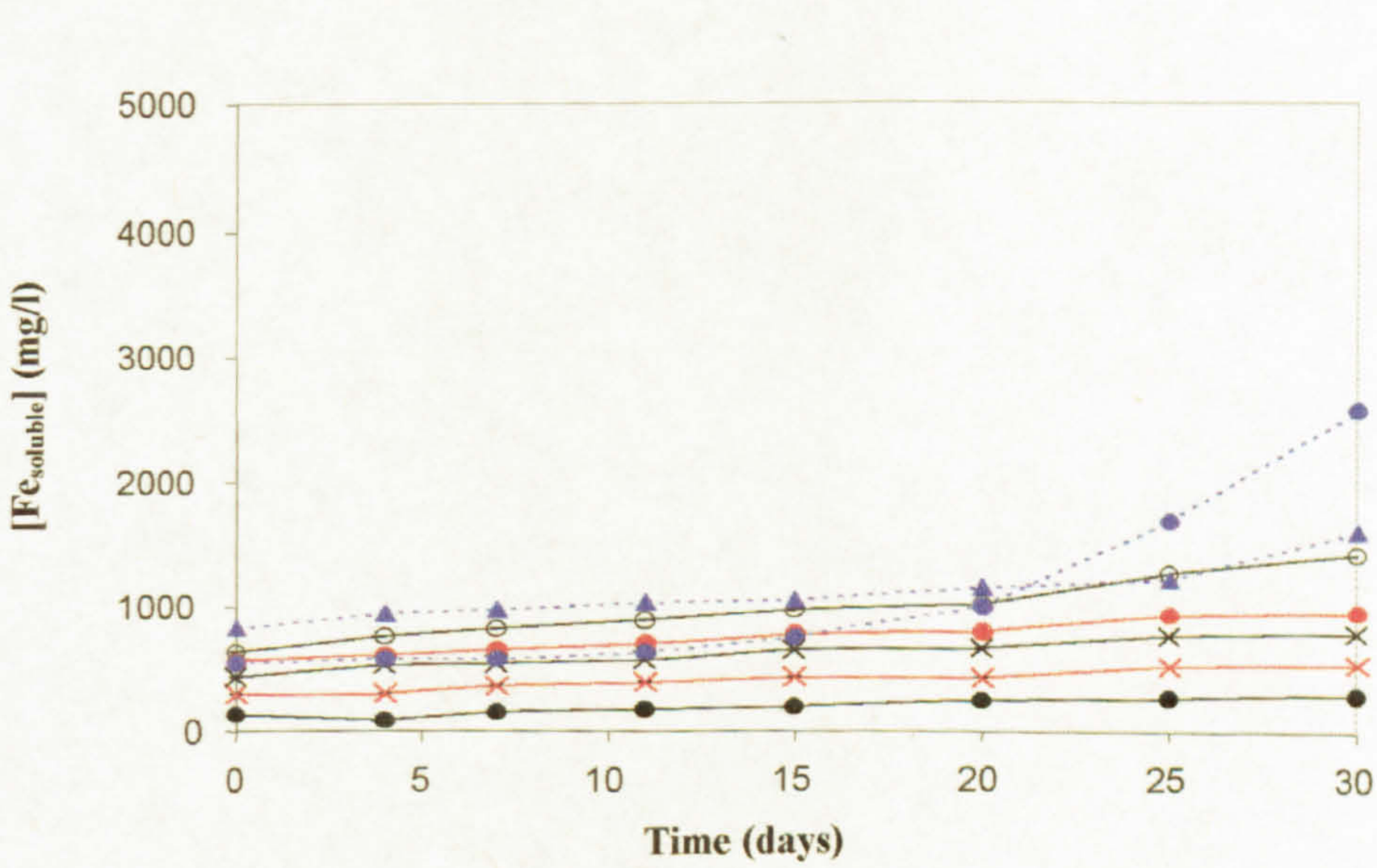


Figure 6.2: Oxidation of pyrite concentrate (solid lines) and rock pyrite (broken lines) by *L. ferrooxidans*^{T}. Key: ●, 0.1% pyrite concentrate; ×, 0.5% pyrite concentrate; ×, 1.0% pyrite concentrate; ●, 1.5% pyrite concentrate; ○, 2.0% pyrite concentrate; ●, 1.0% rock pyrite; ▲, 2.0% rock pyrite.

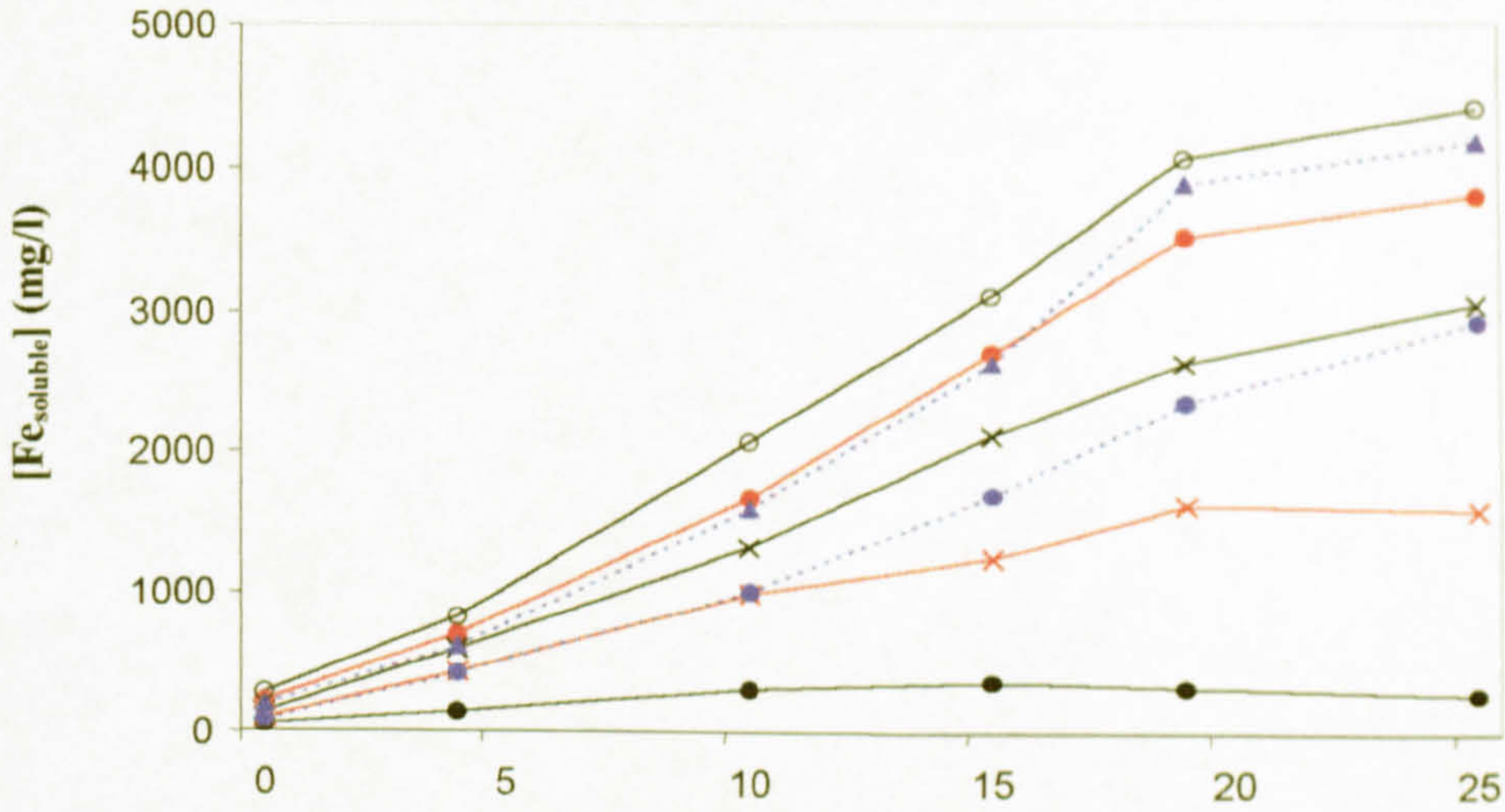


Figure 6.3: Oxidation of pyrite concentrate (solid lines) and rock pyrite (broken lines) by *At. ferrooxidans*[T]. Key: ●, 0.1% pyrite concentrate; ×, 0.5% pyrite concentrate; ×, 1.0% pyrite concentrate; ●, 1.5% pyrite concentrate; ○, 2.0% pyrite concentrate; ●, 1.0% rock pyrite; ▲, 2.0% rock pyrite.

6.3 Attempts to remove "inhibitory compound(s)" present in Mintek pyrite concentrate.

6.3.1 Methods

An attempt was made to remove inhibitory compounds(s) from the pyrite concentrate by washing with acetone or with perchloric acid. Perchloric acid was reported to decompose xanthates absorbed on the mineral surfaces (Hiroyoshi *et al.*, 1997).

In the first experiments, 10 grams of acid-washed pyrite concentrate (section 2.2.1.1.4) was stirred in a beaker containing 500 ml acetone (or distilled water as control) for 30 minutes, harvested by centrifugation, and then washed thoroughly with distilled water to remove any acetone residue. The acetone-washed pyrite concentrate was then dried at

105°C, overnight. Replicate 100 ml flasks, each containing 50 ml of 2% pyrite medium (acetone- or water-washed pyrite concentrate, pH 2.0; section 2.2.1.1.4) were inoculated with *Leptospirillum* MT6 pre-grown in 2% Cae Coch rock pyrite medium, and incubated for 25 days shaken, at 45°C. Samples were removed every 5 days for determination of total soluble iron (section 2.4.3).

In a second experiment, 10 grams of pyrite concentrate was stirred in a beaker containing 500 ml of 1 M perchloric acid for 3 hours, harvested by centrifugation, and washed thoroughly with distilled water to remove any acid residue. The washed pyrite concentrate was then dried at 105°C, overnight. Replicate 100 ml flasks, each containing 50 ml of 2% pyrite medium (non-washed or perchloric acid-washed pyrite concentrate, pH 2.0; section 2.2.1.1.4) were inoculated with *Leptospirillum* MT6 pre-grown in 2% Cae Coch rock pyrite medium, and incubated for 20 days shaken, at 45°C. Samples were removed every 6-7 days for determination of total soluble iron (section 2.4.3).

Replicate 100 ml flasks, each containing 2% Cae Coch pyrite medium (pH 2.0; section 2.2.1.1.4) were used as positive controls.

6.3.2 Results

Although *Leptospirillum* MT6 readily oxidised Cae Coch rock pyrite (positive controls) by day 20, this iron-oxidiser was unable to oxidise both the acetone-washed and the perchloric acid-washed pyrite concentrates as well as water-washed and unwashed pyrite concentrates (negative controls; data not shown). Acetone- and perchloric acid-washing of the pyrite concentrate, therefore, failed to eliminate the "inhibitory compound(s)" present in the pyrite concentrate.

6.4 Determination of MICs (minimum inhibitory concentrations) of flotation reagents

6.4.1 Methods

Universal bottles containing 4.8 ml of media were prepared. These were (i) ferrous iron medium (section 2.2.1.1.1) for *Leptospirillum* MT6; (ii) ferrous iron/yeast extract medium (section 2.2.1.1.3) for *Am. ferrooxidans* ICP and *Sulfobacillus* NC; (iii) tetrathionate medium (section 2.2.1.1.2) for *At. caldus* KU; (iv) "*Ferroplasma*" medium (section 2.2.1.1.5) for *Ferroplasma* MT17. Solutions of Senmin flotation reagents (listed in Table 6.1) were prepared, and added, at different concentrations (initially, 0, 1.0, 10 and 100 μg/ml) to the media. Based on the initial results, a second experiment was set up using an extended range of concentrations of the flotation reagents. The universal bottles were inoculated with 0.2 ml of active cultures and incubated at 45°C, shaken. Flotation reagent stock solutions were prepared either with distilled water or with ethanol (SK series, AP407, AF25, 6005A); those prepared with distilled water were sterilised through 0.2 μm membrane filters (Whatman, England). In order to check that the bacteria used were not inhibited by the concentrations of ethanol used in these experiments, control experiments (using 1-5%, v/v ethanol) were set up.

Table 6.1: Flotation chemicals used in this study (Senmin reagents, supplied by BHP Billiton, South Africa).

Chemical Class	Commercial name	Chemical name
1. Xanthates	**SEX**	Sodium Ethyl Xanthate
	SNPX	Sodium *n*-Propyl Xanthate
	SIBX	Sodium Isobutyl Xanthate
	PNBX	Potassium *n*-Butyl Xanthate
	PAX	Potassium Amyl Xanthate
	X222	Mixture of Xanthates
2. Dithiocarbamates	**SK100**	Sodium (Alkyl) dithiocarbamate
	SK294	Sodium (Alkyl) dithiocarbamate
3. Thionocarbamate	**SK700**	Isopropylthionocarbamate
4. Dithiophosphate	**AF25**	Dithiophosphates (mixture)
5. Mercaptobenzthiazole	**SK50**	Sodium-2-Mercaptobenzthiazole
6. Mixtures of different classes	**SK708**	(Alkyl) thionocarbamate and Sodium Di-(Alkyl) Dithiophosphate
	SK756	Sodium (Alkyl) dithiocarbamate and Sodium Di-(Alkyl) Dithiophosphate
	AP407	Sodium-2-Mercaptobenzthiazole and Sodium Di (Alkyl) Dithiophosphate
7. Frother	**6005A**	Aromatic acid, Diakyl ester 1,1,3 Triethoxybutane

Depending on rate of ferrous iron oxidation/growth of control cultures (incubated in the absence of flotation chemicals), cultures of *Leptospirillum* MT6, *At. caldus*, *Sulfobacillus* NC and *Am. ferrooxidans* were incubated for 4-7 days and *Ferroplasma* MT17, due to its relatively slow ferrous iron oxidation rate, was incubated for 6-10 days until any effects of the flotation chemicals (compared with control cultures) were apparent. Growth of iron-oxidising organisms was monitored by measuring ferrous iron concentrations (section 2.4.2.2) and growth of *At. caldus* by cell counts, using a Thoma counting chamber (section 2.2.2.2.1).

6.4.2 Results

Concentrations of ethanol that inhibited the growth of *Leptospirillum* MT6, *Ferroplasma* MT17, *At. caldus* KU, *Sulfobacillus* NC and *Am. ferrooxidans* ICP were 1%, 1%, 2%, 1% and 5% (v/v), respectively (data not shown). Therefore, stock solutions of flotation reagents (dissolved in ethanol) were prepared to ensure that concentrations of ethanol were always below inhibitory levels.

Ferrous iron oxidation by *Leptospirillum* MT6 in the presence of different concentrations of X222, and numbers of *At. caldus* in the presence of different concentrations of 6005A, are shown in Figures 6.4 and 6.5. No inhibition of ferrous iron oxidation by *Leptospirillum* MT6 was observed in the presence of 0.5 μg/ml X222; however, at concentrations above 1.0 μg/ml, iron oxidation by this iron-oxidiser was increasingly inhibited (Figure 6.4). Growth of *At. caldus* was very similar in media containing 6005A at 0-100 μg/ml, but was inhibited in the presence of 200 μg/ml 6005A (Figure 6.5).

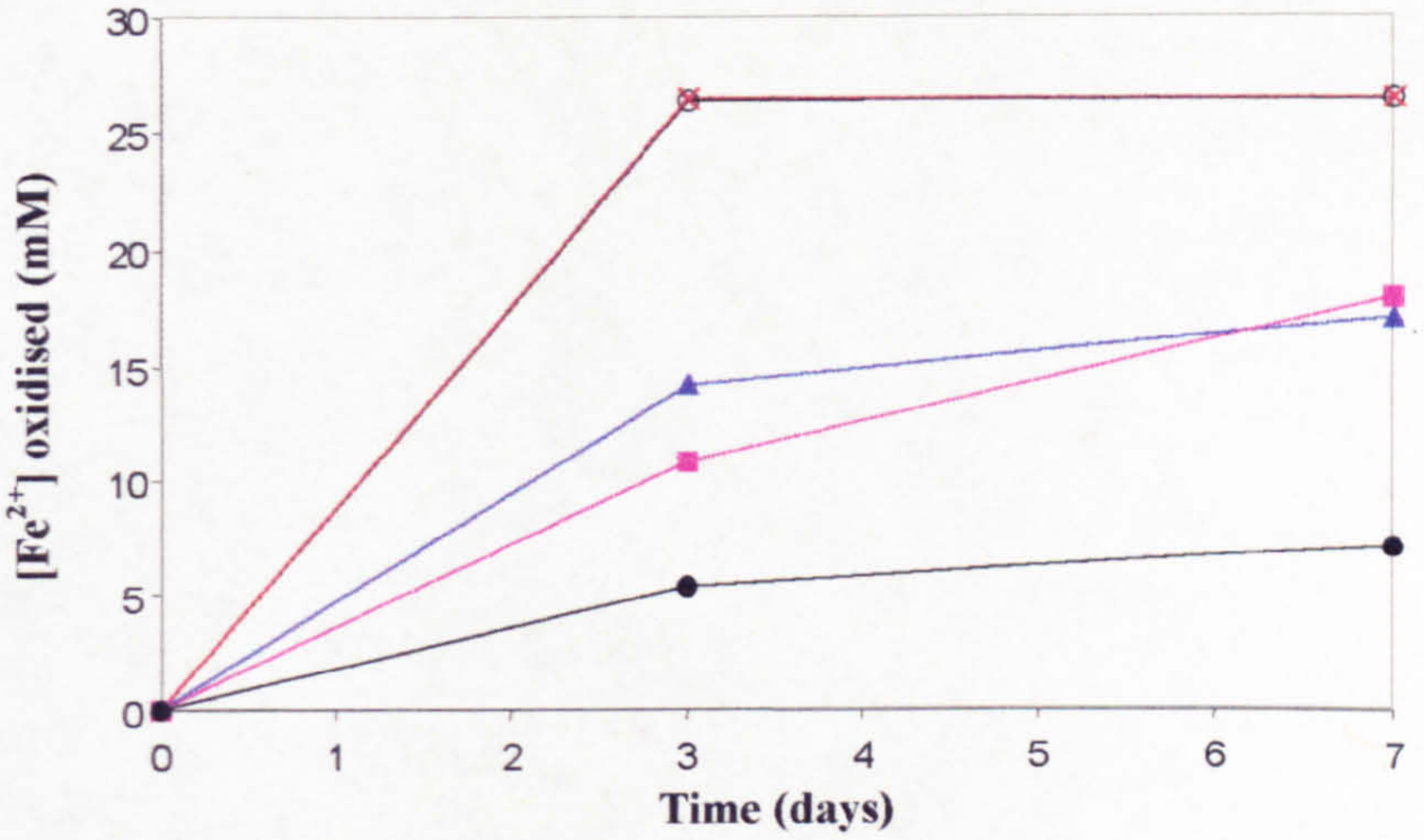


Figure 6.4: Ferrous iron oxidation by *Leptospirillum* MT6 in the presence of different concentrations of X222. Key: ○, 0 µg/ml; ×, 0.5 µg/ml; ▲, 1.0 µg/ml; ■, 2.0 µg/ml; ●, 5.0 µg/ml.

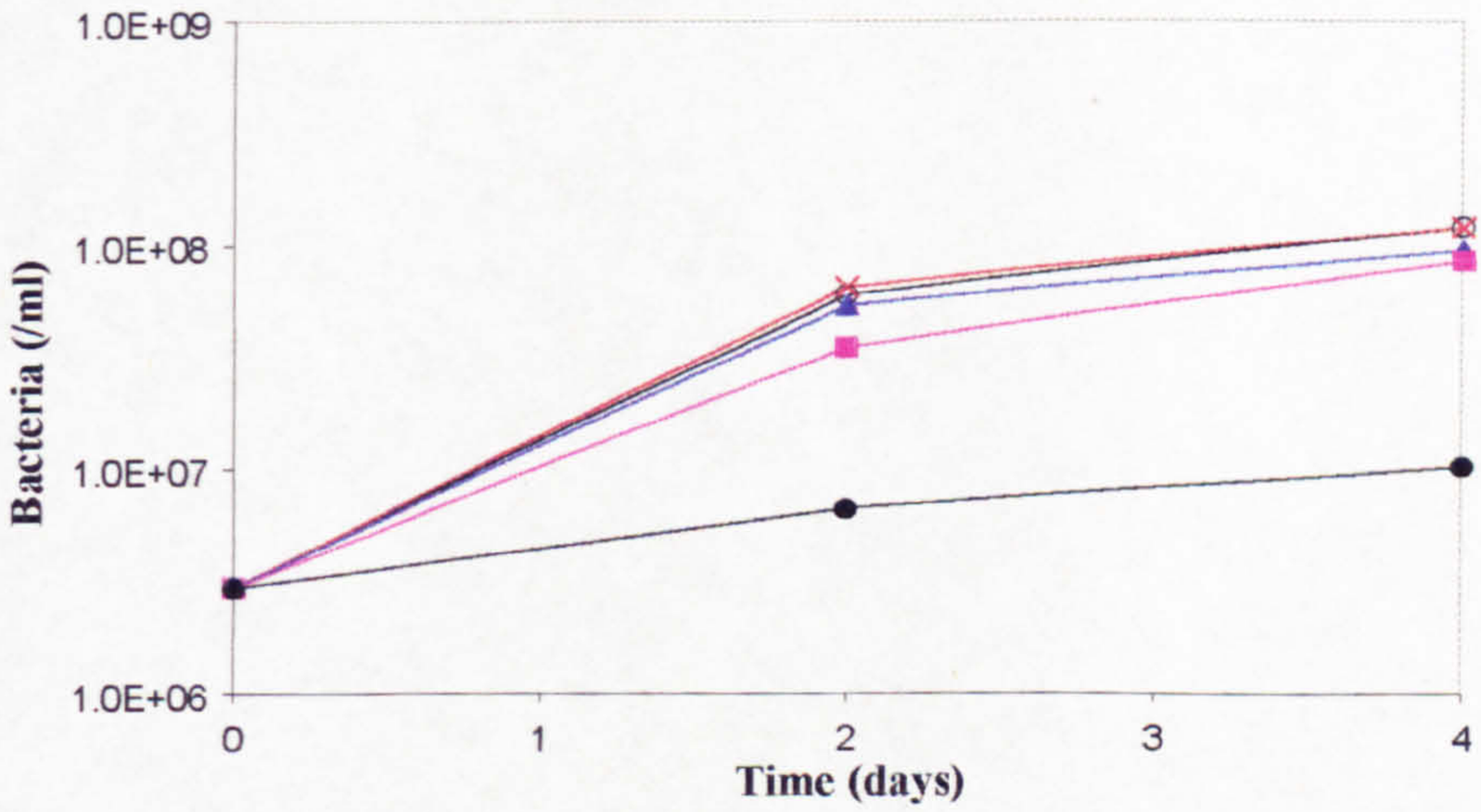


Figure 6.5: Growth of *At. caldus* KU in the presence of different concentrations of 6005A. Key: ○, 0 µg/ml; ×, 1.0 µg/ml; ▲, 10 µg/ml; ■, 100 µg/ml; ●, 200 µg/ml.

As shown in Figures 6.4 and 6.5, the MICs of different flotation reagents were determined for each acidophile (Table 6.2), as concentrations at which ferrous iron oxidation or growth was totally or partially inhibited. Where the flotation reagents caused a very minor lag before ferrous iron oxidation or growth commenced (e.g., growth of *At. caldus* with 100 µg/ml 6005A, Figure 6.5), this was not recorded as an inhibitory concentration.

Table 6.2: The MICs* of flotation reagents to some moderately thermophilic acidophiles. X/Y represents X; concentration (μg/ml) at which no inhibition was observed, Y; concentration (μg/ml) at which inhibition was observed.

Flotation reagents	*Leptospirillum* MT6	*Ferroplasma* MT17	*At. caldus* KU	*Sulfobacillus* NC	*Am. ferrooxidans* ICP
1. Xanthates					
SEX	50/75	100/200	100/200	500/1000	100/200
SNPX	50/75	100/200	100/200	200/300	100/200
SIBX	25/50	100/200	100/200	500/1000	100/200
PNBX	0.5/1.0	10/25	200/500	100/200	100/200
PAX	0/0.5	10/25	100/200	100/200	200/500
X222	0.5/1.0	7.5/10	100/200	75/100	100/200
2. Dithiocarbamates					
SK100	1.0/2.5	0/0.5	100/200	10/25	25/50
SK294	1.0/2.5	1.0/2.5	>200	10/25	50/75
3. Thionocarbamates					
SK700	25/50	25/50	50/75	25/50	75/100
4. Dithiophosphates					
AF25	1.0/2.5	0.1/0.5	50/75	2.5/5.0	5.0/7.5
5. Mercapto-benzthiazole					
SK50	1.0/2.5	0.5/1.0	10/25	10/25	25/50
6. Mixture of different classes					
SK708 (3+4)	0.5/1.0	0.5/1.0	25/50	10/25	10/25
SK756 (2+4)	0.5/1.0	1.0/2.5	>200	10/25	10/25
AP407 (5+4)	0.1/0.5	1.0/2.5	7.5/10	2.5/5.0	7.5/10
7. Frother					
6005A	100/200	7.5/10	100/200	200/500	500/1000

* The colour of numbers indicates the range of MICs, as below.

MIC≤0.5	0.5<MIC≤2.5	2.5<MIC≤10	10<MIC≤50	50<MIC≤200	200≤MIC

It was shown that the toxicity of flotation reagents to the moderately thermophilic acidophiles tested differed markedly. *Leptospirillum* MT6 was generally the most sensitive acidophile to the majority of the flotation reagents (except Senfroth), followed by *Ferroplasma* MT17. Among the flotation reagents tested, AF25 and AP407 appeared to be very toxic to wide range of acidophiles, with MICs for even the generally less sensitive organisms (*At. caldus*, *Sulfobacillus* NC and *Am. ferrooxidans*) being ≤10 μg/ml (except AF25 with *At. caldus*). Overall, the most toxic collectors were AF25 (a mixture of different dithiophosphates) and AP 407 (a mixture of a dithiophosphate and sodium-2-mercaptobenzthiazole), followed by SK50 (sodium-2-mercaptobenzthiazole) and then the dithiocarbamates. As noted, *Leptospirillum* MT6 and *Ferroplasma* MT17 were the most sensitive acidophiles with MICs of these reagents ≤2.5 μg/ml. The toxicity of SK700 (isopropylthionocarbamate) did not differ much between the different acidophiles. The toxicity of the xanthates to the acidophiles was quite variable. The potassium xanthates (PNBX and PAX) and X222 (which was a mixture of different xanthates) were highly toxic to *Leptospirillum* MT6 and *Ferroplasma* MT17, though less so to the other three moderate thermophiles. In contrast, the sodium xanthates (SEX, SNPX and SIBX) were generally less toxic to all the acidophiles. Senfroth, the only frother tested, was one of the least toxic flotation reagents tested, except to *Ferroplasma* MT17 (7.5<MIC≤10).

6.5 Potential elimination of "inhibitory compound(s)" of Mintek pyrite concentrate by pre-oxidation with *Sulfobacillus* NC.

To confirm the hypothesis that the putative inhibitory compound(s) present in the pyrite concentrate could be eliminated by *Sulfobacillus* NC, pyrite concentrate was pre-oxidised with *Sulfobacillus* NC prior to inoculation with *Leptospirillum* MT6.

6.5.1 Methods

Sulfobacillus NC was pre-grown in 2% pyrite medium (Mintek pyrite concentrate, pH 2.0; section 2.2.1.1.4) with or without the addition of 0.02% (w/v) yeast extract. One hundred millilitre flasks, each containing 50 ml of 2% pyrite medium (Mintek pyrite concentrate, pH 2.0) were inoculated with the pre-grown *Sulfobacillus* NC and incubated for 1, 3, 5, 10 days; flasks containing uninoculated medium were used as controls (triplicate flasks in each case). An additional three inoculated flasks containing pyrite medium plus 0.02% yeast extract were incubated for 5 days. After incubation, each flask was vortexed in order to remove attached cells from pyrite concentrate, and the pyrite concentrate recovered by very gentle centrifugation. The concentrate was then resuspended in 50 ml of autotrophic basal-salts (pH 2.0; section 2.2.1) and autoclaved. *Leptospirillum* MT6, pre-grown in 1% pyrite medium (Cae Coch rock pyrite, pH 2.0; section 2.2.1.1.4) was inoculated to 2 of the 3 flasks for each pre-incubation (1 of the 3 flasks was used as an uninoculated control) and the flasks were incubated for 30 days at 45°C, shaken at 130 rpm. Samples were removed every 10 days for determination of total soluble iron (section 2.4.3). In addition, cultures were analysed after 30 days for dissolved organic carbon (DOC; section 2.4.7).

6.5.2 Results

Oxidation of pyrite concentrate (following pre-treatment with *Sulfobacillus* NC or not) by *Leptospirillum* MT6 and in uninoculated controls, is shown in Figures 6.6 and 6.7.

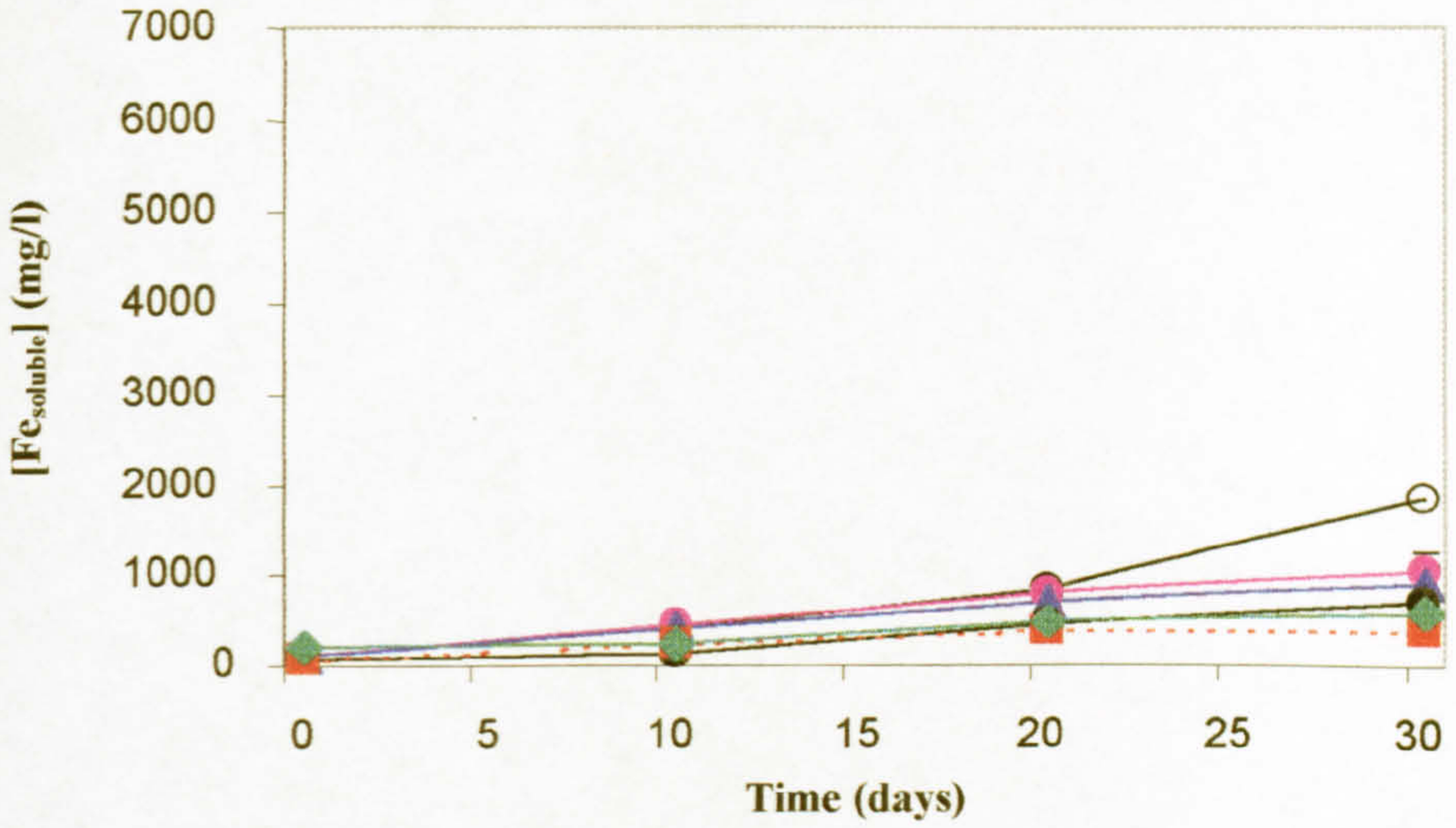


Figure 6.6: Oxidation of pyrite concentrate (pre-oxidised by *Sulfobacillus* strain NC) in uninoculated control cultures. Key: ○, not pre-oxidised (no incubation after inoculation with *Sulfobacillus* NC); ●, pre-oxidised for 1 day; ▲, pre-oxidised for 3 days; ●, pre-oxidised for 5 days; ■, pre-oxidised for 5 days with 0.02% yeast extract (broken line); ◆, pre-oxidised for 10 days.

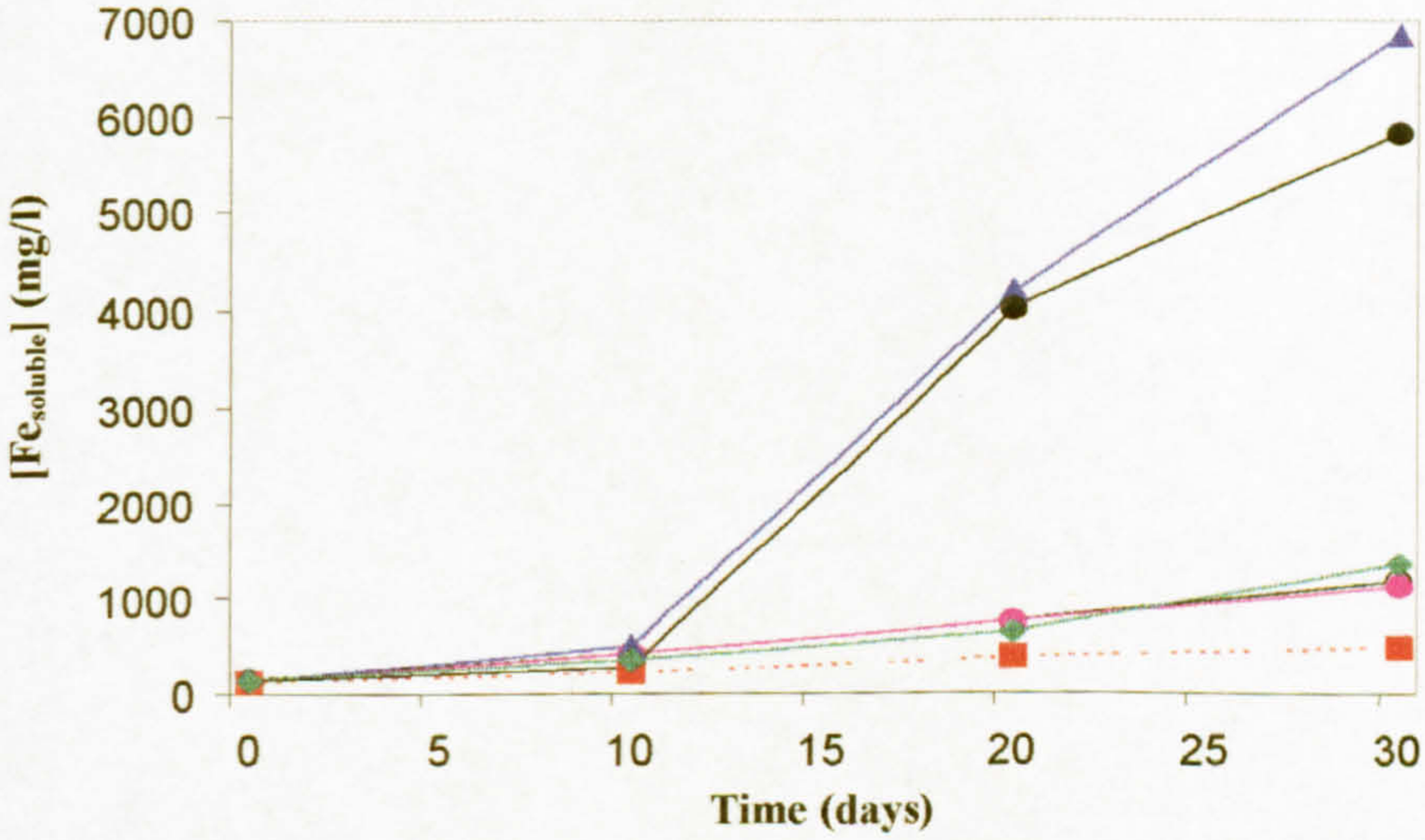


Figure 6.7: Oxidation of pyrite concentrate (pre-oxidised by *Sulfobacillus* strain NC) by *Leptospirillum* MT6. Key: ○, not pre-oxidised (no incubation after inoculation with *Sulfobacillus* NC); ●, pre-oxidised for 1 day; ▲, pre-oxidised for 3 days; ●, pre-oxidised for 5 days; ■, pre-oxidised for 5 days with 0.02% yeast extract (broken line); ◆, pre-oxidised for 10 days.

In uninoculated control cultures, no significant oxidation of the pyrite concentrate was observed (Figure 6.6), confirming that *Sulfobacillus* NC did not survive the autoclaving process. As expected, the pyrite concentrate that had not previously been exposed to

Sulfobacillus NC was not oxidised by *Leptospirillum* MT6. There was also no oxidation of pyrite concentrate, pre-oxidised by *Sulfobacillus* NC for 1 day, 5 days (with yeast extract), or 10 days. In contrast, pyrite oxidation by *Leptospirillum* MT6 did occur with pyrite concentrate, pre-oxidised for either 3 or 5 days by *Sulfobacillus* NC.

DOC concentrations in the cultures at day 30 are shown in Figure 6.8.

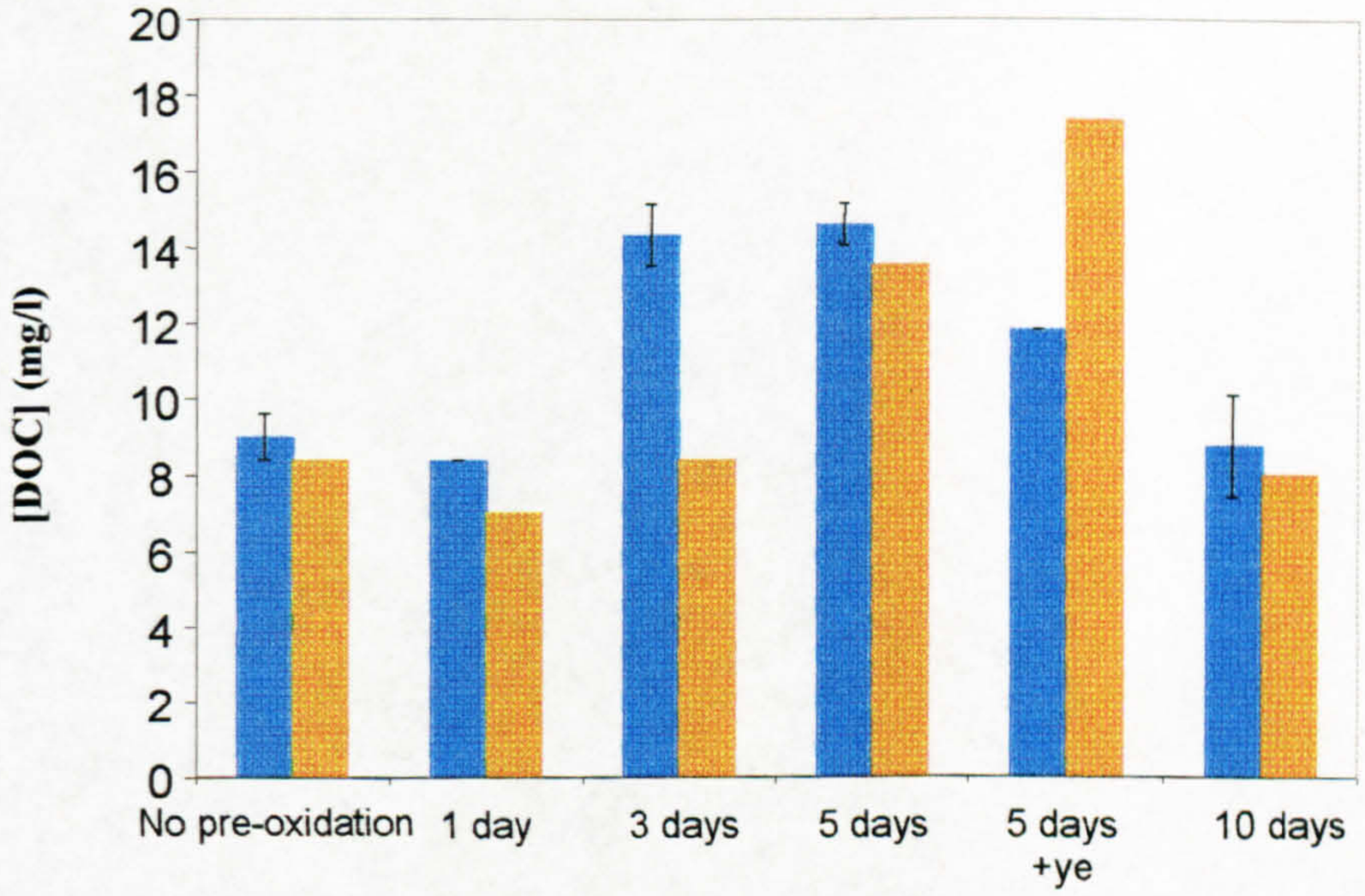


Figure 6.8: DOC concentrations in cultures at day 30. Key: ■, inoculated with *Leptospirillum* MT6; ■, not inoculated with *Leptospirillum* MT6.

DOC concentrations in cultures where pyrite concentrate oxidation by *Leptospirillum* MT6 did not occur were similar to each other (7-9 mg/l). DOC concentrations of the cultures in which *Leptospirillum* MT6 oxidised the pyrite concentrate were ~15 mg/l. Exceptions to this were those cultures where the pre-treatment had included the addition of yeast extract, and also, for some unknown reason, where the pyrite concentrate had been pre-treated for 5 days without yeast extract and subsequently not inoculated with *Leptospirillum* MT6.

6.6 Effect of pre-treating rock pyrite with X222 on subsequent pyrite oxidation by *Leptospirillum* MT6

The aim of this experiment was to examine whether or not Cae Coch rock pyrite, which was readily oxidised by *Leptospirillum* MT6, could be rendered non-leachable by treatment with the flotation reagent X222.

6.6.1 Methods

Suspensions of 1 g of Cae Coch rock pyrite in 10 ml of sterile distilled water (adjusted to pH 2.0) containing different concentrations (0, 1, 10, 50, and 100 µg/ml) of X222 (sterilised through 0.2 µm membrane filters), were shaken in 100 ml flasks at 37°C for 4 hours. The pyrite was then recovered by centrifugation and resuspended in 50 ml of sterile autotrophic basal salts (pH 2.0), inoculated with *Leptospirillum* MT6, and incubated for 25 days, shaken, at 45°C. Samples were removed every 5 days for determination of total soluble iron (section 2.4.3).

6.6.2 Results

All of the treated and control pyrites were oxidised at similar rates by *Leptospirillum* MT6 (data not shown). Pre-treatment of pyrite with X222, therefore, did not result in inhibition of oxidation by this bacterium.

6.7 Discussion

Shake flask experiments with Mintek pyrite concentrate (chapter 4) had shown that *Leptospirillum* MT6 was unable to oxidise this material in pure culture. However, in mixed cultures containing *Sulfobacillus* NC, *Leptospirillum* MT6 was always noted to be the dominant iron-oxidising bacterium present. It was hypothesised, therefore, that

the reason for this might that residual flotation chemicals were present in the concentrate in levels that inhibited *Leptospirillum* MT6, but that these were at least partially removed (possibly metabolised) by mixotrophic *Sulfobacillus* NC. The experiments described in this chapter sought to provide support for this hypothesis, and also to look more generally at the toxicity of flotation reagents used commercially in mineral recovery, to moderately thermophilic acidophilic prokaryotes. The suggestion that, by lowering the concentration of the pyrite concentrate, it would be possible to get to a point below the threshold concentration of the inhibitory substance(s) while still having sufficient pyrite concentrate present for oxidation to be observed was tested. However, even at mineral suspensions as low as 0.1%, no pyrite concentrate oxidation by *Leptospirillum* MT6 was observed. Interestingly, the mesophilic type strain of *L. ferrooxidans* was also unable to oxidise the pyrite concentrate at pulp densities of 0.1-2.0%, though the type strain of *At. ferrooxidans* had no problem in oxidising this material. Since *Leptospirillum* MT6 was able to oxidise ferrous iron present in mineral-free pyrite concentrate liquor media following autoclaving, whereas the pyrite concentrate itself was not oxidised; the "inhibitory compound(s)" appeared, therefore, to be associated with the mineral surfaces (as would be supposed) and affect attached cells.

MICs of flotation reagents were determined using five species of moderately thermophilic acidophiles. The toxicity of flotation reagents to different acidophiles was found to differ significantly, *Leptospirillum* MT6 and *Ferroplasma* MT17 being more sensitive to these chemicals than *Sulfobacillus* NC, *Am. ferrooxidans* ICP and *At. caldus*. Relatively high MICs displayed by the mixotrophic acidophiles, *Sulfobacillus* NC and *Am. ferrooxidans* ICP, possibly resulted from an ability to metabolise the flotation chemicals though this hypothesis was not tested. On the other hand, relatively

high MICs of *At. caldus* may have been due to an inherent resistance to these chemicals. Among the flotation reagents tested, the dithiophospates were, overall, the most toxic, and the sodium xanthates the least (except for the single frothing reagent tested, Senfroth 6005A, which was highly toxic only to *Ferroplasma* MT17).

Inhibition of ferrous iron oxidation by *At. ferrooxidans* by different flotation chemicals was reported by Tuovinen (1978); sodium butyl xanthate and Dowfroth 250 were the least toxic (1% and 2% inhibition at 500 μg/ml, respectively) and potassium ethyl xanthate was the most (85% inhibition at 100 μg/ml) to this mesophile. The effect of xanthate flotation chemicals on leaching of chalcopyrite by *At. ferrooxidans* was studied by Loon and Madgwich (1995). Among the xanthates tested (isopropyl-, isobutyl-, amyl-, ethyl-), isopropyl xanthate was the least toxic and amyl xanthate the most. Copper solubilisation was depressed by 30% by isopropyl-, 53% by isobutyl- and ethyl, and 77% by amyl xanthate at 10 mM (Loon and Madgwick, 1995). Contrasting results were found by Valdivia and Chaves (2001) who reported that the toxicity of amyl xanthate and isopropyl xanthate to *At. ferrooxidans* was less than ethyl xanthate. These sulfide collectors are not stable under the growth conditions of acidophilic bacteria, therefore, it is difficult to compare in detail the different toxic concentrations of the various chemicals in different conditions (Tuovinen, 1978).

Different degrees of inhibition by flotation reagents of ferrous iron and thiosulfate oxidation have been reported, suggesting a growth-substrate specific mechanism of toxicity and possible pH-related effects on the chemical decomposition and formation of intermediates (Tuovinen, 1978). Valdivia and Chaves (2001) found that, in the presence of xanthates, there was a reduction in the percentage of cell attachment, and that cell growth and oxidative activity decreased initially, although *At. ferrooxidans* gradually developed tolerance to the xanthates.

Although acetone- and perchloric acid-washing of the pyrite concentrate failed to remove the "inhibitory compound(s)", pre-treatment of pyrite concentrate with *Sulfobacillus* NC (3-5 days) did allow pure cultures of *Leptospirillum* MT6 to oxidise the concentrate. *Leptospirillum* MT6 did not oxidise pyrite concentrate pre-oxidised for 5 days in the presence of yeast extract, suggesting that *Sulfobacillus* NC utilised the yeast extract rather than the supposed residual flotation chemicals. Surprisingly, a 10-day pre-treatment with *Sulfobacillus* NC did not result in successful oxidation of the concentrate by *Leptospirillum* MT6. Although the amount of pyrite concentrate oxidised by *Sulfobacillus* NC was not determined, pyrite oxidation by pure cultures of *Sulfobacillus* NC was normally limited (in yeast extract-free medium) and this Gram-positive bacterium would not have been expected to solubilise all of the FeS_2 present in 10 days. Therefore, there should still have been a sufficient residual pyrite in pre-treated concentrate for *Leptospirillum* MT6. Also, the DOC concentrations in cultures following 10-day pre-treated pyrite concentrate were not higher than those in other cultures, threfore, there was no evidence that there was any inhibition of *Leptospirillum* MT6 growth due to organic materials, and the reason for lack of oxidation by *Leptospirillum* MT6 of the pyrite concentrate, pre-treated with *Sulfobacillus* NC for 10 days, is not known. Higher DOC concentrations, presumably resulting from the growth of *Leptospirillum* MT6, were observed in cultures with 3 or 5-day pre-oxidised pyrite concentrate. However, the highest DOC concentrations were observed with 5-day pre-oxidised (in the presence of yeast extract) pyrite concentrate; this possibly resulted from a greater amount of *Sulfobacillus* NC biomass (due to yeast extract) attached to the mineral surface that lysed during autoclaving process. The reason why the DOC concentration was also relatively large in the uninoculated 5-day pre-oxidised pyrite concentrate, was not clear.

Treatment of rock pyrite with X222 did not result in inhibition of pyrite oxidation by *Leptospirillum* MT6, probably because this flotation reagent is a mixture of xanthates used for selective coating of economically important sulfide minerals, therefore, did not coat the rock pyrite (~80% of which is FeS_2 and the rest is mostly quartz). This was not too surprising, as flotation reagents are designed to separate the more valuable metal-containing minerals (such as chalcopyrite) from those of little economic value (such as pyrite). Further experimental work is required in this area.

Together with the results from chapter 4, it is likely that, like *Sulfobacillus* NC, other Gram-positive acidophiles which caused enhanced pyrite concentrate oxidation in mixed cultures with *Leptospirillum* MT6, might also be used for pre-treatment of potentially recalcitrant sulfide concentrates, or else be included in leaching consortia with *Leptospirillum* spp.. *Am. ferrooxidans* ICP would appear to be a suitable acidophile in this context, since its MICs of the flotation reagents were also quite large. This finding would be useful especially when "flotation chemical sensitive" acidophiles, such as *Leptospirillum* MT6 and *Ferroplasma* MT17 are used as the primary sulfide oxidisers in mineral processing systems.

Chapter 7

Ferric Iron Sensitivity in *Sulfobacillus* spp. and Related Gram-Positive, Iron-Oxidising Bacteria

7.1 Introduction

The diversity of microorganisms that catalyse ferrous iron oxidation is now known to be extensive. Among these microorganisms, Gram-positive iron-oxidising acidophiles that have been described fall into two main phylogenetic groups: the low G+C spore-forming Gram-positives, and those which (from 16S rRNA gene analysis) occur within the class *Actinobacteria* (Hallberg and Johnson, 2001a). Gram-positive iron-oxidising acidophiles that were first isolated were all found to be moderate thermophiles (temperature range ~40-60°C); however, more recently mesophilic species from both groups have been characterised (Johnson, *et al.*, 2001a; Hallberg and Johnson, 2001a). While most characterised low G+C Gram-positives are *Sulfobacillus* spp., other isolates appear, from their 16S rRNA gene sequences, to be novel genera (Johnson *et al.*, 2001a).

In a study of moderately thermophilic iron-oxidising acidophiles isolated from two sites in Yellowstone National Park, Wyoming, Johnson *et al.* (2001b) noted that oxidation of ferrous iron in shake flask cultures poised initially at pH 2.0 did not go to completion. Further experiments showed that this was not due to limiting concentrations of any nutrient or growth factor, and that the same cultures were able to oxidise all of the ferrous iron present if the initial culture pH was lowered to 1.8. The extent to which iron oxidation was retarded in the pH 2.0 cultures varied between isolates.

This chapter describes a detailed study of the phenomenon of pH-related ferric iron inhibition of *Sulfobacillus*-like bacteria.

7.2 Ferrous iron oxidation in shake flask cultures

7.2.1 Methods

A total of fifteen moderately thermophilic and six mesophilic iron-oxidising acidophiles were used in these experiments; these are listed in Table 7.1.

Table 7.1: Acidophilic, iron-oxidising bacteria used in ferric iron toxicity experiments.

Moderate thermophiles	Source/origin	Reference
Leptospirillum MT6	Bioleaching plant: South Africa	This study
Sb. thermosulfidooxidans^T	Sulfide mineral leach dumps	Karavaiko *et al.*, 1988
Sb. thermosulfidooxidans TH1	Thermal spring, Iceland	Brierley *et al.*, 1978
Sb. acidophilus ALV	Self-heating coal spoil, England	Norris and Barr, 1985
"*Sb. yellowstonensis*" YTF1	Frying Pan hot spring, Yellowstone N.P.	Ghauri and Johnson, 1991
Sulfobacillus YTF3	Sylvan hot springs, Yellowstone N.P.	Johnson *et al.*, 2001b
Sulfobacillus YTF5	Sylvan hot springs, Yellowstone N.P.	Johnson *et al.*, 2001b
Sulfobacillus YTF17	Sylvan hot springs, Yellowstone N.P.	Johnson *et al.*, 2001b
Sulfobacillus Y002	Thermal spring, Gibbon river area, Yellowstone N.P.	This study
Sulfobacillus Y006	Thermal spring, Gibbon river area, Yellowstone N.P.	This study
Sulfobacillus Y0015	Frying Pan hot spring, Yellowstone N.P.	This study
Sulfobacillus Y0016	Frying Pan hot spring, Yellowstone N.P.	This study
Sulfobacillus Y0017	Frying Pan hot spring, Yellowstone N.P.	This study
Am. ferrooxidans^T (ICP)	Icelandic geothermal site	Clark and Norris, 1996
Am. ferrooxidans TH3	Copper leach dump, New Mexico	Norris and Barr, 1985
Isolate GSM	Golden Sunlight Mine, Montana	Johnson *et al.*, 2001a
Mesophiles		
At. ferrooxidans^T	Acid mine drainage, eastern U.S.A.	Temple and Colmer, 1951
L. ferrooxidans^T	Copper mine, Armenia	Markosyan, 1972
"*Sb. montserratensis*" L15	Thermal pool, Montserrat, W.I.	Yahya *et al.*, 1999
Sulfobacillus Riv14	Thermal pool, Montserrat, W.I.	Yahya *et al.*, 1999
Isolate SLC 66	Weathering sulfidic regolith, Utah	Johnson *et al.*, 2001a
"*Fm. acidophilum*" T23	Acid mine drainage, Wales	Johnson *et al.*, 2001a

Liquid medium (pH 1.8 or 2.2) containing 25 mM ferrous sulfate and 0.02% (w/v) yeast extract (section 2.2.1.1.3) was used routinely in these experiments. Growth of most Gram-positive iron-oxidising acidophiles tends to be superior in yeast extract-amended than in "inorganic" media (where a form of reduced sulfur is usually required, together with ferrous iron). Although the Gram-negative bacteria, *At. ferrooxidans* and *Leptospirillum* spp., do not require either yeast extract or reduced sulfur, the same ferrous iron/yeast extract medium was used for uniformity of experiments, after firstly ascertaining that 0.02% yeast extract did not inhibit growth of these acidophiles. Each bacterium was pre-grown in ferrous iron/yeast extract medium at pH 1.8, and inoculated (~5%, v/v) into duplicate 100 ml flasks, each containing 50 ml of the same medium that had been adjusted to either pH 1.8 or 2.2. Cultures were incubated, shaken (150 rpm) at either 45°C (moderate thermophiles) or 30°C (mesophiles). Aliquots were removed periodically to determine ferrous iron, total soluble iron and pH.

In a second series of shake flask experiments, three different growth media were prepared. The first contained 25 mM ferrous sulfate/0.02% yeast extract (as above), the second 20 mM ferrous sulfate/5 mM ferric sulfate/0.02% yeast extract, and the third 15 mM ferrous sulfate/10 mM ferric sulfate/0.02% yeast extract. The pH of the media was adjusted to either 1.8 or 2.3, and then filter-sterilised (through 0.2 μm cellulose nitrate filters; Whatman, U.K.). These were dispensed (25 ml of each) into duplicate 100 ml sterile flasks, inoculated (as above) with those acidophiles whose growth was found to be curtailed at pH 2.2 in the first experiments, shaken, at 45° or 30°C. Ferrous iron concentrations were determined immediately after inoculation, and again 3 and 5 days later.

Ferrous iron was determined using the ferrozine colorimetric method (section 2.4.2.2). Total soluble iron was determined by centrifuging 1.5 ml sample aliquots (13,000 rpm,

1 min), adding excess ascorbic acid to the supernatant (to reduce ferric iron to ferrous) and repeating the ferrozine analysis. Concentrations of soluble ferric iron were determined from the difference between total soluble and ferrous iron.

7.2.2 Results

The concentrations of soluble ferric iron present in cultures (of initial pH 2.2) at the point at which iron oxidation was observed to halt completely, or show a marked retardation, are shown in Table 7.2. As the total initial ferrous iron concentrations in media were ~27-28 mM, the range of ferric iron concentrations also includes values >25 mM.

Most of the moderately thermophilic and mesophilic iron-oxidising bacteria oxidised >99% of the ferrous iron provided in liquid cultures where the initial pH was 1.8. The only exceptions to this were *"Fm. acidophilum"* T23 and isolate SLC 66, though these could be successfully grown in media adjusted to an initial pH of 1.9-2.0. In contrast, for cultures with an initial pH of 2.2, only the Gram-negative bacteria and the *Actinobacteria* completely oxidised the available iron. Cultures of the *Sulfobacillus* spp. and other low G+C Gram positive bacteria all displayed incomplete iron oxidation, though the amounts of ferrous iron that were oxidised in these cultures varied widely between different bacteria. The most sensitive isolates were the mesophilic *Sulfobacillus* spp. and *Sulfobacillus* YTF3, 5 and 17, while strains of both *Sb. thermosulfidooxidans* and *Sb. acidophilus* were less readily inhibited by soluble ferric iron.

Table 7.2: Concentrations of soluble ferric iron present in shake flask cultures (original pH 2.2) displaying partial or complete inhibition of ferrous iron oxidation.

<5 mM Fe^{3+}	**6-10 mM Fe^{3+}**	**11-15 mM Fe^{3+}**	**16-20 mM Fe^{3+}**	**21-25 mM Fe^{3+}**	**No inhibition (>25 mM Fe^{3+})**
Sb. montserrat-ensis L15	*Sulfobacillus* Y0017	*Sulfobacillus* Y0015	Isolate Y002	*Sb. thermo-sulfidooxidans*^{T}	*At. ferrooxidans*^{T}
Sulfobacillus Riv14	Isolate SLC66	*Sulfobacillus* Y0016	Isolate Y006	*Sb. thermosul-fidooxidans* TH1	*L. ferrooxidans*^{T}
Sulfobacillus YTF3			Isolate GSM	*Sb. acidophilus* ALV	*Leptospirillum* MT6
Sulfobacillus YTF5			*Sulfobacillus* YTF1		*Am. ferrooxidans*^{T}
Sulfobacillus YTF17					*Am. ferrooxidans* TH3
					"Fm. acidophilum" T23

Representative trends of ferrous iron oxidation that were observed in the various cultures are shown in Figure 7.1.

In the case of *Sb. thermosulfidooxidans* TH1 (pH 2.2 cultures; Figure 7.1a) ferrous iron oxidation was accompanied by corresponding increases in soluble ferric iron for the first 22 hours of incubation. The pH of the cultures increased during this time, due to proton consumption (equation [7.1]):

$$Fe^{2+} + 0.25O_2 + H^+ \rightarrow Fe^{3+} + 0.5H_2O \quad [7.1].$$

However, after 22 hours hydrolysis of ferric iron resulted in the formation of solid phase ferric compounds, and a decrease in culture pH. Equation [7.2] illustrates this for the formation of ferric hydroxide, though a variety of amorphous and (semi-)crystalline ferric mineral are known to form under such conditions, including ferrihydrite, schwertmannite and jarosites.

$$Fe^{3+} + H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad [7.2].$$

There was a notable decrease in the rate of ferrous iron oxidation from 22 hours of incubation, though iron oxidation did not stop entirely. After about 64 hours, continued hydrolysis had resulted in the culture pH decreasing to <2.3, and further lowering of soluble ferric iron concentrations. The rate of ferrous iron oxidation increased again at this point, though it was still slower than that observed in the earlier (0-22 hour) growth phase.

A similar scenario was observed with isolate GSM (Figure 7.1b), except that iron oxidation came to a virtual halt after 22 hours and did not restart. In the case of isolate YTF3, virtually no oxidation of iron was detected over a protracted (118 hour) incubation period, in contrast to the cultures at initial pH of 1.8, where complete oxidation of ferrous iron occurred (Figure 7.1c). In these cultures, the 2.5 mM soluble ferric iron present at time 0 was predominantly that contained in the inoculum.

Figure 7.1d shows the contrasting situation with the type strain of *At. ferrooxidans*, where all of the available iron was oxidised, regardless of initial culture pH and pH fluctuations. No hydrolysis of the ferric iron produced was detected in these cultures over 65 hours, which was due to the lower (30°C) temperature incubation temperature used for this (and other) mesophile.

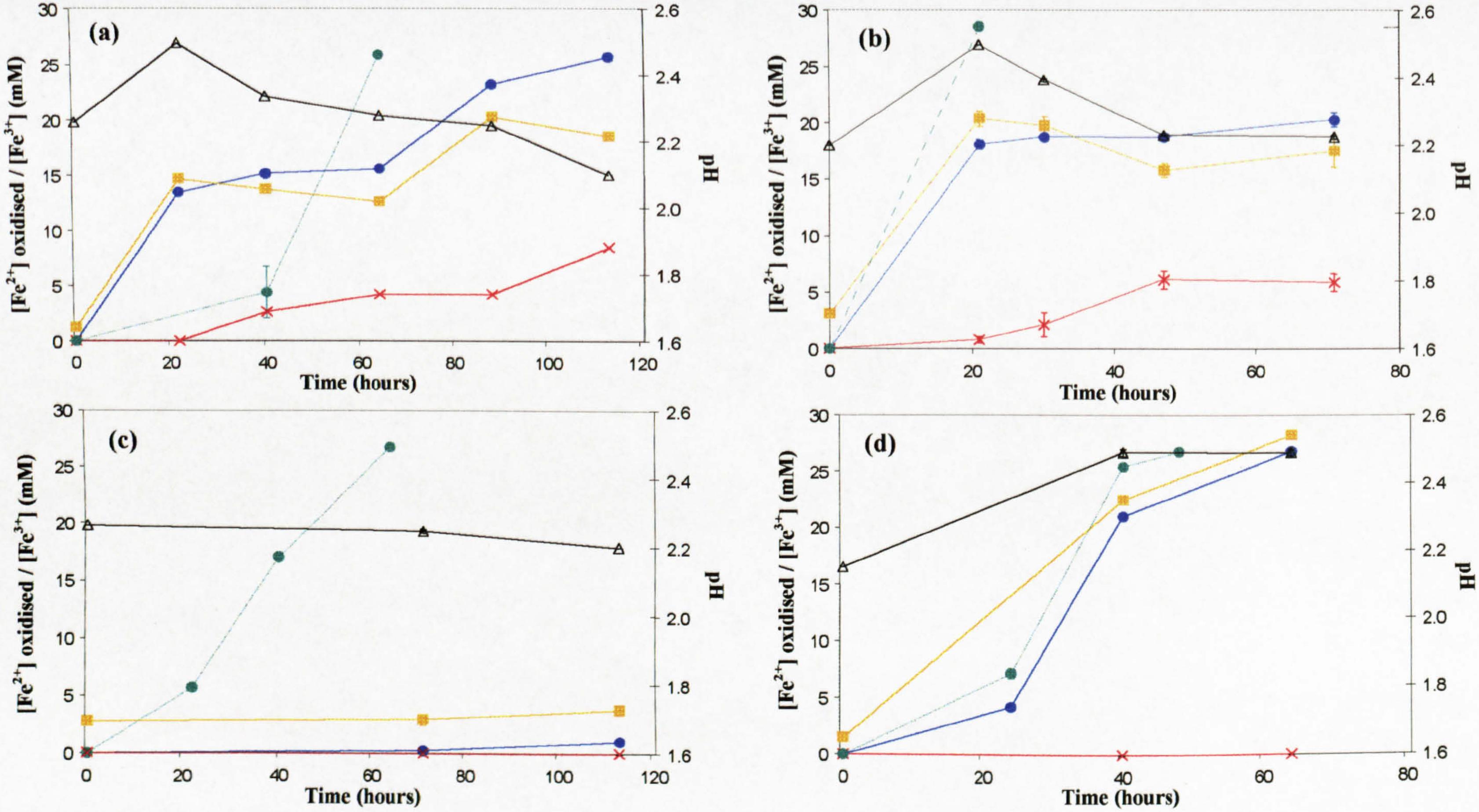


Figure 7.1: Oxidation of ferrous iron in media with initial pH 1.8 (broken lines, ●) and pH 2.2 (solid lines, ●), concentrations of soluble (■)/insoluble (×) ferric iron, and pH (△) changes at pH 2.2 in cultures of *Sb. thermosulfidooxidans* TH1 (a), isolate GSM (b), *Sulfobacillus* YTF3 (c) and *At. ferrooxidans*^{T} (d).

In the second series of experiments, all Gram-positive acidophiles tested were found to oxidise ferrous iron in pH 1.8 media containing 10 mM ferric sulfate (except that isolate SLC66 was able to do so only in media with initial pH 1.9). Concentrations of ferric iron which were found to completely inhibit ferrous iron oxidation in pH 2.3 media are shown in Figure 7.3.

Table 7.3: Concentrations of ferric iron* causing complete inhibition of ferrous iron oxidation by low G+C Gram-positive bacteria in pH 2.3 media.

0 mM Fe^{3+}	5 mM Fe^{3+}	>10 mM Fe^{3+}
"Sb. montserratensis" L15	Y002	*Sb. acidophilus* ALV
Sulfobacillus Riv14	Y006	*Sb. thermosulfidooxidans*^{T}
	Y0017	*Sb. thermosulfidooxidans* TH1
	YTF3	Isolate GSM
	YTF5	Y0015
	YTF17	Y0016
		SLC66
		"Sb. yellowstonensis" YTF1

*The figures refer to amounts of ferric sulfate added to the growth media, and exclude that introduced in the inoculum.

Again, *"Sb. montserratensis"* L15 and *Sulfobacillus* Riv14 were shown to be the most sensitive acidophiles, and were unable to oxidise ferrous iron in media containing no added ferric iron. No oxidation of ferrous iron occurred in media containing 5 or 10 mM ferric iron by isolates Y002, Y006, Y0017, YTF3, YTF5 and YTF17, but these bacteria oxidised iron in media to which no ferric sulfate had been added. All the other

acidophiles tested were able to oxidise ferrous iron in media to which 0-10 mM ferric iron had been added. There were differences between soluble ferric iron concentrations that appeared to inhibit ferrous iron oxidation by isolates Y002 and Y006, in the first (16-20 mM) and second (≤5mM) experiments. This may have been due to the slightly higher culture pH in experiment 2, or possibly due to concentrations of the inhibitory agent (a putative ferric iron complex, as discussed below) only increasing gradually in the first experiment.

Results from the second experiment confirmed that the inhibition of ferrous iron oxidation by these acidophiles was not due to the formation of solid phase ferric iron compounds or culture pH fluctuations, but was caused by soluble ferric iron.

7.3 Phylogenetic analysis of isolate YTF3

In an earlier study (Johnson *et al.*, 2001b) isolate YTF3 was tentatively identified as an *Acidimicrobium*-like Gram-positive acidophile, though DNA from this particular bacterium was not sequenced at that time. Since the pH-related ferric iron sensitivity exhibited by this acidophile in the current work was more similar to that of *Sulfobacillus* spp. than *Acidimicrobium ferrooxidans*, it was considered appropriate to ascertain its phylogeny using the 16S rRNA gene as a marker.

7.3.1 Methods

PCR-amplification and sequencing of the 16S rRNA gene, sequence analysis and phylogenetic tree assembly were carried out as described in sections 2.5.1, 2.5.7 and 2.5.8, respectively.

7.3.2 Results

Phylogenetic analysis of isolate YTF3 confirmed that it is a *Sulfobacillus* sp. (99.2% homology with "*Sb. yellowstonensis*" YTF1), in contrast to the earlier identification that was based solely on physiological criteria (Johnson *et al.*, 2001b). The relationship of isolate YTF3 to other Gram-positive acidophiles, based on 16S rRNA gene sequence data, is shown in Figure 7.2.

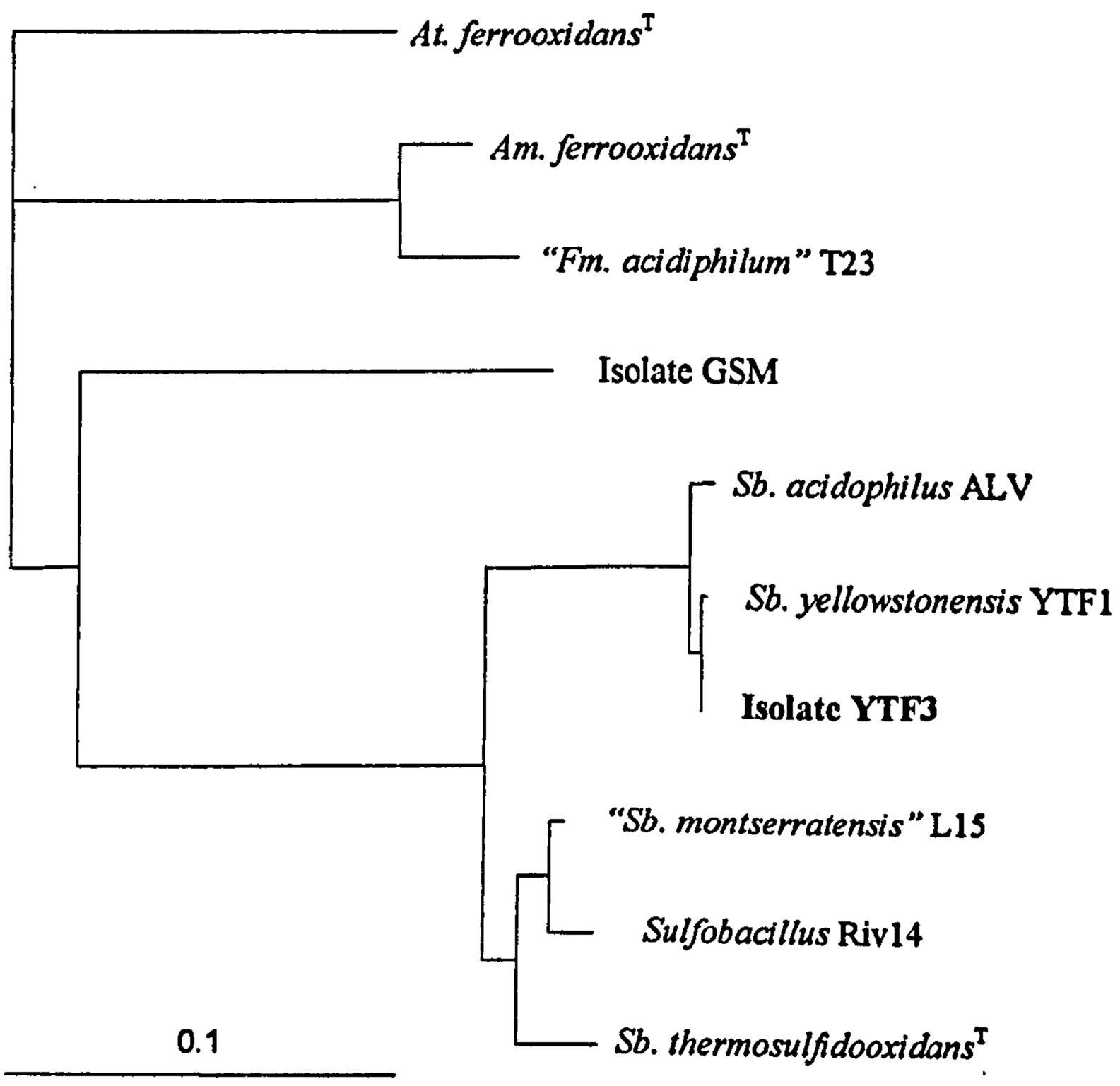


Figure 7.2: Phylogenetic relationships of isolate YTF3 (in bold) to known Gram-positive acidophiles. The phylogenetic tree was rooted with *At. ferrooxidans*. The bar represents 0.1 nucleotides substitution per 100 for the horizontal branch lengths.

7.4 Iron oxidation by isolate YTF3 in pH-controlled bioreactor cultures

7.4.1 Methods

Isolate YTF3 was found to be particularly sensitive to ferric iron inhibition in a previous study (Johnson *et al*, 2001b) and was confirmed to be so in the present work (Table 7.2). This organism was selected for further experimental work, carried out in a bioreactor in which pH was controlled to within 0.05 of a pH unit (section 2.2.1.3). The vessel was part-filled with 1.5 L of 25 mM ferrous sulfate/0.02% yeast extract medium (section 2.2.1.1.3), and inoculated with an active culture of isolate YTF3. The culture was grown at 45°C, stirred (170 rpm) and aerated (0.2 L/min) at pH 1.8. Approximately 85% of the culture liquor was removed and replaced with fresh medium following iron oxidation. Aliquots were withdrawn at regular intervals to determine ferrous iron and total iron concentrations (as described in section 7.2.1), and culture doubling times were evaluated from semi-logarithmic plots of iron oxidised against time. Following several repeated cycles at pH 1.8, a series of experiments was carried out whereby, shortly after the onset of exponential ferrous iron oxidation, the culture pH was raised and maintained (by addition of 1 M NaOH) to pH 2.00, 2.10, 2.15, 2.20 or 2.30 (+/- 0.05 pH unit) and iron concentrations monitored. To determine the effect of raising the pH on the viability of the bacteria, total counts using a Thoma counting chamber (section 2.2.2.2.1) and viable counts by plating onto ferrous iron/tetrathionate overlay medium (section 2.2.1.2.1.2) were made of culture aliquots.

7.4.2 Results

Sulfobacillus YTF3 grew readily in heterotrophic medium in the bioreactor, with the pH maintained at 1.8. Under such conditions, its culture doubling time was 2.1 hours. The

effects of increasing and maintaining culture pH to values between 2.00 and 2.30 following the onset of exponential ferrous iron oxidation (at pH 1.8) are shown in Figure 7.3.

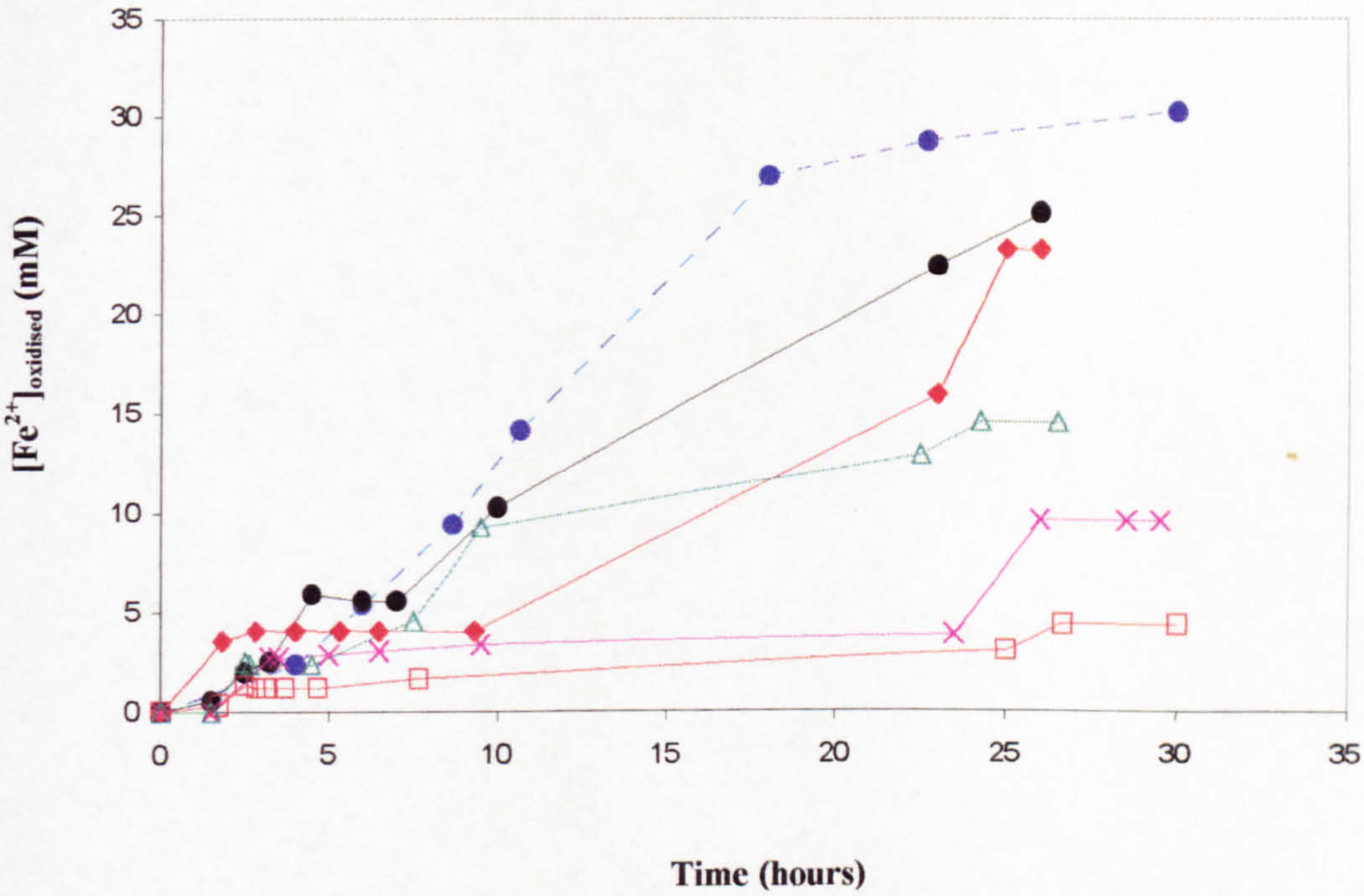


Figure 7.3: The effects of increasing and maintaining culture pH to values between 2.0 and 2.3 following the onset of exponential ferrous iron oxidation (at pH 1.8) on ferrous iron oxidation by isolate YTF3. Key: ●, pH 1.80 (continuous); ●, pH 1.80 → pH 2.00; ◆, pH 1.80 → pH 2.10; △, pH 1.80 → pH 2.15; ×, pH 1.80 → pH 2.20; □, pH 1.80 → pH 2.30.

In all cases (except the constant pH 1.80 control) iron oxidation was inhibited to some extent, though there appeared to be a correlation between the degree of inhibition and the pH to which the cultures were adjusted. Iron oxidation was either slowed down or partially inhibited by increasing culture pH to 2.00, 2.10 or 2.15, but was completely inhibited when the pH was increased to either 2.20 or 2.30. In the culture that was adjusted to pH 2.20, there was a later phase during which iron oxidation re-commenced (from about 23 to 26 hours culture incubation). The effects of these changes in pH on concentrations of ferrous iron and soluble/insoluble ferric iron, in the culture where the

pH was adjusted to 2.20, are shown in Figure 7.4. Hydrolysis resulted in the concentration of soluble ferric iron decreasing from 5.8 mM to 3.0 mM, which then increased to 6.5 mM at 9 hours, at which it remained. This allowed ferrous iron oxidation to re-start (at 23.5 hours), causing the soluble ferric iron concentration to increase to 11.5 mM after 28 hours of incubation, and this, in turn, resulted in cessation of further iron oxidation. A similar scenario was observed in cultures when the culture pH was increased to >2.10 (data not shown).

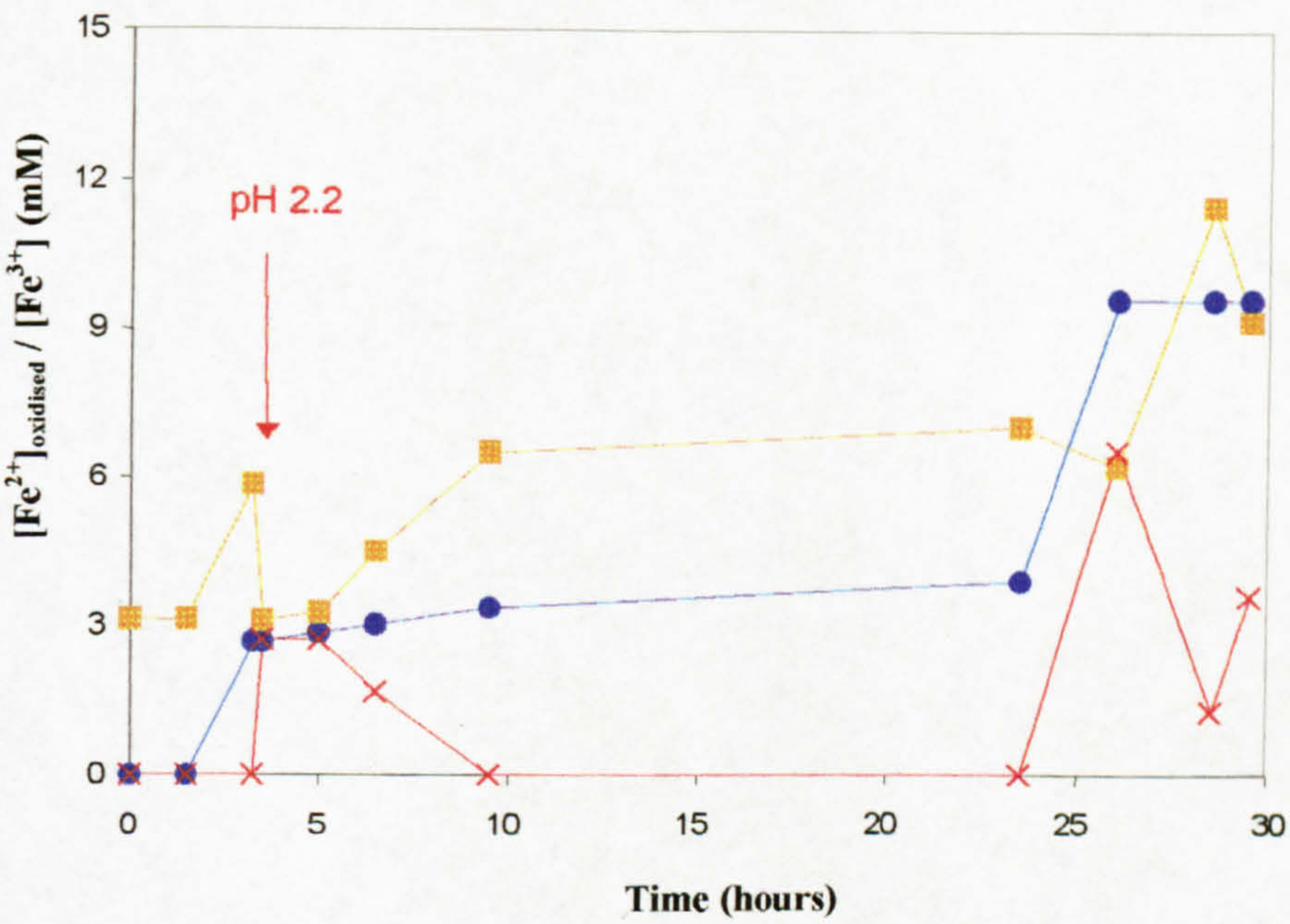


Figure 7.4: Effect of pH changes (1.80 → 2.20) on ferrous iron (●) and soluble (■) /insoluble (×) ferric iron concentrations.

The mortality rates of *Sulfobacillus* YTF3 in bioreactor cultures in which the pH was either increased to either 2.15 or 2.30 after initiation of exponential ferrous iron oxidation (at pH 1.8) are shown in Figure 7.5. In both cases, direct (Thoma) cell counts were about an order of magnitude greater than those obtained from plate counts. Adjustment of culture pH occurred 2.5 hours after the culture was set up, and 7.5 hours later numbers of viable bacteria in the (then) pH 2.30 culture were similar to those

found on earlier sampling occasions. However, on the next sampling occasion (27 hours from the start of the experiment) numbers of viable *Sulfobacillus* YTF3 had decreased by about two orders of magnitude. In contrast, the culture at pH 2.15 contained similar numbers of viable bacteria throughout the 27 hour incubation period.

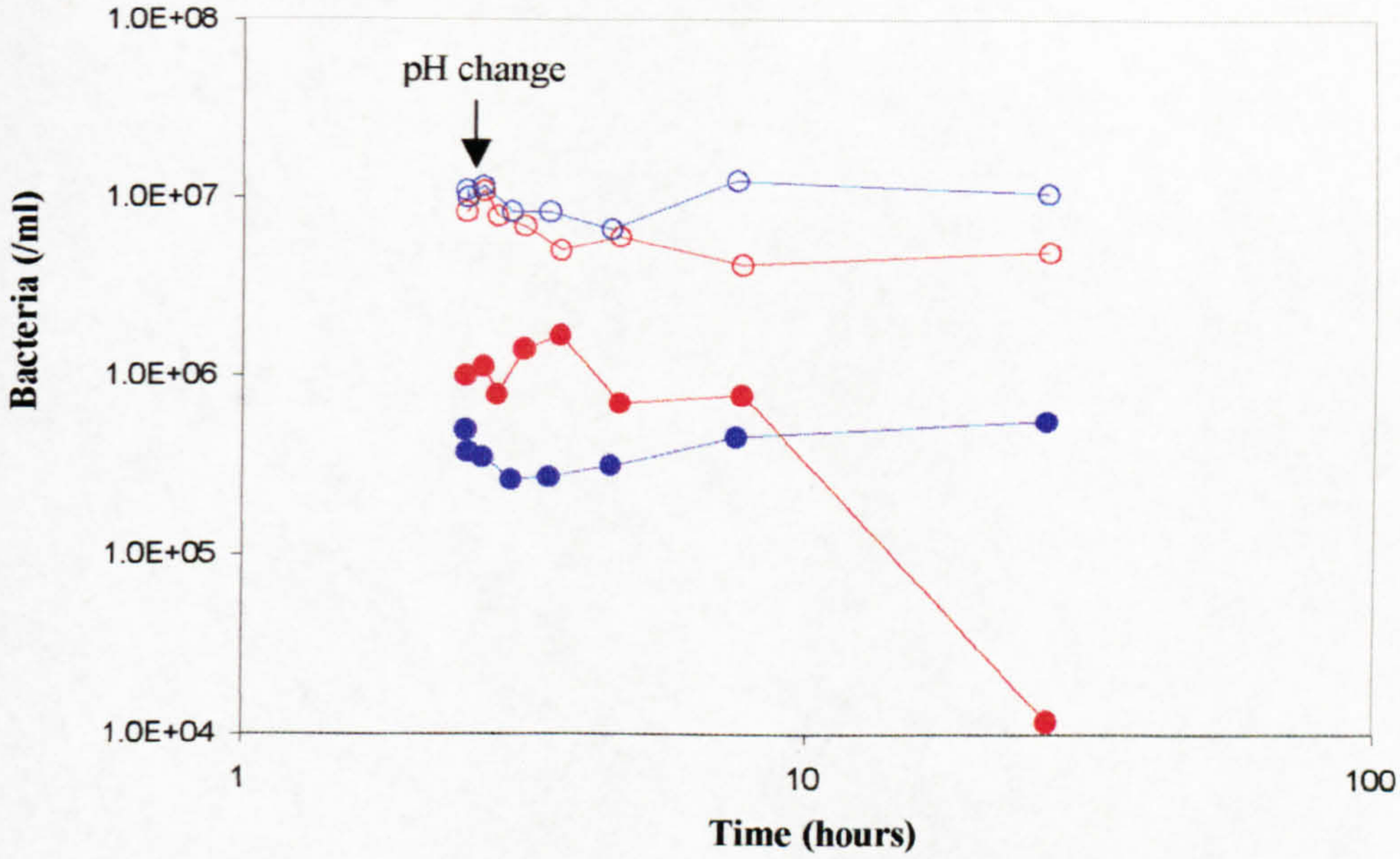


Figure 7.5: Total and viable counts of *Sulfobacillus* YTF3 in bioreactor cultures in which the pH was either increased to pH 2.15 (○, Thoma cell counts; ●, plate counts) or to pH 2.30 (○, Thoma cell counts; ●, plate counts) after initiation of exponential ferrous iron oxidation (at pH 1.80).

7.5 Growth of isolate YTF3 on glucose

7.5.1 Methods

In order to determine whether the observed inhibition of ferrous iron oxidation by isolate YTF3 at pH 2.2-2.3 was due to proton acidity directly, the organism was adapted to grow in liquid medium containing 10 mM glucose/0.02% yeast extract/500 μM ferrous sulfate, at pH ~2.0. This culture was used to inoculate the bioreactor, which contained the same liquid medium. Cultures were grown under pH-controlled conditions at pH 2.50, 2.25, 2.10, 2.00 and 1.70 (at 45°C, aerated and stirred as above).

Culture aliquots were withdrawn at regular intervals, and optical densities measured at 600 nm (section 2.2.2.1). From semi-logarithmic plots of these values against time, culture doubling times were evaluated.

7.5.2 Results

The apparent intensive pH-related ferric iron sensitivity displayed by *Sulfobacillus* YTF3 meant that it was not possible to ascertain the pH optimum and range for this isolate using the ferrous iron/yeast extract medium. However, as with many other *Sulfobacillus* spp. (particularly its nearest relative "*Sb. yellowstonensis*") *Sulfobacillus* YTF3 adapted readily to growth on glucose, in a medium in which the ferrous iron concentration was lowered to 500 μM (lowering the ferrous iron concentration beyond this resulted in much lower yields; data not shown). The effect of culture pH on the growth rates of *Sulfobacillus* YTF3 is shown in Figure 7.6.

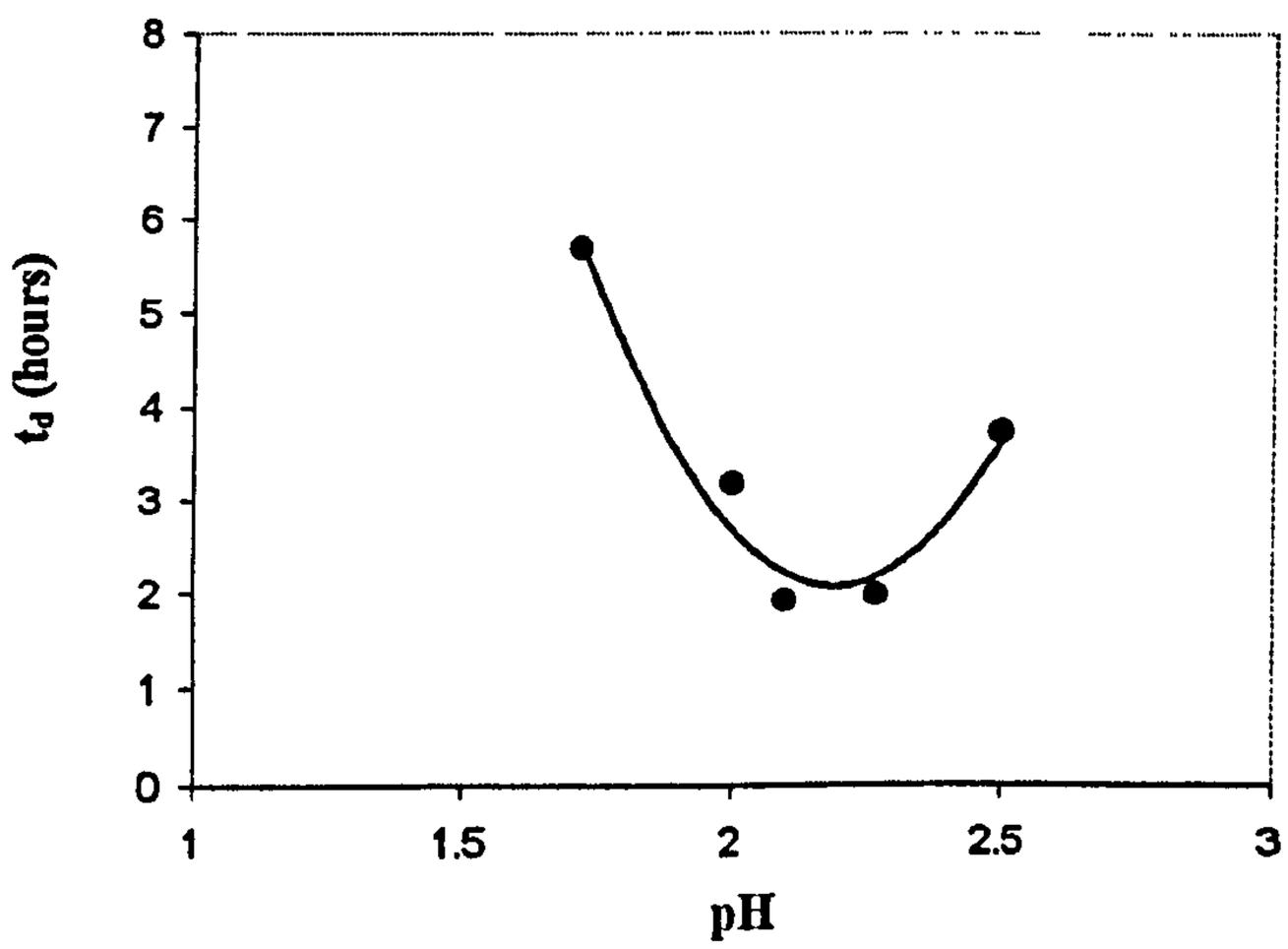


Figure 7.6: Effect of pH on the culture doubling times (t_d's) of *Sulfobacillus* YTF3 (at 45°C) grown in glucose/yeast extract medium.

The pH optimum of this acidophile was found to be pH 2.20, and growth occurred over the full pH range tested (1.7-2.5). Interestingly, fastest growth of *Sulfobacillus* YTF3 on glucose (corresponding to a culture doubling time of 2 hours) was similar to that found using ferrous iron/yeast extract medium, though at different pH values (pH 2.20 and 1.80, respectively).

7.6 Discussion

In contrast to Gram-negative iron-oxidising bacteria and *Actinobacteria, Sulfobacillus* spp. and other low G+C Gram positive iron-oxidising bacteria all displayed limited ferrous iron oxidation in cultures initially poised at pH 2.2. This trait varied widely between the different bacteria; the most "sensitive" isolates being the mesophilic *Sulfobacillus* spp. and *Sulfobacillus* YTF3, 5 and 17, while the "least sensitive" were *Sb. thermosulfidooxidans* and *Sb. acidophilus*. Strain variation might cause different degrees of sensitivity, since there was marked difference between *Sulfobacillus* YTF3 and *"Sb. yellowstonensis"* YTF1, though these two organisms share 99.2% homology in their 16S rRNA gene sequences. It appeared that this inhibition of iron oxidation was due to ferric iron, but that this was modified by culture pH, since ferrous iron oxidation went to completion when cultures were poised initially at pH 1.8 (or 1.9) and all of the ferric iron (>25 mM) remained in solution. Also, the inhibition was not a direct effect of solid phase ferric iron compounds, since inhibition of ferrous iron oxidation was observed in pH 2.3 media containing soluble ferric sulfate, before any ferric iron precipitates had formed.

Sulfobacillus YTF3 was selected for further experimental work, in view of its apparent marked sensitivity to ferric iron. The pH optimum of *Sulfobacillus* YTF3 was found to be pH 2.20, and growth occurred over the full pH range tested (1.70-2.50) in glucose

medium, suggesting that inhibition of ferrous iron oxidation by this organism in iron/yeast extract medium at pH 2.2-2.3 was not related directly to proton acidity (pH). Correlation between the degree of inhibition of iron oxidation and the pH to which the cultures were adjusted was found with the *Sulfobacillus* YTF3 culture in the pH-controlled bioreactor. Ferrous iron oxidation by *Sulfobacillus* YTF3 was completely inhibited when the pH was increased to either 2.20 or 2.30, and raising the culture pH to 2.30 in the presence of 25 mM ferric iron was found to cause a severe decrease in the proportion of viable cells in this culture, compared to when the pH was increased to 2.15.

The fastest growth of *Sulfobacillus* YTF3 on glucose at pH 2.2 (corresponding to a t_d of 2 hours) that was similar to that found using ferrous iron/yeast extract at pH 1.8 implying that this organism could, in theory, have a culture doubling of <2 hours at pH 2.2 in ferrous iron/yeast extract media in the absence of any inhibition of soluble ferric sulfate.

It has been reported that the solution chemistry of ferric iron is much more complex than that of ferrous iron, and the trivalent cation is known to form stable complexes with ligands such as sulfate and hydroxide (Welham *et al.*, 2000). In aqueous, sulfate-free solutions, the dominant form of soluble ferric iron below pH 3 is Fe^{3+}, with the cationic complexes $Fe(OH)^{2+}$ and $Fe(OH)_2^{+}$ becoming increasingly important at pH 3-5. In contrast, in sulfate-containing aqueous solutions, uncomplexed ferric iron is generally of minor significance, except at pH <1.0, and ferric sulfate complexes dominate over the pH range where mineral leaching generally occurs (pH 1-3; Figure 7.7).

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Figure 7.7: Ferric speciation with pH for a unity ligand concentration (Welham *et al.*, 2000).

The two soluble ferric sulfate complexes described by Welham *et al.* (2000) are $FeSO_4^+$ and $Fe(SO_4)_2^-$. Of these, the disulfate complex is of particular note in that it becomes the increasingly dominant species at pH>1.0 (Figure 7.7) and, unlike the other ferric iron complexes, it is anionic. In general terms, acidophilic microorganisms are tolerant of concentrations of metal cations (e.g. copper and zinc) that are inhibitory or lethal to neutrophilic microorganisms. In contrast, acidophiles tend to display far greater sensitivity to anions such as molybdate and nitrate (Alexander *et al.*, 1987). The reason

for the pH-related ferric iron inhibition of *Sulfobacillus* spp. and related acidophiles found in this and previous work might be related to their sensitivity to the anionic ferric complex, $Fe(SO_4)_2^-$. If this is the case, it is interesting that other iron-oxidising bacteria, including the Gram-positive *Actinobacteria* as well as the Gram-negative acidophiles *At. ferrooxidans* and *L. ferrooxidans* do not appear to be inhibited by ferric iron at pH 2.2-2.3. It would also be interesting to investigate whether non iron-oxidising bacteria related to *Sulfobacillus* (*Alicyclobacillus*-like bacteria) are similarly hypersensitive to ferric iron at pH 2.2-2.3.

Such sensitivity may have important implications where Gram-positive bacteria are involved for commercial mineral processing. If so, it would be more appropriate to operate mineral processing operations using these "sensitive" microorganisms at lower pH, or else as consortia of moderate thermophiles (including "non-sensitive" acidophiles) rather than as pure cultures.

Chapter 8

"Rapid" Means of Identification of Acidophilic Bacteria by Amplified Ribosomal DNA Restriction Enzyme Analysis (ARDREA)

8.1 Introduction

A range of different autotrophic and heterotrophic microorganisms may be responsible for, or associated with, the solubilisation of metals from sulfide minerals in acidic environments. To study the ecological relationship of these microorganisms and the population dynamics during the bioleaching processes, specific methods for their quick identification and enumeration are useful. The aim of the present study was to develop and apply a simple and quick ARDREA method to differentiate these bacterial populations.

8.2 Development of the ARDREA method, using the 16S rRNA gene sequences from known acidophilic bacteria

8.2.1 Methods

The 16S rRNA gene sequences of a variety of acidophilic bacteria were obtained from DNA database (GenBank) to develop the ARDREA method. The selected moderate thermophilic and mesophilic bacteria were divided into five "differentiation groups" (differentiation groups No.1–5 in Tables 8.1-8.5), within which different bacterial species or strains could be differentiated using two different restriction enzymes. The GenBank accession numbers for the 16S rRNA gene sequences of each bacterium used

in this study are also shown in Tables 8.1-8.5. The restriction enzyme sites were searched using the programme, DNAstar. Since some of the 16S rRNA gene sequences used in this study were shorter than others, a 16S rRNA gene alignment was made to work out the actual length of the restriction fragments within the 1500bp sized 16S rRNA gene, to construct each of the diagrammatic restriction enzyme maps in Figures 8.1-8.8. Alongside theoretical application of the ARDREA method, experiments were conducted in which amplified 16S rRNA genes obtained from Acidophile Culture Collection at the University of Wales, Bangor, were digested with restriction enzymes, and fragments sizes analysed. Each of these bacteria was purified from a single colony, either on ferrous iron overlay medium (section 2.2.1.2.1.1) or on ferrous iron/tetrathionate overlay medium (section 2.2.1.2.1.2), followed by cultivation in appropriate liquid media (sections 2.2.1.1.1 and 2.2.1.1.3). The 16S rRNA genes of bacteria were amplified (section 2.5.1) and concentrated if necessary using QIAquick PCR Purification Kits (QIAGEN), according to the manufacturer's instruction. The appropriate amount of DNA was digested with restriction enzymes, as instructed by the manufacturer. The restriction enzymes used in this study are listed in Table 8.6. DNA fragments were separated in a 2% agarose gel using high-resolution blend agarose (type 3:1; Amresco; section 2.5.2).

Table 8.1: Differentiation Group No.1: Details of 4 groups of moderate thermophiles that may be differentiated using *Eco*72I and *Bsa*AI.

Moderate thermophiles	Characteristics				16S rRNA gene Acc. No.
	Carbon metabolism	Gram stain	Cell shape	Comment	
i) ***Leptospirillum*** **MT6**	Autotroph	–	Vibrioid cells Spiral forms	Ferrous iron and FeS_2 oxidiser	AF513709
ii) ***Sulfobacillus*** **spp.**	Facultative autotroph	+	Straight rods Spore formers	Ferrous iron, sulfur, and FeS_2 oxidiser	
Sb. thermosulfidooxidans^T					X91080
Sb. acidophilus^T					AF050169
"Sb. yellowstonensis" YTF1					AY007665
iii) Isolate GSM	Facultative autotroph	+	Straight rods Spore formers	Ferrous iron, sulfur, and FeS_2 oxidiser	AY007662
iv) ***Acidimicrobium*** **spp.**	Facultative autotroph	+	Rods, Filaments	Ferrous iron and FeS_2 oxidiser	
Am. ferrooxidans TH3					AI1001580
Am. ferrooxidans^T (ICP)					U75647

Table 8.2: Differentiation Group No.2: Details of 4 groups of mesophiles that may be differentiated using *Ban*II and *Xcm*I.

Mesophiles	Characteristics				16S rRNA gene Acc. No.
	Carbon metabolism	Gram stain	Cell shape	Comment	
i) ***At. ferrooxidans***	Autotroph	–	Straight rods	Ferrous iron, sulfur and FeS_2 oxidiser	
DSM9465					Y11595
ATCC23270^T					AJ278718
ii) ***Leptospirillum*** **spp.**	Autotroph	–	Vibrioid cells Spiral forms	Ferrous iron and FeS_2 oxidiser	
L. ferrooxidans					
DSM2705^T					X86776
L. ferriphilum					
ATCC49881^T					AF356829
DSM9468					X72852
DSM2391					AJ237903
Strain La					AJ237902
Strain MT6					AF513709
iii) ***"T. ferrooxidans"***	Autotroph	–	Straight rods	Ferrous iron and FeS_2 oxidiser	
Strain m-1					AF387301
iv) ***"Fm. acidiphilum"*** **T23**	Heterotroph	+	Rods	Ferrous iron and FeS_2 oxidiser	AF251436

Table 8.3: Differentiation Group No.3: Details of 3 groups of mesophiles that may be differentiated using *Alw*I and *Xcm*I.

Mesophiles	Characteristics				16S rRNA gene Acc. No.
	Carbon metabolism	Gram stain	Cell shape	Comment	
i) Isolate SLCs	Heterotroph	+	Straight rods	Ferrous iron oxidiser	
SLC1			Spore formers		-
SLC2					-
SLC66					AY040739
ii) *Sulfobacillus* spp.	Facultative	+	Straight rods	Ferrous iron,	
"Sb. montserratensis" L15	autotroph		Spore formers	sulfur and	AY007663
Sulfobacillus Riv14				FeS_2 oxidiser	AY007664
iii) *"Fm. acidiphilum"* T23	Heterotroph	+	Rods	Ferrous iron and FeS_2 oxidiser	AF251436

Table 8.4: Differentiation Group No.4: Details of 2 groups of Gram-positive bacteria that may be differentiated using *Apa*I and *Hsp*92I.

Gram-positive bacteria	Characteristics				16S rRNA gene Acc. No.
	Carbon Metabolism	Gram stain	Cell shape	Comment	
i) Mesophiles:					
Isolate SLCs	Heterotroph	+	Straight rods	Ferrous iron and FeS_2 oxidiser	
SLC1					-
SLC2					-
SLC66					AY040739
ii) Moderate Thermophiles:					
Isolate GSM	Facultative autotroph	+	Straight rods Spore formers	Ferrous iron, sulfur and FeS_2 oxidiser	AY007662
Alicyclobacillus spp.	Heterotroph	+	Straight rods	ω-alicyclic membrane	
Alb. acidophilus YTH1			Spore formers	fatty acid-producer	AF031645
Alb. acidocaldarius					X60742
Alb. acidoterrestris					X60743
Alb. cycloheptanicus					X51928

Table 8.5: Differentiation Group No.5: Details of 2 groups of *Sulfobacillus* spp. that may be differentiated using *Sna*BI and *Bsm*BI.

Sulfobacillus spp.	Characteristics				16S rRNA gene Acc. No.
	Carbon Metabolism	Gram stain	Cell shape	Comment	
i) *Sulfobacillus* GroupI:	Facultative	+	Straight rods	Ferrous iron,	
Sb. acidophilus^T (NAL)	autotroph		Spore formers	sulfur and	AF050169
"Sb. yellowstonensis" YTF1				FeS_2 oxidiser	AY007665
ii) *Sulfobacillus* GroupII:	Facultative	+	Straight rods	Ferrous iron,	
Sb. thermosulfidooxidans^T	autotroph		Spore formers	sulfur and	X91080
"Sb. montserratensis" L15				FeS_2 oxidiser	AY007663
Sulfobacillus Riv14					AY007664

Table 8.6: Details of restriction enzymes used in this study.

Enzyme	Recognition site (5'→3')	Site complexity	Optimal Temp.	Manufacturer
*Eco*72I	5'---CAC▼GTG---3' 3'---GTG▲CAC---5'	6.0	37°C	MBI Fermentas
*Bsa*AI	5'---YAC▼GTR---3' 3'---RTG▲CAY---5'	5.0	37°C	NEW ENGLAND
*Ban*II	5'---GRGCY▼C---3' 3'---C▲YCGRG---5'	5.0	37°C	Promega
*Xcm*I	5'---CCA(N)$_5$▼(N)$_4$TGG---3' 3'---GGT(N)$_4$▲(N)$_5$ACC---5'	6.0	37°C	NEW ENGLAND
*Alw*I	5'---GGATC(N)$_4$▼---3' 3'---CCTAG(N)$_5$▲---5'	5.0	37°C	NEW ENGLAND
*Apa*I	5'---GGGCC▼C---3' 3'---C▲CCGGG---5'	6.0	37°C	Promega
Hsp92I	5'---GR▼CGYC---3' 3'---CYGC▲RG---5'	5.0	37°C	Promega
*Sna*BI	5'---TAC▼GTA---3' 3'---ATG▲CAT---5'	6.0	37°C	Promega
*Bsm*BI	5'---CGTCTC(N)$_1$▼---3' 3'---GCAGAG(N)$_5$▲---5'	6.0	55°C	NEW ENGLAND

8.2.2 Results

The diagrammatic restriction enzyme maps and the actual restriction patterns on agarose gels are shown in Figures 8.1-8.8. As predicted from virtual restriction sites analyses, it was shown that those bacteria can be differentiated from each other by their restriction patterns on 2% agarose gels.

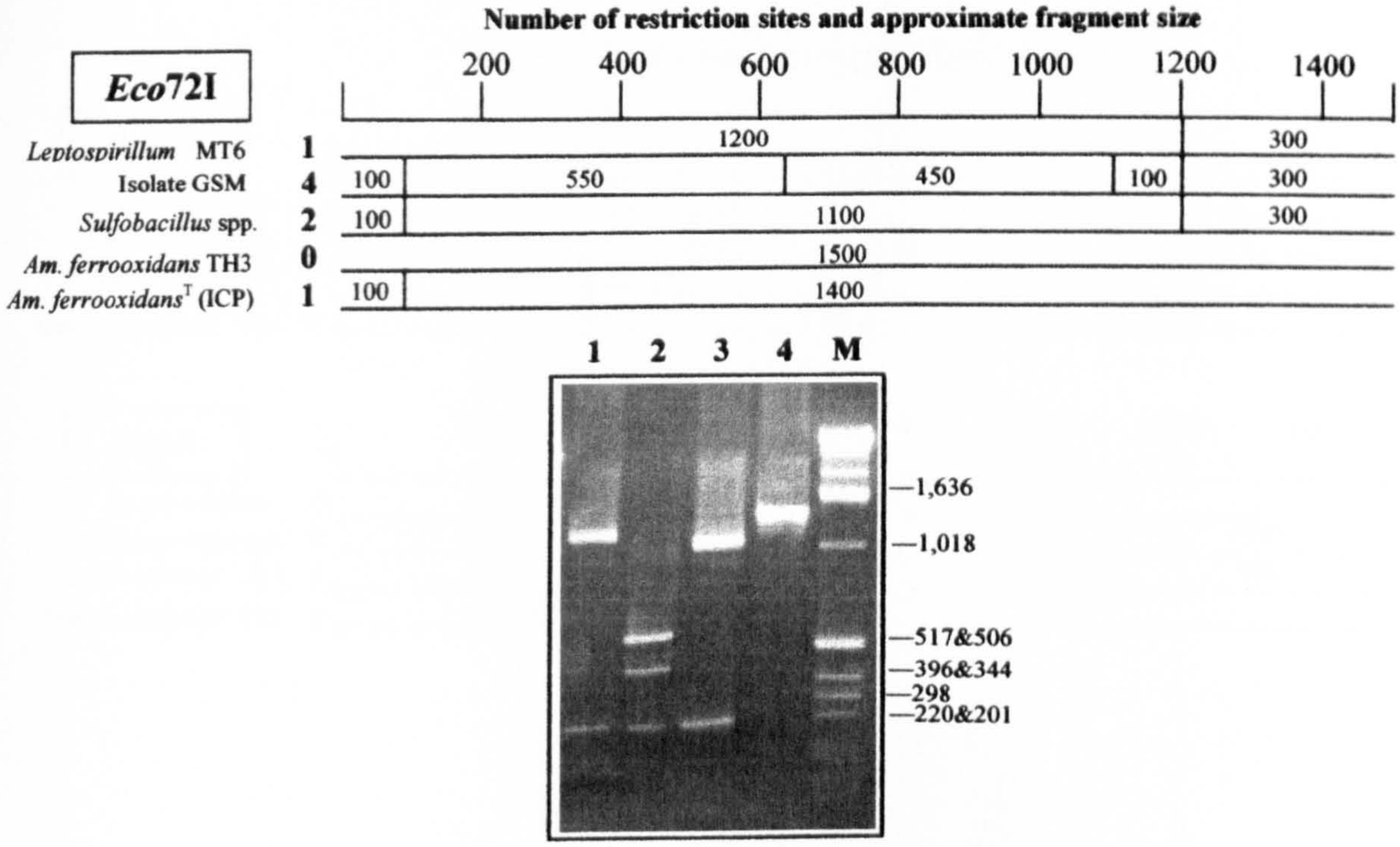


Figure 8.1: Theoretical diagrammatic restriction enzyme map and electrophoretic analysis of 16S rRNA gene of moderately thermophilic bacteria (Table 8.1) digested with *Eco*72I. Lane M, 1kb DNA ladder (Gibco BRL); lane 1, *Leptospirillum* MT6; lane 2, isolate GSM; lane 3, *Sb. thermosulfidooxidans*^{T}; lane 4, *Am. ferrooxidans* TH3.

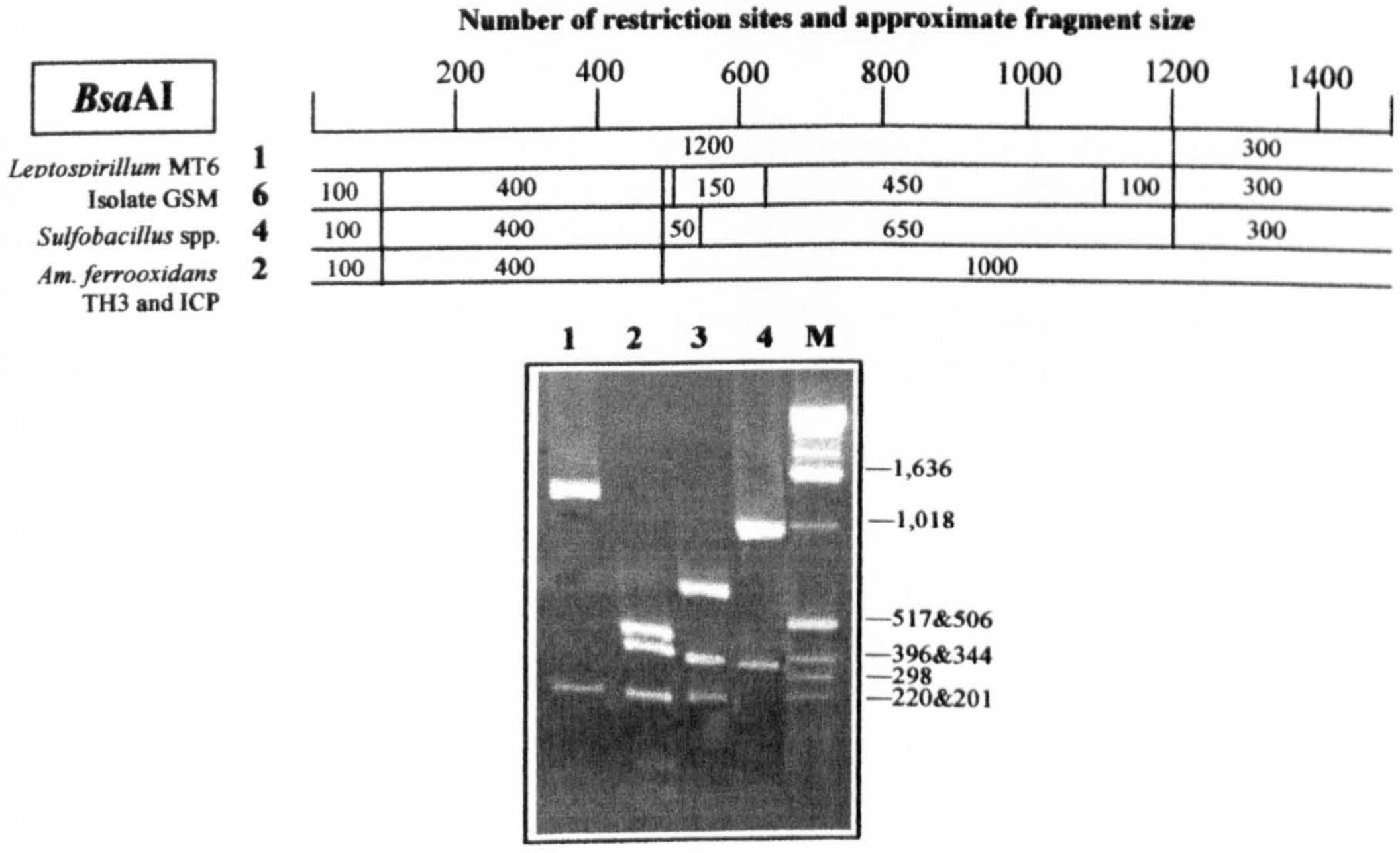


Figure 8.2: Theoretical diagrammatic restriction enzyme map and electrophoretic analysis of 16S rRNA gene of moderately thermophilic bacteria (Table 8.1) digested with *Bsa*AI. Lane M, 1kb DNA ladder (Gibco BRL); lane 1, *Leptospirillum* MT6; lane 2, isolate GSM; lane 3, *Sb. thermosulfidooxidans*^{T}; lane 4, *Am. ferrooxidans* TH3.

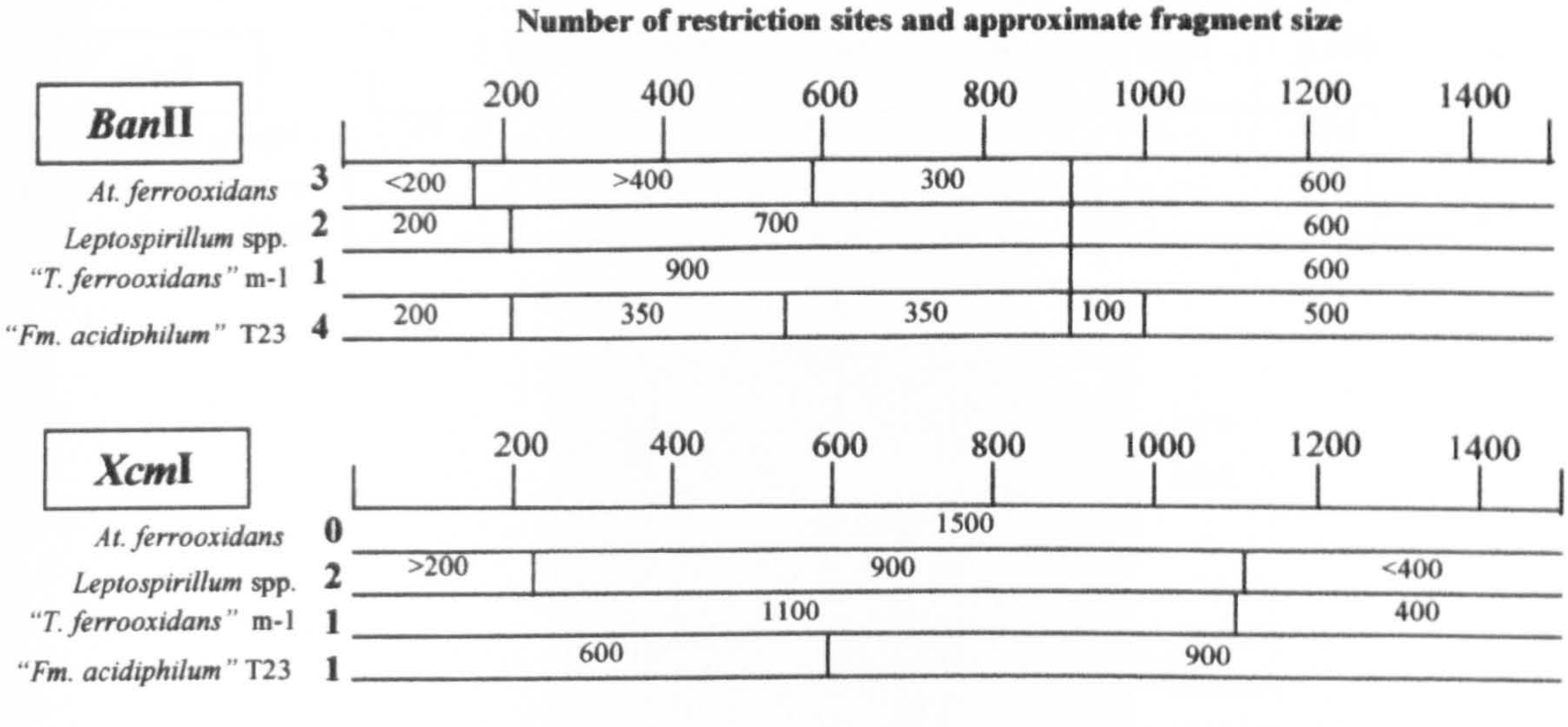


Figure 8.3: Theoretical diagrammatic restriction enzyme maps and electrophoretic analysis of 16S rRNA gene of mesophilic bacteria (Table 8.2) digested with *Ban*II or *Xcm*I. Lanes M, 1kb DNA ladder (Gibco BRL); lane 1, *At. ferrooxidans*^{T}/*Ban*II; lane 2, *L. ferrooxidans*^{T}/*Ban*II; lane 3, strain m-1/*Ban*II; lane 4, *At. ferrooxidans*^{T}/*Xcm*I; lane 5, *L. ferrooxidans*^{T}/*Xcm*I; lane 6, strain m-1/*Xcm*I ("*Fm. acidiphilum*" T23 may also be differentiated though the electrophoretic analysis was not performed for this bacterium).

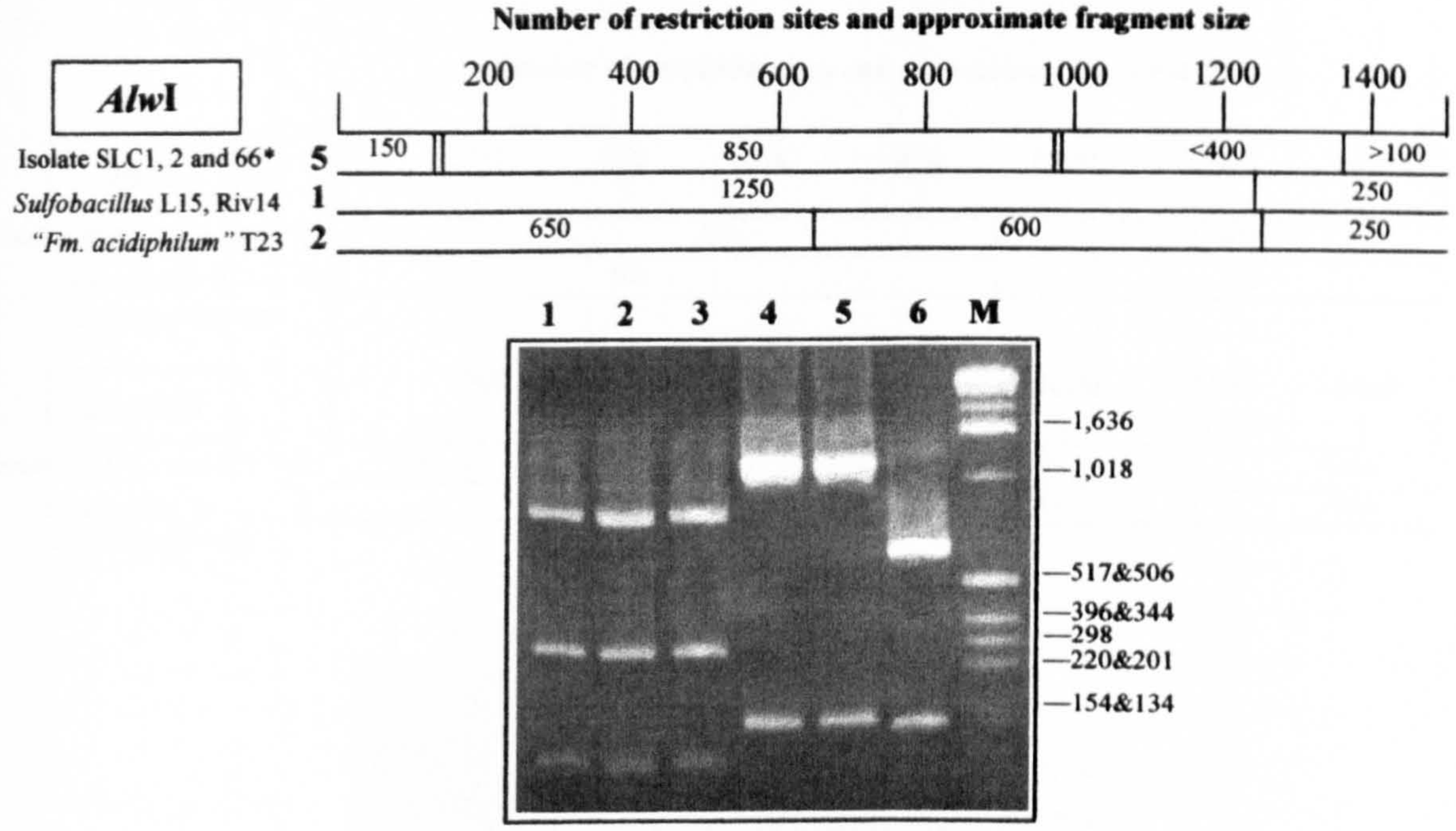


Figure 8.4: Theoretical diagrammatic restriction enzyme map and electrophoretic analysis of 16S rRNA gene of mesophilic bacteria (Table 8.3) digested with *Alw*I. Lane M, 1kb DNA ladder (Gibco BRL); lane 1, SLC1; lane 2, SLC2; lane 3, SLC66; lane 4, *Sulfobacillus* Riv14; lane 5, *"Sb. montserratensis"* L15; lane 6, *"Fm. acidiphilum"* T23. *16S rRNA gene sequences of isolates SLC1 and SLC2 were available in the laboratory though they are not deposited in GenBank.

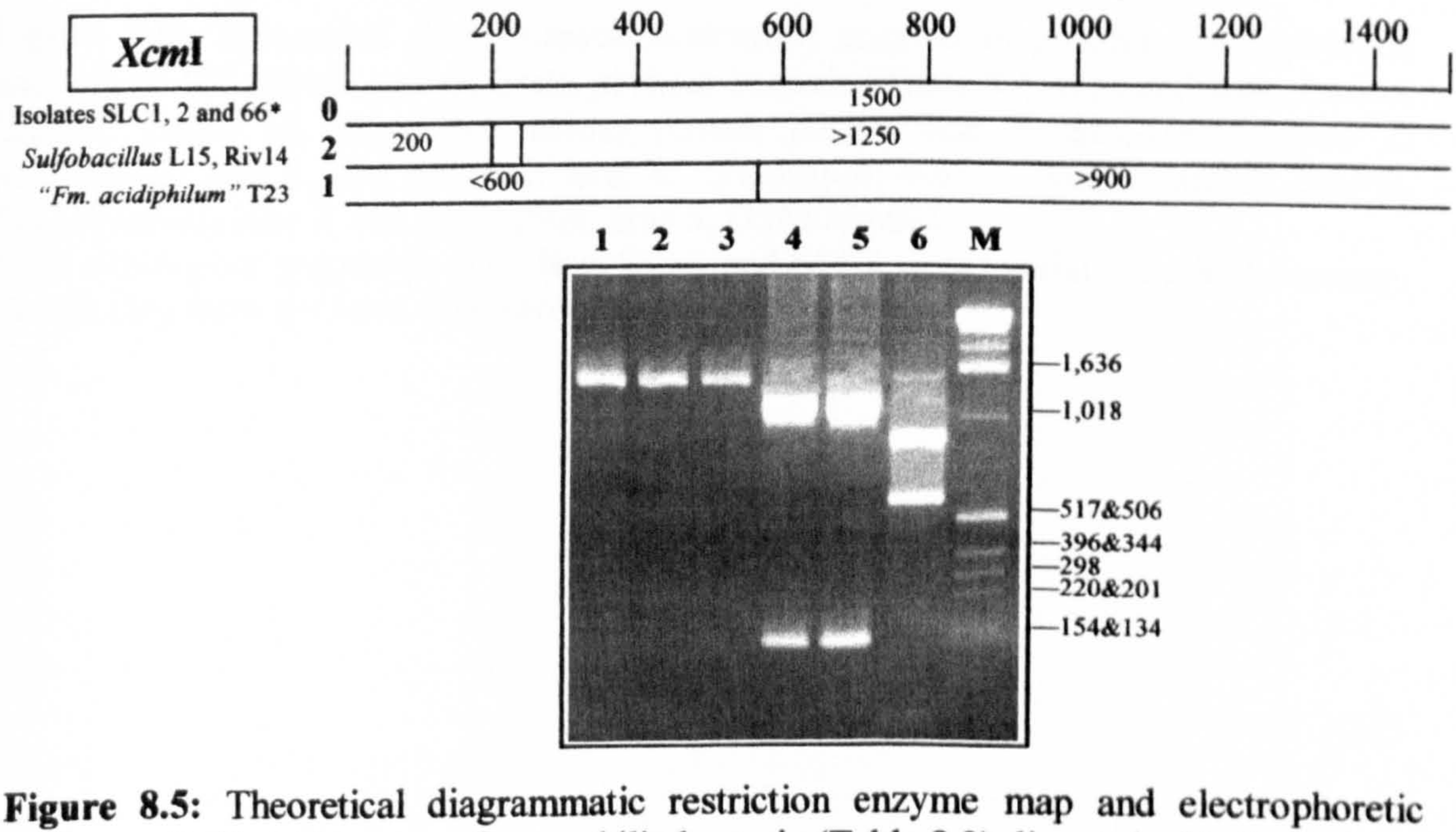


Figure 8.5: Theoretical diagrammatic restriction enzyme map and electrophoretic analysis of 16S rRNA gene of mesophilic bacteria (Table 8.3) digested with *Xcm*I. Lane M, 1kb DNA ladder (Gibco BRL); lane 1, SLC1; lane 2, SLC2; lane 3, SLC66; lane 4, *Sulfobacillus* Riv14; lane 5, *"Sb. montserratensis"* L15; lane 6, *"Fm. acidiphilum"* T23. *16S rRNA gene sequences of isolates SLC1 and SLC2 were available in the laboratory though they have not been deposited in GenBank.

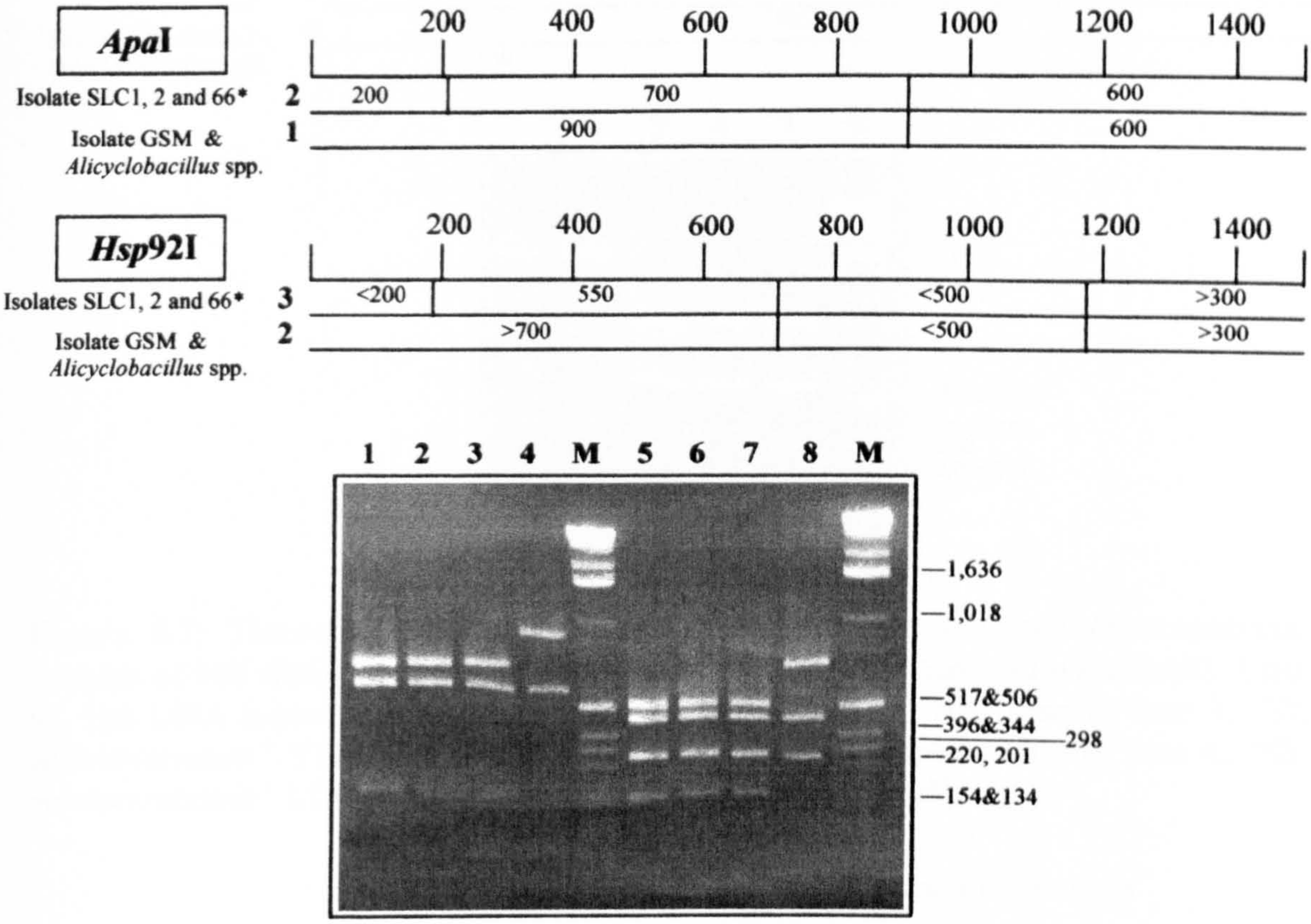


Figure 8.6: Theoretical diagrammatic restriction enzyme maps and electrophoretic analysis of 16S rRNA gene of Gram-positive bacteria (Table 8.4) digested with *Apa*I or *Hsp*92I. Lanes M, 1kb DNA ladder (Gibco BRL); lane 1, SLC1/*Apa*I; lane 2, SLC2/*Apa*I; lane 3, SLC66/*Apa*I; lane 4, GSM/*Apa*I; lane 5, SLC1/*Hsp*92I; lane 6, SLC2/*Hsp*92I; lane 7, SLC66/*Hsp*92I; lane 8, GSM/*Hsp*92I.
*16S rRNA gene sequences of isolates SLC1 and SLC2 were available in the laboratory though they have not been deposited in GenBank.

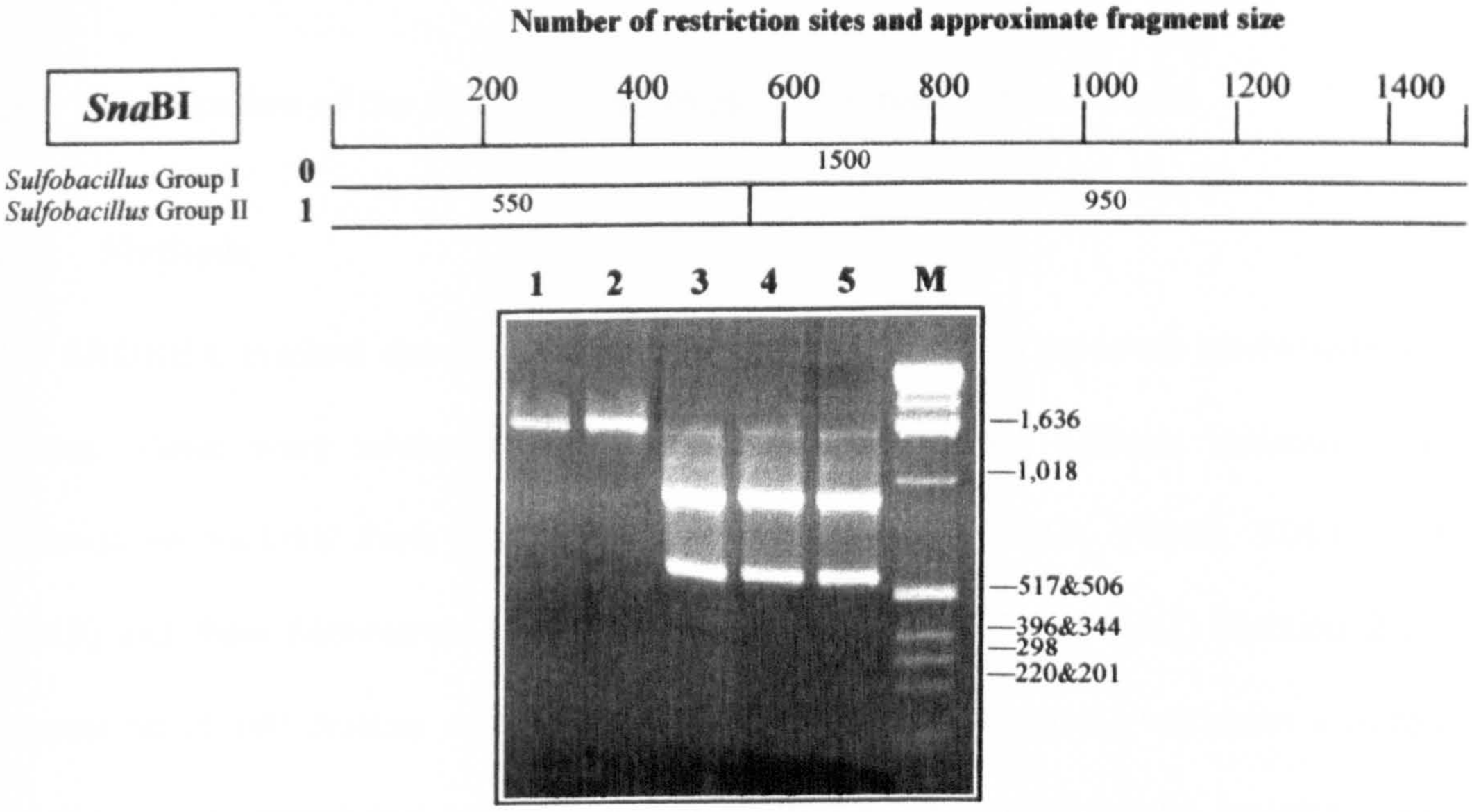


Figure 8.7: Theoretical diagrammatic restriction enzyme map and electrophoretic analysis of 16S rRNA gene of *Sulfobacillus* spp. (Table 8.5) digested with *Sna*BI. Lane M, 1kb DNA ladder (Gibco BRL); lane 1, *Sb. acidophilus*^{T} NAL/*Sna*BI; lane 2, "*Sb. yellowstonensis*" YTF1/*Sna*BI; lane 3, *Sb. thermosulfidooxidans*^{T}/*Sna*BI; lane 4, "*Sb. montserratensis*" L15/*Sna*BI; lane 5, *Sulfobacillus* Riv14/*Sna*BI.

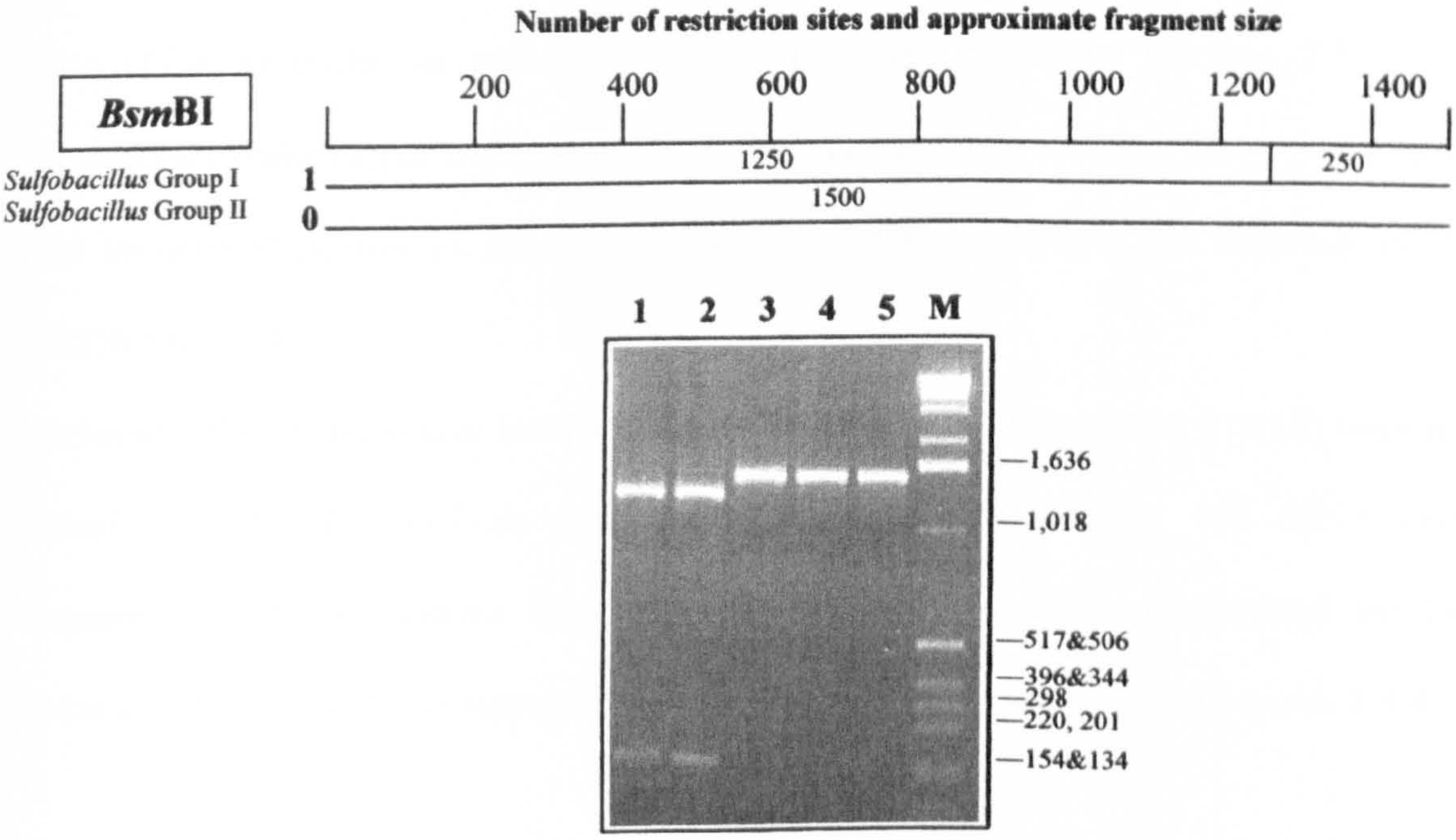


Figure 8.8: Theoretical diagrammatic restriction enzyme map and electrophoretic analysis of 16S rRNA gene of *Sulfobacillus* spp. (Table 8.5) digested with *Bsm*BI. Lane M, 1kb DNA ladder (Gibco BRL); lane 1, *Sb. acidophilus*^{T} NAL/*Bsm*BI; lane 2, "*Sb. yellowstonensis*" YTF1/*Bsm*BI; lane 3, *Sb. thermosulfidooxidans*^{T}/*Bsm*BI; lane 4, "*Sb. montserratensis*" L15/*Bsm*BI; lane 5, *Sulfobacillus* Riv14/*Bsm*BI.

8.3 Application of the ARDREA method to environmental isolates

8.3.1 Methods

The ARDREA method developed in section 8.2 was applied to some environmental isolates. These were moderately thermophilic iron-oxidising bacteria isolated from Yellowstone National Park (Y002, Y005, Y006, Y0010, Y0015, Y0016, Y0017 and Y0018) and from Montserrat (G1, G2, GG6/1, GG6/3, 8/30 and Riv2) (section 2.1). Preparation of cell lysates, amplification of 16S rRNA gene, and restriction enzyme digestion were carried out as described in section 8.2.1. As all of the isolates were moderately thermophilic iron-oxidisers, the restriction enzymes, *Bsa*AI and *Eco*72I, were tested on the isolates first for Differentiation Group No.1 (Table 8.1) and then the enzymes *Sna*BI and *Bsm*BI for Differentiation Group No.5 (Table 8.5). The 16S rRNA gene (PCR products) of some of the isolates were amplified (section 2.5.1) and sequenced (Yellowstone isolates by Dr. Francisco Roberto; INEEL, Idaho Falls, USA and Montserrat isolates as described in section 2.5.8) to confirm the accuracy of the ARDREA results.

Although other Yellowstone isolates (Y004, Y008, Y0012, Y0013 and Y0014) were not tested for ARDREA analysis since they were not iron-oxidisers, 16S rRNA gene sequences of these isolates were also determined (sequences determined by Dr. Francisco Roberto). A phylogenetic tree was constructed as described in section 2.5.8.

8.3.2 Results

8.3.2.1 Yellowstone isolates

The electrophoretic analyses of the digested 16S rRNA gene from the iron-oxidising Yellowstone isolates are shown in Figures 8.9-8.12.

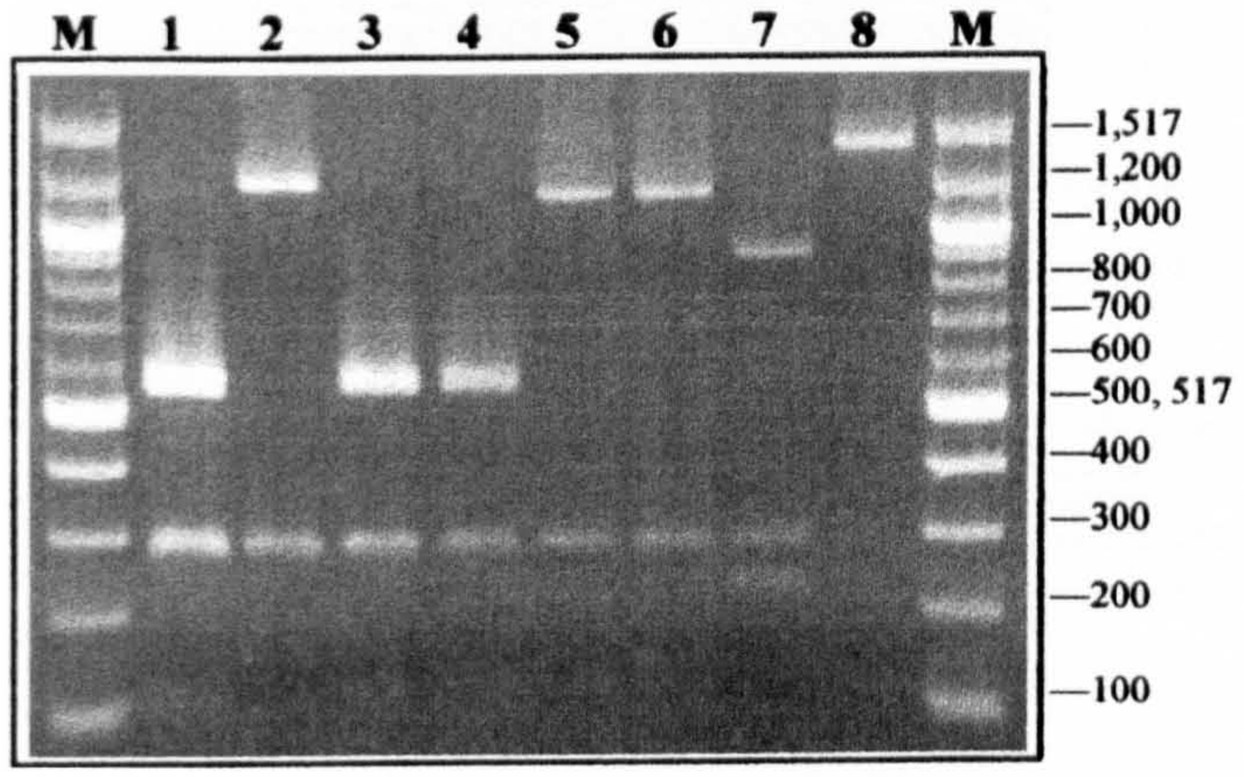


Figure 8.9: Electrophoretic analysis of 16S rRNA gene from Yellowstone isolates digested with *Eco*72I. Lanes M, 100bp DNA ladder (New England Biolabs); lane 1, Y002; lane 2, Y005; lane 3, Y006; lane 4, Y0010; lane 5, Y0015; lane 6, Y0016; lane 7, Y0017; lane 8, Y0018.

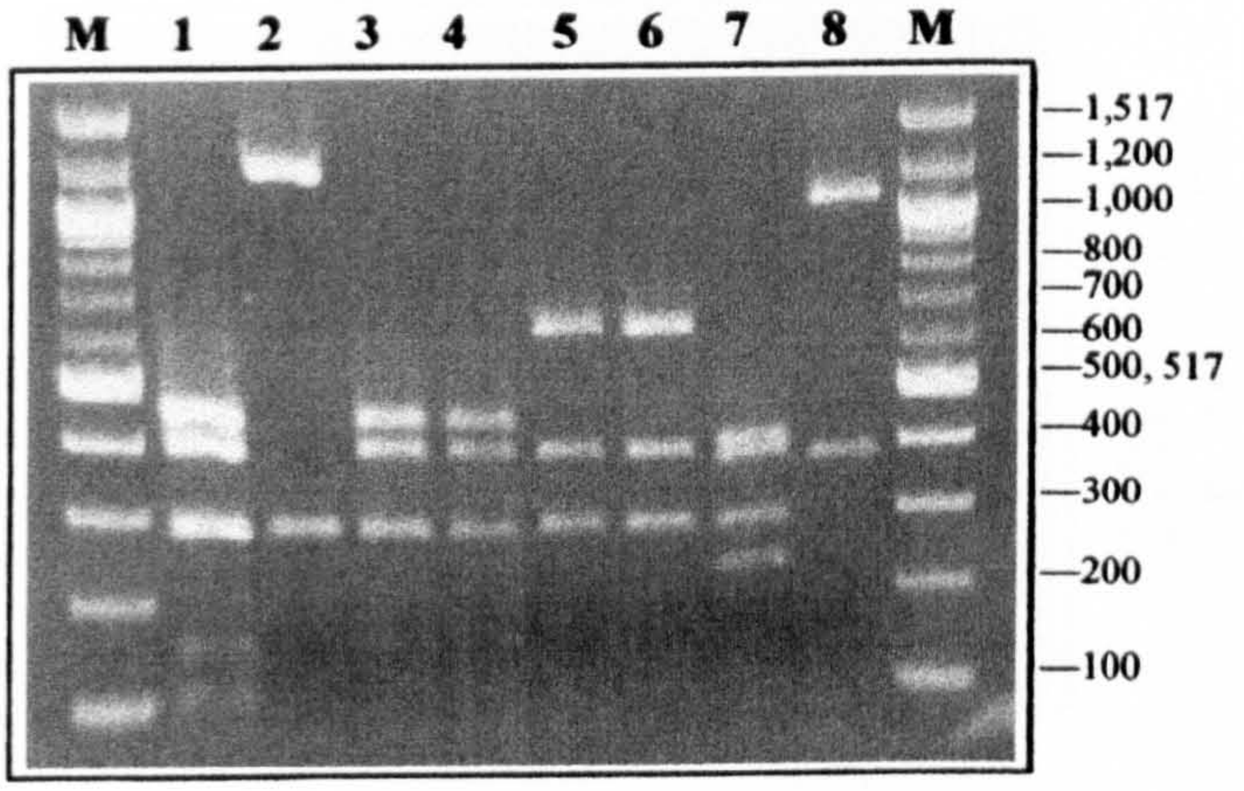


Figure 8.10: Electrophoretic analysis of 16S rRNA gene from Yellowstone isolates digested with *Bsa*AI. Lanes M, 100bp DNA ladder (New England Biolabs); lane 1, Y002; lane 2, Y005; lane 3, Y006; lane 4, Y0010; lane 5, Y0015; lane 6, Y0016; lane 7, Y0017; lane 8, Y0018.

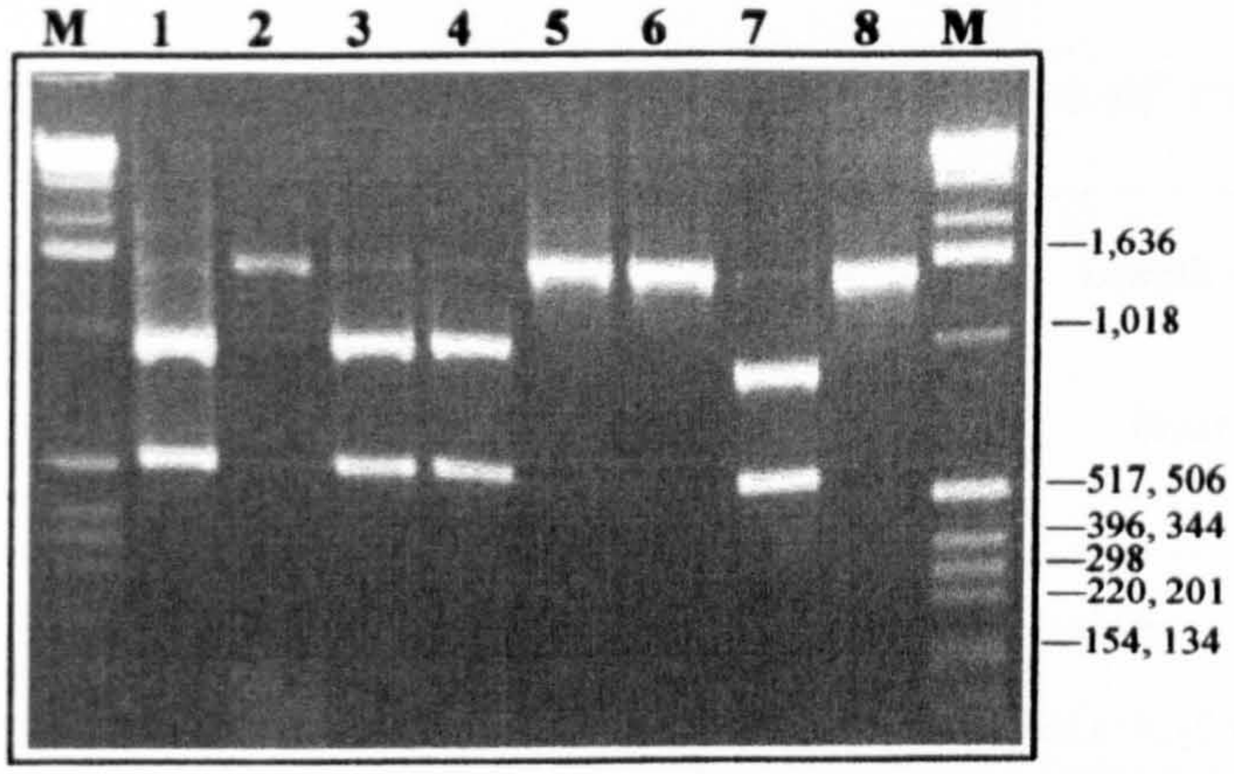


Figure 8.11: Electrophoretic analysis of 16S rRNA gene from Yellowstone isolates digested with *Sna*BI. Lanes M, 1kb DNA ladder (Gibco BRL); lane 1, Y002; lane 2, Y005; lane 3, Y006; lane 4, Y0010; lane 5, Y0015; lane 6, Y0016; lane 7, Y0017; lane 8, Y0018.

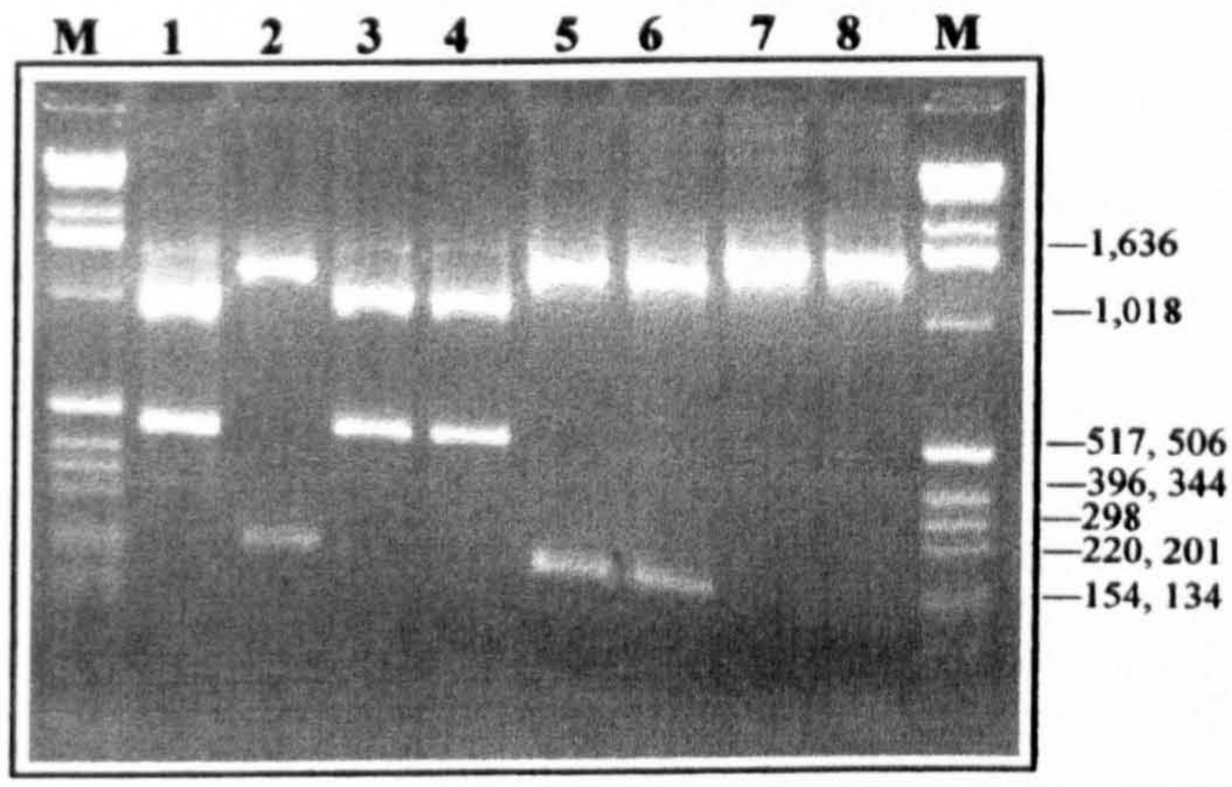


Figure 8.12: Electrophoretic analysis of 16S rRNA gene from Yellowstone isolates digested with *Bsm*BI. Lanes M, 1kb DNA ladder (Gibco BRL); lane 1, Y002; lane 2, Y005; lane 3, Y006; lane 4, Y0010; lane 5, Y0015; lane 6, Y0016; lane 7, Y0017; lane 8, Y0018.

The restriction patterns that the Yellowstone isolates exhibited with the four restriction enzymes (*Eco*72I, *Bsa*AI, *Sna*BI and *Bsm*BI), the lengths of the determined 16S rRNA gene sequences, and the names of most homologous organisms determined by comparison of 16S rRNA gene sequences, are summarised in Table 8.7.

Table 8.7: Restriction patterns and the putative identities of the Yellowstone isolates.

Isolate	Restriction patterns				Length of 16S rRNA gene determined : Accession number Most homologous organism (%) : Accession number
	Differentiation No.1 (Table 8.1)		Differentiation No.5 (Table 8.5)		
	*Eco*72I	*Bsa*AI	*Sna*BI	*Bsm*BI	
Y002	New pattern (=Y006 & 10)	GSM pattern	*Sulfobacillus* Group II	New pattern (=Y006 & 10)	1423bp SLC66 (95.3%): AY040739 [Y006&Y0010 (99.9%)]
Y005	*Leptospirillum* MT6 pattern	*Leptospirillum* MT6 pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	1404bp: AY140237 *Methylobacterium fujisawaense* (96.3%): AJ250801
Y006	New pattern (=Y002 & 10)	GSM pattern	*Sulfobacillus* Group II	New pattern (=Y002 & 10)	1425bp SLC66 (95.3%): AY040739 [Y0010 (99.9%)]
Y0010	New pattern (=Y002 & 6)	GSM pattern	*Sulfobacillus* Group II	New pattern (=Y002 & 6)	1333bp: AY140235 SLC66 (95.3%): AY040739
Y0015	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	1397bp *"Sb. yellowstonensis"* YTF1 (99.0%): AY007665 [Y0016 (99.2%)] [*Sulfobacillus* NC (chapter 3) (99.6%)] [*Sulfobacillus* YTF3 (chapter 7) (99.7%)]
Y0016	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	1394bp *"Sb. yellowstonensis"* YTF1 (98.9%): AY007665 [*Sulfobacillus* NC (chapter 3) (99.5%)] [*Sulfobacillus* YTF3 (chapter 7) (99.6%)]
Y0017	New pattern	New pattern	*Sulfobacillus* Group II	*Sulfobacillus* Group II	1295bp: AY140239 *"Sb. montserratensis"* L15 (98.0%): AY007663
Y0018	*Acidimicrobium* TH3 pattern	*Acidimicrobium* TH3 pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group II	1262bp: AY140240 *Am. ferrooxidans* TH3 (97.6%): M79434

Isolates Y002, Y006, and Y0010 showed the same restriction patterns with all of the four enzymes tested. Isolates Y002, Y006 and Y0010 showed the "GSM pattern" with *Bsa*AI, but a new pattern with *Eco*72I, suggesting that these three isolates belong to

none of the four groups of moderately thermophiles listed in Table 8.1. It was later confirmed, using 16S rRNA gene sequences, that these three isolates had the highest homology to isolate SLC66 (95.3%), which is a mesophilic iron-oxidising bacterium (Table 8.7). Isolates Y002, Y006 and Y0010 were closely related to each other, with 99.9% homology.

The restriction patterns of isolate Y005 with *Bsa*AI and *Eco*72I were the same as those of *Leptospirillum* MT6; however, the cellular morphology of Y005 was very different from *Leptospirillum* spp. in that it grew as long filaments, forming 1-2 mm diameter flocs in liquid media. Therefore, it was speculated that this isolate might belong to none of the four groups of moderate thermophiles listed in Table 8.1. From 16S rRNA gene sequence analysis, the most homologous organism to Y005 was shown to be *Methylobacterium fujisawaense*, with 96.3% homology (Table 8.7).

Isolates Y0015 and Y0016 exhibited *Sulfobacillus* restriction patterns with both *Bsa*AI and *Eco*72I. In addition, when digested with *Sna*BI and *Bsm*BI, these two isolates had the same pattern as those of *Sulfobacillus* group I (defined in Table 8.5); therefore, these bacteria were suggested to belong to *Sulfobacillus* group I, which was shown to be correct from subsequent 16S rRNA gene sequence analysis (Table 8.7). Isolates Y0015 and Y0016 had 99.2% homology to each other.

Isolate Y0017 had new restriction patterns with both *Bsa*AI and *Eco*72I, suggesting that this isolate belongs to none of the four groups of moderate thermophiles listed in Table 8.1. The 16S rRNA gene of Y0017 had 98.0% homology with the mesophile "*Sb. montserratensis*" L15. The ARDREA method therefore differentiated this isolate from known moderate thermophilic *Sulfobacillus* spp. (listed in Table 8.1).

The restriction patterns of Y0018 with *Eco*72I and *Bsa*AI were the same as those of *Acidimicrobium* TH3 (Table 8.7). From 16S rRNA gene sequence analysis, Y0017 was

confirmed to be an *Acidimicrobium* sp., with 97.6% homology with *Am. ferrooxidans* TH3 (Table 8.7).

In addition, the 16S rRNA gene sequences of heterotrophic (non iron-oxidising) moderately thermophilic Yellowstone isolates were determined (Table 8.8). Other than isolate Y004, which had the highest homology (97.6%) with bacterium K1 (a putative *Alicyclobacillus* sp.), all isolates were most closely related to *Acidisphaera rubrifaciens* with 93-94% homology. Also, Y008, 12, 13 and 14 were closely related to each other with 97.9-99.9% homology.

Table 8.8: Results of the 16S rRNA gene determination of heterotrophic Yellowstone isolates.

Heterotrophic isolates	Length of 16S rRNA gene determined: Accession number	Most homologous organism (%): Accession number
Y004	1405bp: AY140236	Bacterium K1 (97.6%): Z21979
Y008	1340bp: AY140238	*Acidisphaera rubrifaciens* (93.3%): D86512
Y0012	1384bp	*Acidisphaera rubrifaciens* (93.4%): D86512
Y0013	1290bp	*Acidisphaera rubrifaciens* (93.5%): D86512
Y0014	1364bp	*Acidisphaera rubrifaciens* (93.7%): D86512

8.3.2.2 Montserrat isolates

Electrophoretic analysis of 16S rRNA gene from the iron-oxidising Montserrat isolates, digested with *Eco*72I and *Bsa*AI, are shown in Figures 8.13 and 8.14. Those which had the *Sulfobacillus* patterns with these enzymes were then tested with *Sna*BI and *Bsm*BI (Figure 8.15).

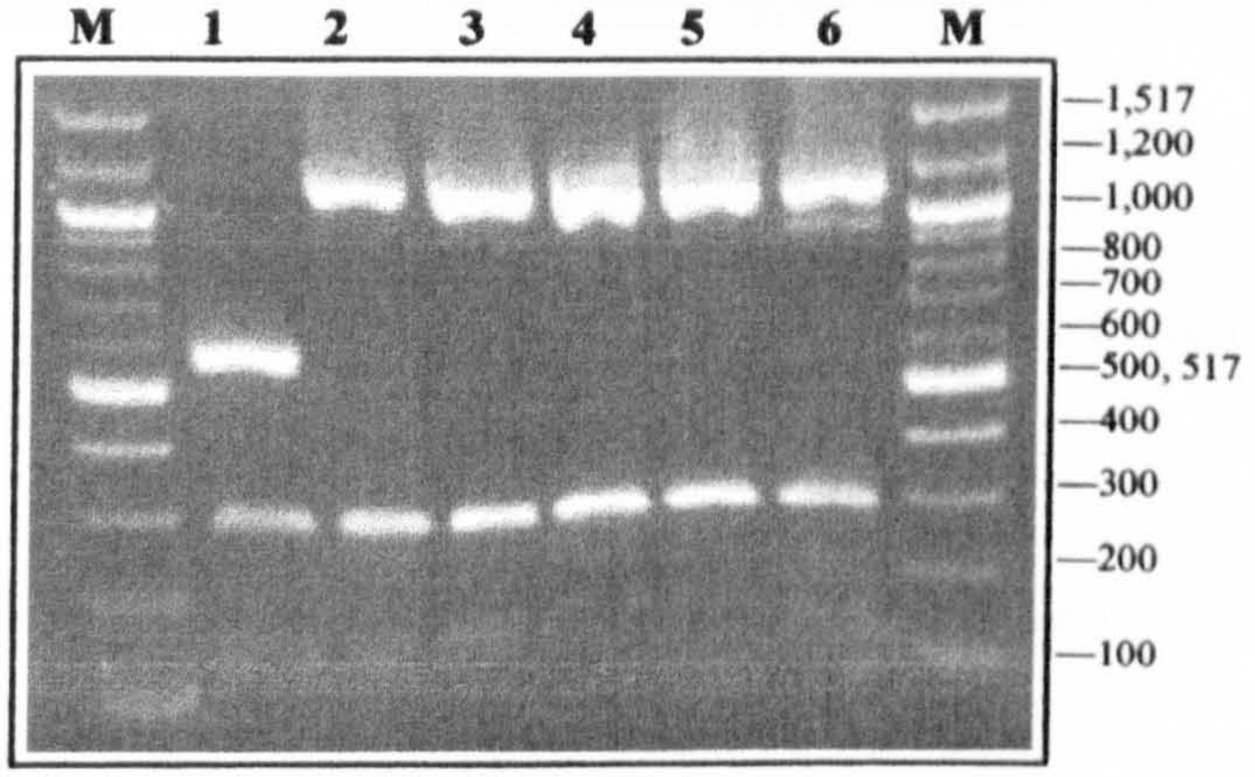


Figure 8.13: Electrophoretic analysis of 16S rRNA gene of Montserrat isolates digested with *Eco*72I. Lanes M, 100bp DNA ladder (New England Biolabs); lane 1, G1; lane 2, G2; lane 3, GG6/1; lane 4, GG6/3; lane 5, 8/30; lane 6, Riv2.

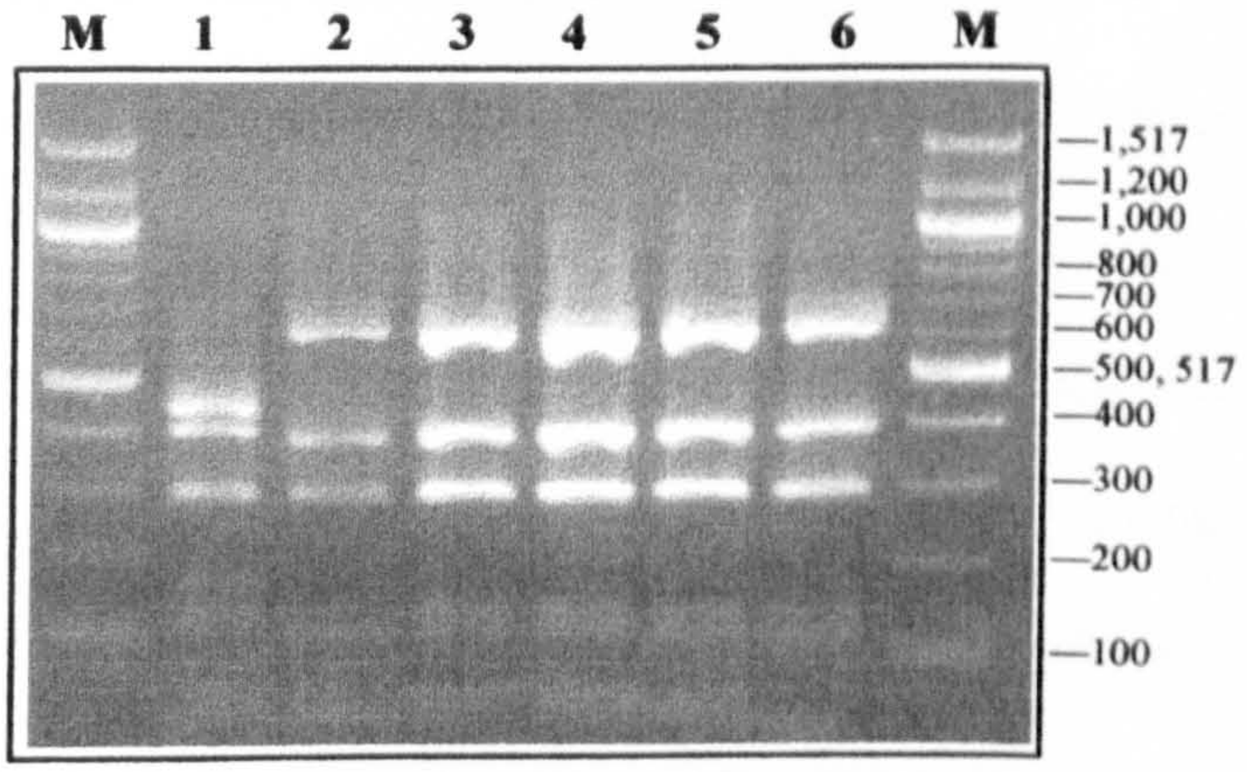


Figure 8.14: Electrophoretic analysis of 16S rRNA gene of Montserrat isolates digested with *Bsa*AI. Lanes M, 100bp DNA ladder (New England Biolabs); lane 1, G1; lane 2, G2; lane 3, GG6/1; lane 4, GG6/3; lane 5, 8/30; lane 6, Riv2.

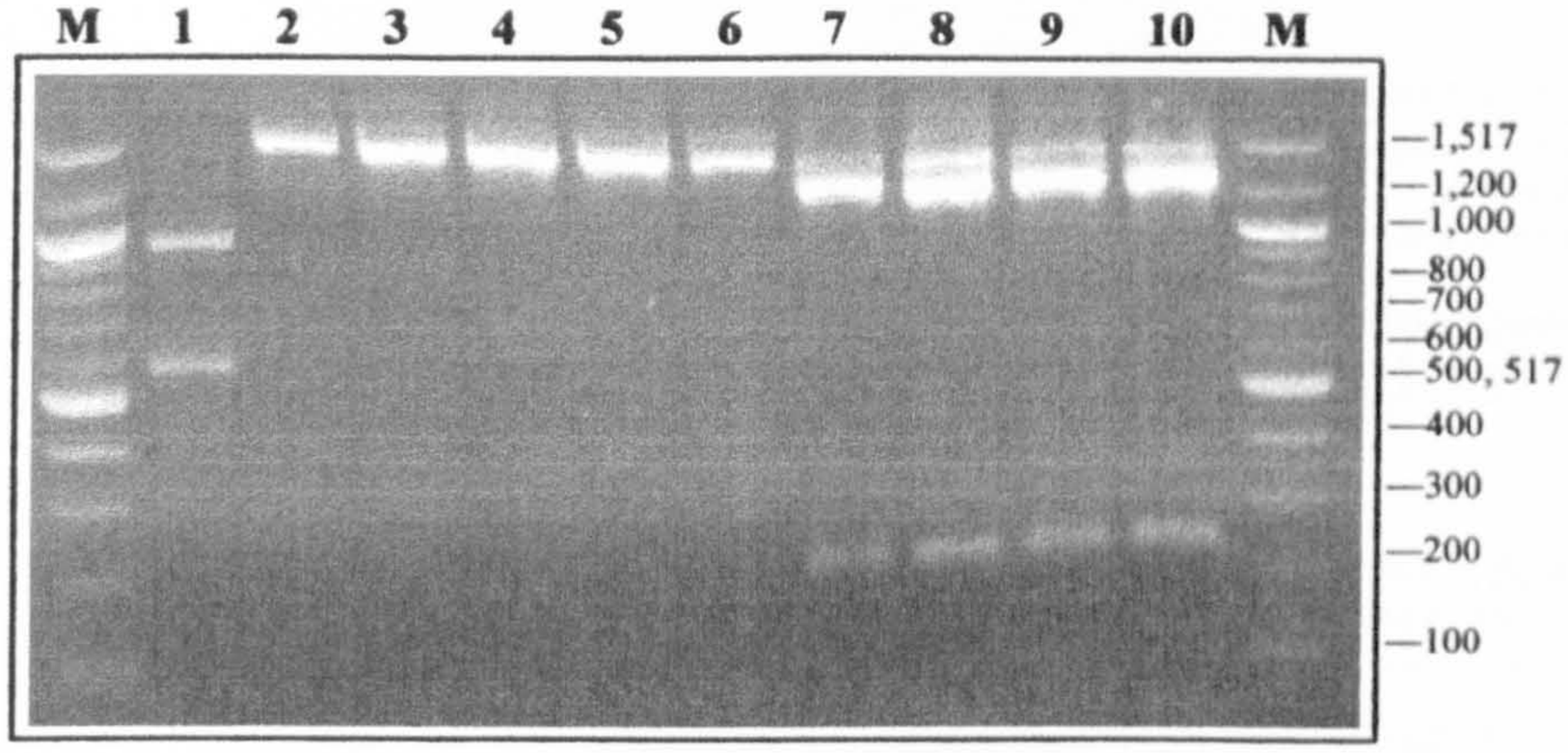


Figure 8.15: Electrophoretic analysis of 16S rRNA gene of Montserrat isolates digested with *Sna*BI or *Bsm*BI. Lanes M, 100bp DNA ladder (New England Biolabs); lane 1, G2/*Sna*BI; lane 2, GG6/1/*Sna*BI; lane 3, GG6/3/*Sna*BI; lane 4, 8/30/*Sna*BI; lane 5, Riv2/*Sna*BI; lane 6, G2/ *Bsm*BI; lane 7, GG6/1/ *Bsm*BI; lane 8, GG6/3/ *Bsm*BI; lane 9, 8/30/ *Bsm*BI; lane 10, Riv2/ *Bsm*BI.

The restriction patterns that the Montserrat isolates exhibited with the four (or two) restriction enzymes are summarised in Table 8.9.

From the ARDREA results, it was suggested that isolate G1 is an organism which does not belong to any of the four groups of moderate thermophiles listed in Table 8.1, and that isolate G2 and the other four isolates (GG6/1, GG6/3, 8/30 and Riv2) belong to *Sulfobacillus* group II and *Sulfobacillus* group I (defined in Table 8.5), respectively. Determination of 16S rRNA gene sequences of isolates G1, G2 and also of isolate GG6/1 (as a representative of the four isolates which had the *Sulfobacillus* group I pattern; GG6/1, GG6/3, 8/30 and Riv2) confirmed the accuracy of the ARDREA results. Isolates G1, G2 and GG6/1 were found to be most closely related to SLC66 (95.4%), *Sb. thermosulfidooxidans*^{T} (99.6%) and "*Sb. yellowstonensis*" YTF1 (98.7%), respectively. Isolate G1 was also closely related to Y002 (99.9%), Y006 (99.9%) and Y0010 (100%) and isolate GG6/1 to Y0015 (98.9%) and Y0016 (98.9%).

Table 8.9: Restriction patterns and the putative identities of the Montserrat isolates.

Isolate	Restriction patterns				Length of 16S rRNA gene determined :Accession number Most homologous organism (%) :Accession number
	Differentiation No.1 (Table 8.1)		Differentiation No.5 (Table 8.5)		
	*Eco*72I	*Bsa*AI	*Sna*BI	*Bsm*BI	
G1	New pattern (=Y002,6&10)	GSM pattern	n.d.	n.d.	1411bp SLC66 (95.4%): AY040739 [Y002&Y006 (99.9%) Y0010 (100%)]
G2	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group II	*Sulfobacillus* Group II	1411bp: AY140233 *Sb. thermosulfidooxidans*^{T} (99.6%): X91080
GG6/1	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	1433bp: AY140234 *"Sb. yellowstonensis"* YTF1 (98.7%): AY007665 [Y0015&Y0016 (98.9%)] [*Sulfobacillus* NC (chapter 3) (100%)] [*Sulfobacillus* YTF3 (chapter 7) (99.6%)]
GG6/3	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	n.d.
8/30	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	n.d.
Riv2	*Sulfobacillus* pattern	*Sulfobacillus* pattern	*Sulfobacillus* Group I	*Sulfobacillus* Group I	n.d.

n.d.: not determined

A phylogenetic tree was constructed using the 16S rRNA gene sequences of the isolates determined in this study (Figure 8.16).

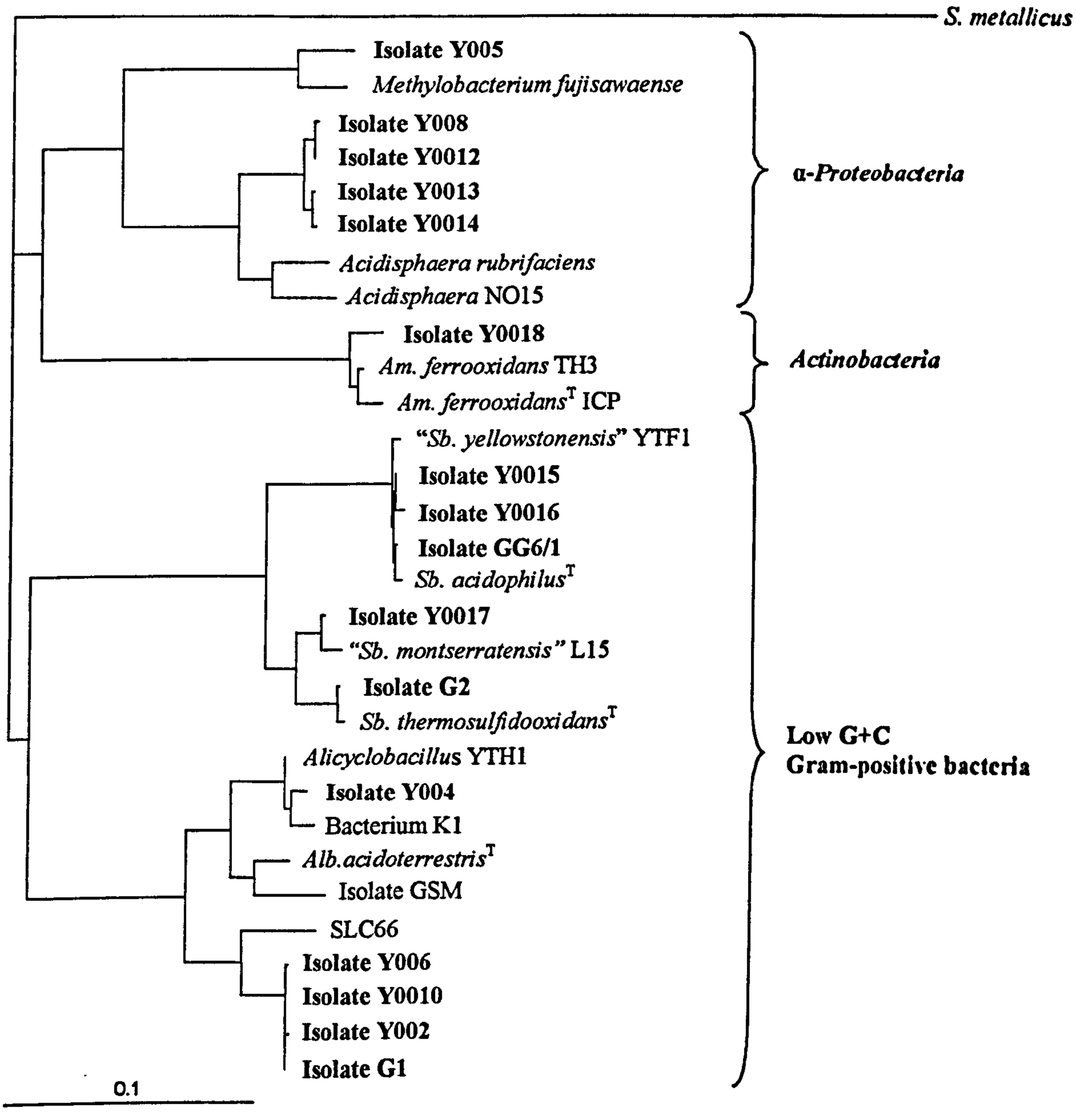


Figure 8.16: Phylogenetic relationships of the "Yellowstone" and "Montserrat" isolates (in bold) to known acidophilic prokaryotes. The phylogenetic tree was rooted with *S. metallicus*. The bar represents 0.1 nucleotides substitution per 100 for the horizontal branch lengths.

8.4 Discussion

The ARDREA method was developed to identify a range of different acidophilic bacteria in environmental and other samples. There was good agreement between predictive models and actual experiments, and restriction fragments were readily separated and identified on 2% agarose gels. It was found necessary to use a 100 bp DNA ladder rather than 1 kb ladder as a marker on agarose gel, since some restriction fragments, especially small size (<~300 bp) fragments, corresponded to the 100 bp DNA ladder, but not to the 1 kb DNA ladder.

General schemes for identification of moderately thermophilic and mesophilic iron-oxidising isolates using ARDREA are shown in Figures 8.17 and 8.18, respectively. Since the environmental isolates tested in this study displayed wide diversity as described above, and some of the isolates were not within the categories of the original "Differentiation Groups" (Table 8.1-8.5), the final schemes were constructed including additional theoretical routes to differentiate such isolates. In addition, an ARDREA method to differentiate *L. ferriphilum* and *L. ferrooxidans* described by Coram and Rawlings (2002) is incorporated in Figure 8.18.

"*Sb. montserratensis*" L15 and *Sulfobacillus* Riv14-like isolates, together with isolateY0017 (Low G+C Gram-positive pattern C), can be separated from the other *Sulfobacillus* spp. (Low G+C Gram-positive pattern A) when tested first with *Eco*72I and *Bsa*AI (Figure 8.17). However, if isolates are identified as *Sulfobacillus* spp., and their amplified 16S rRNA genes digested with *Sna*BI and *Bsm*BI, "*Sb. montserratensis*"-like bacteria are not differentiated from *Sb. thermosulfidooxidans* (i.e. both belong to Group II in the scheme presented in Figure 8.17.

SLC-like isolates, which include isolates Y002, Y006, Y0010 and G1, can be separated as a single group (Low G+C Gram-positive pattern D) and differentiated from most other low G+C Gram-positive acidophiles, in the scheme presented. However, since this group of iron-oxidising bacteria displays the same restriction pattern as isolate GSM with one *Bsa*AI, it is important that both restriction enzymes are used. In general, it was found that greater accuracy of the ARDREA approach always resulted when two, rather than a single, restriction enzymes were used.

The "Differentiation Group No.4" (Table 8.4) was developed initially to differentiate the "SLC group" from the "GSM + *Alicyclobacillus* group", which form two different branches in the phylogenetic tree (Figure 8.16). Since the "SLC" group of isolates were all mesophilic (Johnson *et al.*, 2001a) and isolate GSM and *Alicyclobacillus* spp. are all moderate thermophiles, a simple temperature test (e.g. for growth at 45-50°C) would also serve to differentiate these bacteria. However, a cautionary note in interpreting results from such a test arose in the present study, as several moderately thermophilic isolates (Y002, Y006, Y0010 and G1) were found to be most closely related to the SLC isolates. In addition, the bacterium classified as *"Sulfobacillus disulfidooxidans"* (Dufresne *et al.*, 1996) is actually more closely related to *Alicyclobacillus* spp. than to *Sulfobacillus* spp. (the mis-classification was due to erroneous sequence data having been deposited in the databanks for *Sb. thermosulfidooxidans*; Hallberg and Johnson, 2001a). *"Sb. disulfidooxidans"* is mesophilic, which suggests that the genus *Alicyclobacillus* (like *Sulfobacillus*) may include both moderate thermophiles and mesophiles. The situation regarding iron-oxidation in *Alicyclobacillus* spp. is also unclear, as isolate K1 (Karavaiko *et al.*, 2000), which has 94.0% 16S rRNA gene

homology with *Alicyclobacillus cycloheptanicus* is, in contrast with all currently-classified *Alicyclobacillus* spp., an iron-oxidiser.

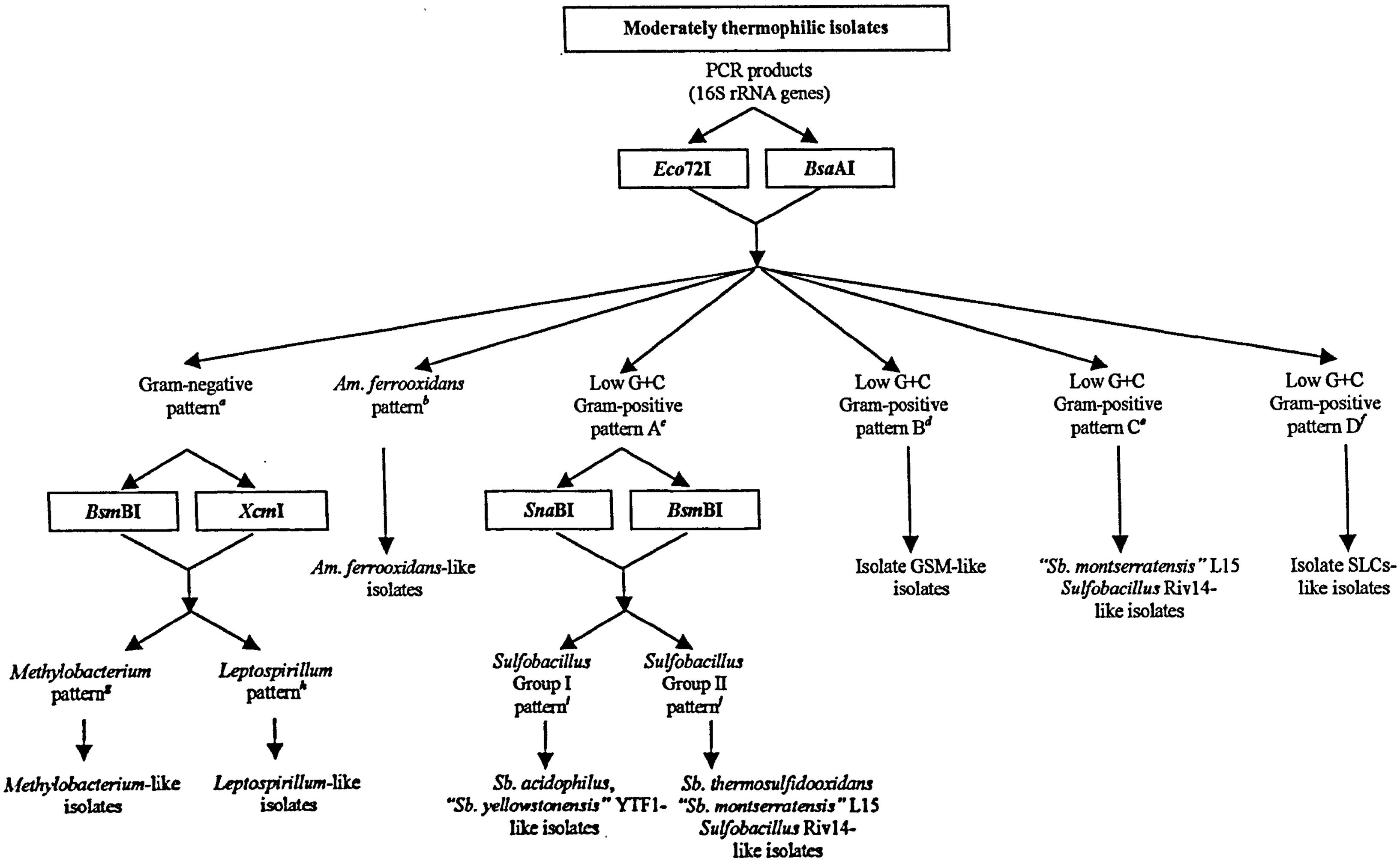
Although the preliminary ARDREA test (using *Eco*721 and *Bsa*A1) fails to differentiate *Methylobacterium*-like isolates (such as Y005) and thermotolerant *Leptospirillum* (e.g. isolate MT6) a second set of digests, using *Bsm*BI and *Xcm*I, may be used to identify these iron-oxidisers (Figure 8.17). Since *Methylobacterium* spp. and *Leptospirillum* spp. display very different cellular morphologies, these bacteria may be more readily differentiated by simple physiological tests.

Coram and Rawlings (2001) found that *L. ferriphilum* and *L. ferrooxidans* may be differentiated using any one of the following restriction enzymes to digest amplified 16S rRNA genes: *Age*I, *Avr*I, *Bfr*I, *Eco*RV, *Mro*I, *Nco*I, *Sma*I, *Ssp*I and *Stu*I (Figure 8.18). Of these enzymes, *Eco*RV was found to be able, in theory, to differentiate isolate MT6 from the two recognised *Leptospirillum* spp., though this was not tested with actual DNA samples.

Overall, the ARDREA method developed in this study was shown to be effective in most cases, at least with those environmental isolates tested. As 16S rRNA gene sequence data from only a single organism was used sometimes to differentiate one group from another, the accuracy of the method might become relatively low in such cases. To compile a more accurate ARDREA method, 16S rRNA gene sequences from increased number of microorganisms should be utilised ideally, when they become available. The ARDREA methods reported so far can only be applied to a few mesophilic acidophilic species and *At. caldus* (Rawlings, 1995 and Kamimura *et al.*, 2001). Rawlings (1995) developed the ARDREA method to differentiate *At.*

ferrooxidans, *At. thiooxidans* and *L. ferrooxidans*. Later, Kamimura *et al.* (2001) described rapid identification of *At. ferrooxidans* by combining ARDREA with physiological analysis. The ARDREA method described here can be applied to a much wider variety of acidophiles. In particular, the ARDREA method was developed for the first time for moderately thermophilic acidophiles in this study. By using this method, a large number of environmental isolates can be identified to a genus or even species level.

Figure 8.17 (following page): General scheme for identification of moderately thermophilic iron-oxidising isolates using ARDREA. Descriptions of *a-j* are commented in Table 8.10.


Moderately thermophilic isolates
PCR products
(16S rRNA genes)
Eco72I
BsaAI
BsmBI
XcmI
SnaBI
BsmBI
Gram-negative pattern[a]
Am. ferrooxidans pattern[b]
Low G+C Gram-positive pattern A[c]
Low G+C Gram-positive pattern B[d]
Low G+C Gram-positive pattern C[e]
Low G+C Gram-positive pattern D[f]
Methylobacterium pattern[g]
Leptospirillum pattern[h]
Sulfobacillus Group I pattern[i]
Sulfobacillus Group II pattern[i]
Am. ferrooxidans-like isolates
Isolate GSM-like isolates
"Sb. montserratensis" L15 Sulfobacillus Riv14-like isolates
Isolate SLCs-like isolates
Methylobacterium-like isolates
Leptospirillum-like isolates
Sb. acidophilus, "Sb. yellowstonensis" YTF1-like isolates
Sb. thermosulfidooxidans "Sb. montserratensis" L15 Sulfobacillus Riv14-like isolates

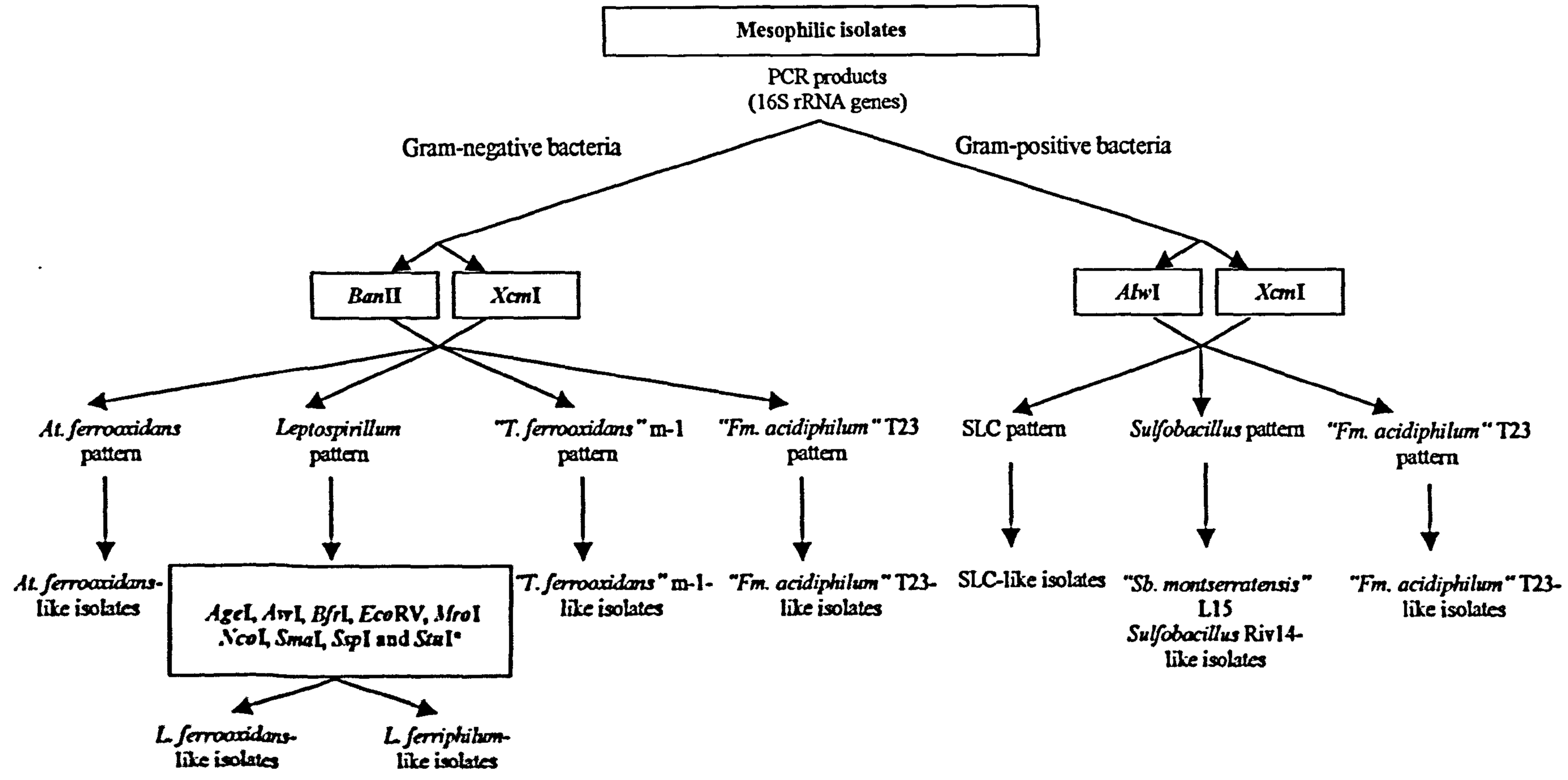


Figure 8.18: General scheme for identification of mesophilic iron-oxidising isolates using ARDREA. The restriction patterns generated with *Ban*II and *Xcm*I (on left) are shown in Figure 8.3. The restriction patterns generated with *Alw*I and *Xcm*I (on right) are shown in Figures 8.4 and 8.5, respectively.
*Digestion with one of these enzymes was reported to be able to differentiate *L. ferriphilum* and *L. ferrooxidans* (Coram and Rawlings, 2001). In addition, *Eco*RV can differentiate *L. ferriphilum*, *L. ferrooxidans* and *Leptospirillum* MT6.

Table 8.10: Restriction pattern name from Figure 8.17 and description of the pattern. *a-j* in this Table correspond to *a-j* in Figure 8.17.

Restriction pattern name	Description of the pattern
[a] Gram-negative pattern	*Eco*72I: 1st line of enzyme map; lane 1 on agarose gel in Figure 8.1 *Bsa*AI: 1st line of enzyme map; lane 1 on agarose gel in Figure 8.2
[b] *Am. ferrooxidans* pattern	*Eco*72I: 4th or 5th line of enzyme map; lane 4 on agarose gel in Figure 8.1 *Bsa*AI: 4th line of enzyme map; lane 4 on agarose gel in Figure 8.2
[c] Low G+C Gram-positive pattern A	*Eco*72I: 3rd line of enzyme map; lane 3 on agarose gel in Figure 8.1 *Bsa*AI: 3rd line of enzyme map; lane 3 on agarose gel in Figure 8.2
[d] Low G+C Gram-positive pattern B	*Eco*72I: 2nd line of enzyme map; lane 2 on agarose gel in Figure 8.1 *Bsa*AI: 2nd line of enzyme map; lane 2 on agarose gel in Figure 8.2
[e] Low G+C Gram-positive pattern C	*Eco*72I: Lane 7 on agarose gel in Figure 8.9 *Bsa*AI: Lane 7 on agarose gel in Figure 8.10
[f] Low G+C Gram-positive pattern D	*Eco*72I: Lane 1&3&4 on agarose gel in Figure 8.9 *Bsa*AI: 2nd line of enzyme map; lane 2 on agarose gel in Figure 8.2
[g] *Methylobacterium* pattern	*Bsm*BI: theoretical pattern include ~1250bp and ~250bp fragments *Xcm*I: theoretical pattern include ~1050bp and ~450bp fragments
[h] *Leptospirillum* pattern	*Bsm*BI: theoretical pattern include two ~750bp fragments *Xcm*I: theoretical pattern include ~900bp, ~350bp and ~250bp fragments
[i] *Sulfobacillus* Group I pattern	*Sna*BI: 1st line of enzyme map; lane 1 and 2 on agarose gel in Figure 8.7 *Bsm*BI: 1st line of enzyme map; lane 1 and 2 on agarose gel in Figure 8.8
[j] *Sulfobacillus* Group II pattern	*Sna*BI: 2nd line of enzyme map; lane 3&4&5 on agarose gel in Figure 8.7 *Bsm*BI: 2nd line of enzyme map; lane 3&4&5 on agarose gel in Figure 8.8

As well as the ARDREA analysis, 16S rRNA genes from several of the more interesting and unusual iron-oxidising bacteria studied were sequenced. From these analyses, it was found that the environmental isolates tested comprised a diverse range of moderately thermophilic acidophiles, including α-*Proteobacteria*, *Actinobacteria* and low G+C Gram-positive bacteria (Figure 8.16). Isolates Y002, Y006, Y0010 and G1 were phylogenetically closely related to each other, and the 16S rRNA gene sequences of these isolates were most homologous (95.3-95.4%) to that of a previously sequenced isolate, SLC66. However, as noted earlier, the Yellowstone and Montserrat isolates were all moderately thermophilic, whereas the optimum temperature of isolate SLC66 (and other similar isolates, SLC1 and SLC2) was found to be 37°C, and no growth occurs at 45°C; Johnson *et al.*, 2001a). Other contrasting features are that isolates Y002, Y006 and Y0010 are all capable of oxidising elemental sulfur, whereas the "SLC isolates" do not, and the thermophiles are more acid-tolerant than the mesophiles (pH minima of 1.3, compared with 1.7; Johnson *et al.*, 2001a).

Isolate Y004 displayed the typical physiological characteristics of *Alicyclobacillus* spp.. Its nearest relative (97.6% 16S rRNA gene homology) in the databanks was the Gram-positive moderate thermophile, K1. However, Y004 does not oxidise ferrous iron while strain K1 has been reported to do so (Karavaiko *et al.*, 2000).

Although isolate Y0017 has been described as a moderate thermophile, the maximum temperature of this isolate is relatively low (50°C; Johnson, unpublished data). Interestingly, this iron-oxidiser is most closely related (98.0% 16S rRNA gene homology) to "*Sb. montserratensis*" L15. The latter acidophile is mesophilic, with a temperature optimum of 37°C and temperature maximum of 43°C (Johnson *et al.*, 2001a). One other distinct feature of "*Sb. montserratensis*" L15 is its extreme acidophily (pH minimum 0.7, making it the most acid-tolerant of all known iron-oxidising

bacteria). In contrast, the pH minimum of isolate Y0017 was found be much higher (1.3; D B. Johnson, unpublished data).

Isolates Y0015 and Y10016 were both found to be most closely related to "*Sb. yellowstonensis*" YTF1, and all three of these bacteria were isolated from the same sampling site in Yellowstone National Park (Frying Pan Hot Spring). These bacteria shared a number of physiological traits, such as temperature maxima of about 60°C; D. B. Johnson, unpublished data).

Isolate Y0018 was found to be most closely related to *Am. ferrooxidans* TH3 with 97.6% 16S rRNA gene homology. Both the former and the latter were found to have the same temperature maximum of 55°C, and were able to reduce ferric iron and unable to oxidise sulfur (Clark and Norris, 1996a; Johnson *et al.*, 2001a; D. B. Johnson, unpublished data). The pH minimum of isolate Y0018 was found to be 1.0 (D. B. Johnson, unpublished data) though that of the latter was not determined.

Phylogenetic analysis of the Yellowstone isolates did, however, reveal two seemingly novel groups of thermo-acidophiles. The first of these were isolates Y008, Y0012, Y0013 and Y0014, which were found to be obligately heterotrophic bacteria (D. B. Johnson, unpublished data). Data searches revealed that the closest known relative of these isolates is *Acidisphaera rubrifaciens* (93-94% 16S rRNA gene homology). Currently, there is only one designated species of *As. rubrifaciens* (Hiraishi *et al.* 2000) though a bacterium (NO-15) sharing 94.5% rRNA gene homology was isolated from acid mine drainage in Norway (Johnson *et al.*, 2001c). The Yellowstone isolates were even more distantly related to NO-15 (91.9% gene homology) than to the original strain. However, both the original strain and the Norwegian isolate are mesophilic (temperature optimum 30-35°C) whereas the Yellowstone isolates were found to grow at up to 65°C

(making them more thermotolerant than some *Alicyclobacillus* spp.). Given this fact, and the relatively low 16S rRNA gene homology, it is clear that the Yellowstone isolates represent a novel species of acidophilic bacteria.

The other interesting discovery was the isolation of a bacterium that appears to be the first methylotrophic iron-oxidising acidophile to be described. This bacterium was isolated directly from enrichment cultures of Yellowstone samples, where it formed small "fuzzy" colonies on ferrous iron-overlay plates incubated at 45°C (D. B. Johnson, unpublished data). Liquid cultures of Y005 routinely used ferrous sulfate/yeast extract medium, as the *Sulfobacillus* and *Acidimicrobium* isolates. In liquid medium, this isolate grew as 1-2 mm-diameter flocs, which were composed of filamentous bacteria. Phylogenetic analysis of Y005 confirmed that its nearest relative (96.3% 16S rRNA gene homology) is the α-*Proteobacterium*, *Methylobacterium fujisawaense*, though this has a very contrasting physiology to isolate Y005 in that it is a mesophilic non-iron-oxidising neutrophile (Green *et al.*, 1988). There are no known acidophilic strains of *Methylobacterium*, though the mesophilic heterotroph *Acidomonas methanolica* is a methylotroph (Urakami *et al.*, 1989) and several strains of *Acidiphilium* can grow using methanol as sole carbon source (D. B. Johnson, unpublished data). Neither *Acidomonas* nor *Acidiphilium* can, however, oxidise ferrous iron.

Chapter 9

General Discussion and Conclusions

The current study has focused on moderately thermophilic (or thermotolerant) acidophilic microorganisms and, in particular, their role in the oxidation of the sulfide mineral, pyrite. Commercial mineral processing using microorganisms ("biomining") has developed into an important and expanding area of biotechnology. Whilst temperatures in heap leaching and *in situ* leaching operations are not controllable (and may vary widely), stirred tank leaching of mineral concentrates currently generally operate at 40-55°C (though there are projections to use higher temperature systems), and these temperatures would favour the growth of moderate thermophiles.

The project began by characterising four apparently diverse moderately thermophilic, acidophilic microorganisms that had been isolated from an aerated stirred tank bioleaching pilot plant operated by Mintek, South Africa. The microbial inoculum used in that operation was essentially the "BacTech" culture, which had previously been used in a biomining operation (the Youanmi mine) in Australia (Miller, 1997). These isolates (MT1, MT6, NC, and MT16/MT17) were found, from analysis of their 16S rRNA genes, to be most closely related to *At. caldus*^{T} (99.5%), *L. ferriphilum*^{T} (99.5%), "*Sb. yellowstonensis*" YTF1 (98.9%), and *Fp. acidiphilum*^{T} (99.6%), respectively. Despite these high 16S rRNA gene sequence homologies, there were significant differences in some physiological characteristics between the novel acidophiles, *Leptospirillum* MT6 and *Ferroplasma* MT17, and other *Leptospirillum* spp. and *Ferroplasma* spp., such as the much faster growth rate of isolate MT6 than all previously characterised *Leptospirillum* spp. Experimental data also showed that the *Ferroplasma* isolates

(MT16 and MT17) could metabolise tetrathionate, which is the first time this has been reported.

Following their physiological and phylogenetic characterisation, these and other moderately thermophilic acidophiles, were tested for pyrite oxidation. The relative efficiencies of "logically designed" consortia of moderate thermophiles, compared to pure cultures were assessed. In all of these consortia, it was important to include an iron-oxidising acidophile, as it is known that the oxidation of some sulfide minerals (including pyrite) is primarily mediated by ferric iron, which is produced by ferrous iron-oxidisers. Ferric iron attack on pyrite results in the (ultimate) production of elemental sulfur and/or various reduced inorganic sulfur compounds (RISCs; Schippers *et al.*, 1996; Schippers and Sand, 1999). These are not oxidised by bacteria such as *Leptospirillum* or *Am. ferrooxidans* (though they can be oxidised by the ferric iron which is produced by these microorganisms), but they are metabolised by other moderate thermophiles such as *Sulfobacillus* spp. and *At. caldus*. Although *Sulfobacillus* spp. are potentially important organisms in leaching environments, due to their ability to oxidise both ferrous iron and RISCs, the actual importance of these acidophiles appears, at least in aerated stirred tanks, to be minor. Foucher *et al.* (2001) analysed microbial populations in a stirred tank bioreactor and an aerated column reactor processing cobaltiferous pyrite operating at 35°C. Among the three bacteria identified (*L. ferrooxidans*, *At. caldus* and *"Sulfobacillus thermosulfidooxidans"*), *Sb. thermosulfidooxidans*, (later confirmed to be more closely related to *"Sb. montserratensis"*; P. d'Hughes, personal communication) initially accounted for ~30% of the mixed culture but became less abundant (~5-10% of total bacteria) as mineral oxidation progressed. Similarly, *At. caldus* appeared to out-compete *Sulfobacillus* spp.

in the Mintek stirred tanks and generally greatly outnumber the Gram-positive bacteria (chapter 3). Both *Leptospirillum* and *At. caldus* are autotrophs, and fix carbon dioxide, some of which, due to cell lysis and exudation, ends up as soluble (dissolved) organic carbon. A third group of candidate acidophiles to be included in bioleaching consortia would therefore be heterotrophs (or mixotrophs). These acidophiles could, in theory, benefit the autotrophs by (i) metabolising organic compounds, thereby reducing or eliminating any potentially inhibitory effects and, (ii) their production of carbon dioxide (mineral processing bioreactors are often fed with CO_2-enriched air). In addition, inclusion of heterotrophs (or mixotrophs) that also catalyse the oxidation of iron (and/or sulfur) could, in theory, result in more accelerated sulfide mineral oxidation.

The initial pyrite oxidation experiments were carried out in shake flasks. These had the advantage of allowing a large number of replicated microbial cultures to be screened, but suffered from the lack of control of some parameters, most notably pH. The main iron-oxidiser used in mixed cultures was the *Leptospirillum* isolate (MT6). Some interesting data were obtained with both ground rock pyrite and a pyrite concentrate. It was found, for example, that oxidation of the ground rock pyrite was suppressed in mixed cultures of *Leptospirillum* MT6+*Ferroplasma* MT17 and (to a lesser extent) *Leptospirillum* MT6+*Sulfobacillus* NC, compared to pure cultures of *Leptospirillum* MT6.

One other notable result from the shake flask pyrite oxidation experiments was the finding that pure cultures of *Leptospirillum* MT6 were unable to oxidise the pyrite concentrate, though this acidophile could oxidise ground rock pyrite and also commercially-available pyrite, obtained from Strem Chemicals (data not shown). When mixed cultures of *Leptospirillum* MT6 and the Gram-positive isolate *Sulfobacillus* NC

were grown on the pyrite concentrate, the *Leptospirillum* isolate emerged as the dominant iron-oxidiser. This suggested that there was an inhibitory agent associated with the pyrite concentrate which was at least partially eliminated by *Sulfobacillus* NC (Figure 9.1). Since *Leptospirillum* MT6 was able to oxidise ferrous iron present in mineral-free pyrite concentrate liquor media following autoclaving, whereas the pyrite concentrate itself was not oxidised (chapter 6), the "inhibitory compound(s)" appeared to affect the cells attached to mineral surfaces, A similar phenomenon was also described by Valdivia and Chaves (2001).

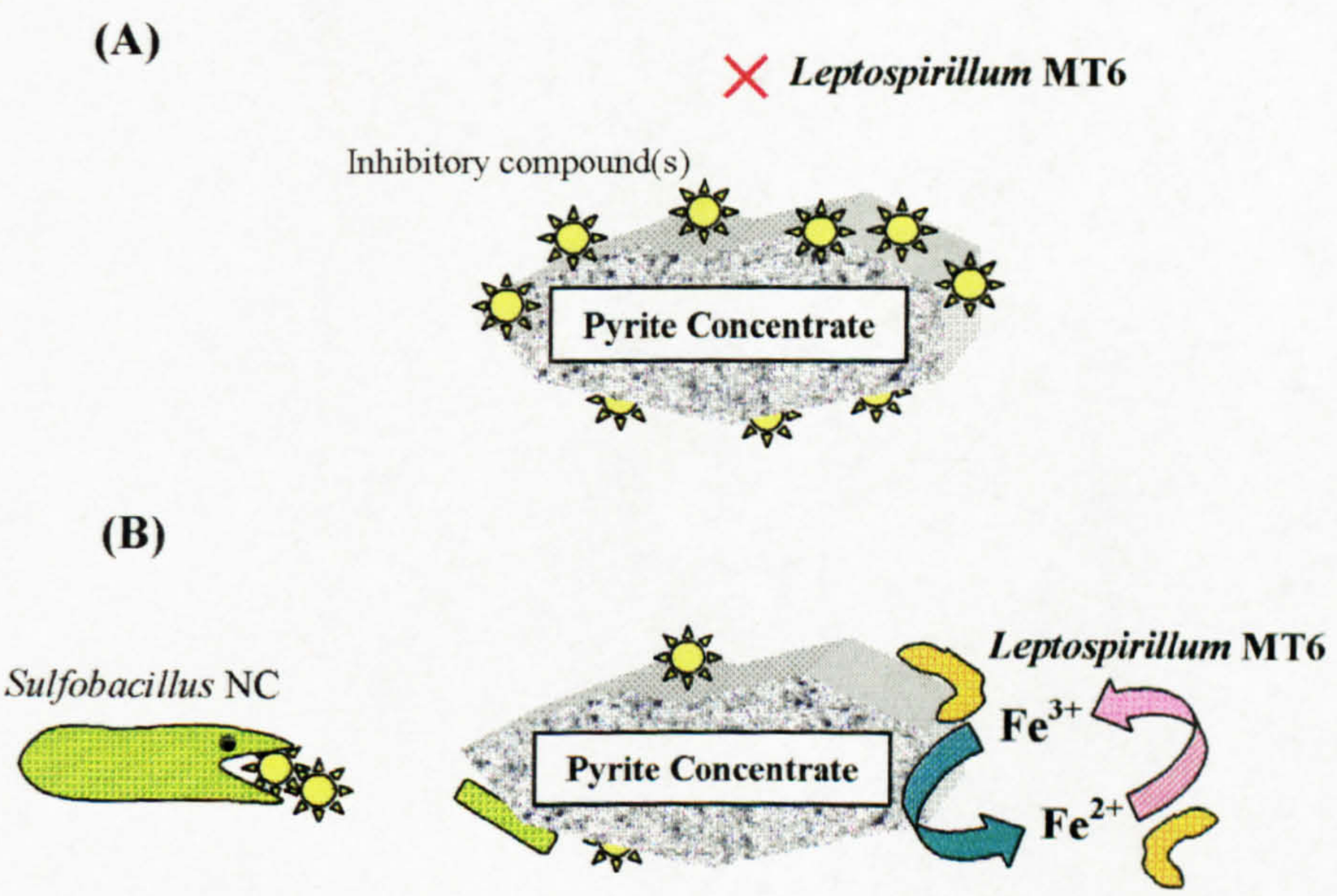


Figure 9.1: Possible interaction between *Leptospirillum* MT6 and *Sulfobacillus* NC during oxidation of pyrite concentrate. (A) Pure culture of *Leptospirillum* MT6. (B) Mixed culture of *Leptospirillum* MT6 and *Sulfobacillus* NC.

Pre-oxidation of pyrite concentrate by *Sulfobacillus* NC prior to inoculation with *Leptospirillum* MT6 was also found to be effective to remove the inhibitory agent, whereas attempts to remove the inhibition chemically by pre-washing with either acetone or perchloric acid were both unsuccessful. Interestingly, the autotroph *At.*

caldus was also unable to remove this "inhibitory compound(s)", suggesting that the latter, present on the surface of the pyrite concentrate (which was usually pre-treated with xanthates; Mariekie Gericke, Mintek; *personal communication*) may be metabolised by heterotrophic acidophiles as a carbon source.

This observation prompted an investigation into the effects of different flotation reagents (fourteen collectors and one frother) on iron oxidation/growth of five moderately thermophilic, mineral-oxidising acidophiles. Different flotation reagents displayed different degrees of toxicities to the microorganisms tested, with *Leptospirillum* MT6 and *Ferroplasma* MT17 being, in general, more sensitive to these chemicals than *Sulfobacillus* NC, *Am. ferrooxidans* ICP and *At. caldus*. Enhanced oxidation of pyrite concentrate was observed in mixed cultures of *Leptospirillum* MT6 and some Gram-positive acidophiles other than *Sulfobacillus* NC, suggesting that a number of Gram-positive acidophiles might also be able to eliminate "inhibitory compound(s)", thereby allowing *Leptospirillum* MT6 to oxidise the concentrate, though this was not investigated further. *Am. ferrooxidans* ICP might be a suitable acidophile in this context, since its sensitivity to a number of flotation reagents tended to be relatively low.

Data from pyrite oxidation experiments in shake flasks were used to select microbial consortia in later experiments in temperature- and pH-controlled bioreactors using ground rock pyrite as the test sulfide mineral. These involved monitoring rates of mineral oxidation, and relative numbers of the different microorganisms using a plating technique in conjunction with a molecular approach (FISH). The results from the pyrite oxidation studies in bioreactors indicated that mixed populations of acidophiles could accentuate or diminish the rates and extent of pyrite oxidation, relative to pure cultures,

as a result of possible interactions between moderate thermophiles, as illustrated in Figure 9.2.

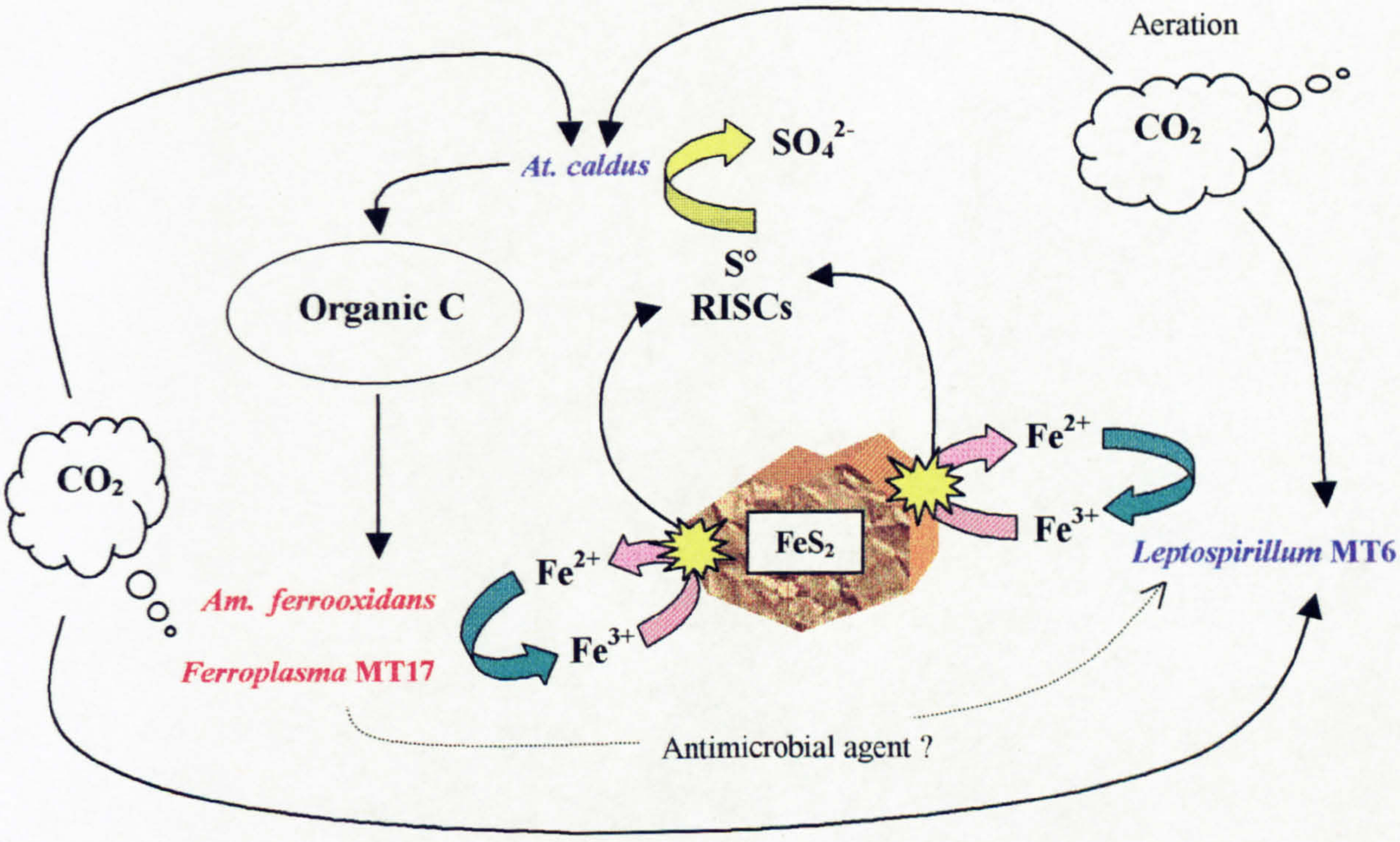


Figure 9.2: Possible interactions of moderate thermophiles in the oxidation of pyrite.

Dopson and Lindström (1999) suggested two possible roles for *At. caldus* when leaching arsenopyrite in co- culture with *Sb. thermosulfidooxidans*. These were: (i) to remove sulfur that can accumulate on the surface of the oxidising mineral (only a portion of this elemental sulfur was removed by *Sb. thermosulfidooxidans*, and sulfur accumulation was less in the mixed culture with *At. caldus*), and (ii) to aid mixotrophic growth of *Sb. thermosulfidooxidans* by releasing organic chemicals. In the current study, *At. caldus* was found to have variable effects on pyrite oxidation in different

mixed cultures. A mixed culture of *Am. ferrooxidans*+*At. caldus* was far more effective than a pure culture of *Am. ferrooxidans*. Since *Am. ferrooxidans* is a mixotrophic non sulfur-oxidiser, both the former and latter roles of *At. caldus* described above were likely to benefit *Am. ferrooxidans*. In contrast, in mixed culture with *Leptospirillum*, *At. caldus* appeared to have a negative impact on pyrite oxidation, possibly due to these two obligate autotrophs competing for CO_2.

Although *Ferroplasma* MT17 also had a negative effect on pyrite oxidation by *Leptospirillum* MT6 (possibly due to production of some active metabolite produced by the archaeon, though this was not investigated further), pyrite oxidation was particularly effective in the mixed culture of *Leptospirillum* MT6+*Ferroplasma* MT17+*At. caldus*. The roles of *At. caldus* described by Dopson and Lindström (1999) were probably also the case in this mixed culture (though *Ferroplasma* MT17 might have contributed to the oxidation of RISCs). *Ferroplasma* MT17 became dominant in the later phase of pyrite oxidation (and at lower pH) possibly due to the DOC, originating from dead bacterial cells, which would have become available for the heterotrophic archaeon.

Possible competition for iron was observed in the mixed culture of *Leptospirillum* MT6+*Am. ferrooxidans*, where pyrite oxidation was slightly less than that by the pure culture of *Leptospirillum* MT6. *At. caldus* was present in equal or greater numbers than the iron-oxidisers in some mixed cultures, presumably due to its ability to utilise RISCs (which yield more energy than the oxidation of ferrous iron).

Inhibition of moderately thermophilic and mesophilic *Sulfobacillus* spp. and related low G+C Gram-positive bacteria by ferric iron also formed part of the present study. Previously, Johnson *et al.* (2001b) had noted that ferrous iron oxidation by *Sulfobacillus*-like moderate thermophiles isolated from two sites in Yellowstone

National Park, Wyoming did not go to completion in shake flask cultures poised initially at pH 2.0, though it did so at lower pH (1.8). Experimental results suggested that this group of Gram-positive acidophiles are particularly sensitive to pH-related ferric iron inhibition, and it was hypothesised that the anionic ferric sulfate complex, $Fe(SO)_2^-$, might be particularly important in this regard. Commercial stirred tanks are generally run at pH <2 and, therefore, this pH-related ferric iron sensitivity is probably not important in such cases. However, in sulfide ore processing operations where the pH is not controlled (mineral heaps, *in situ* operations etc.) it is conceivable that mineral oxidation by these low G+C Gram-positive bacteria would be more likely to be inhibited by ferric iron.

The other part of the current project included investigation and development of ARDREA (Amplified Ribosomal DNA Restriction Enzyme Analysis) using the 16S rRNA gene sequences of known acidophilic bacteria to identify moderately thermophilic (and mesophilic) acidophiles. The ARDREA methods described previously had been applied only to a few mesophilic species and *At. caldus* (Rawlings, 1995; Kamimura *et al.*, 2001), and were further developed, in the current study, to apply to wide variety of moderately thermophilic and mesophilic acidophilic isolates, facilitating identification at the genus or even species level.

The ARDREA method was applied successfully to identify moderate thermophiles isolated from geothermal sites in Yellowstone National Park and Montserrat. It was found that these isolates comprised a diverse range of moderately thermophilic acidophiles, including α-*Proteobacteria*, *Actinobacteria* and low G+C Gram-positive bacteria, and two seemingly novel groups of thermo-acidophiles. One of the latter was most closely related to *Acidisphaera rubrifaciens*, and the other appeared to be a

methylotrophic iron-oxidising acidophile (most closely related to *Methylobacterium fujisawaense*), making it the first mineral-oxidising bacterium of this type to be described.

The results from the current research project indicate that the use of defined mixed populations of moderately thermophilic acidophiles in mineral processing may have benefit in terms of: (i) maximising the oxidation of mineral sulfides (both rates and yields); (ii) minimising costs by utilising organic carbon flow from autotrophic to heterotrophic acidophiles; (iii) compensating disadvantageous characteristics of "ferric iron sensitive" Gram-positive bacteria by including "non-sensitive" acidophiles in mixed cultures; and (iv) minimising or eliminating problems relating to the presence of residual flotation reagents in mineral concentrates.

Mixed cultures that include microorganisms with different physiological characteristics can expand the range of microbial adaptability to variations in physico-chemical parameters, such as pH, temperature, and concentrations of solutes and heavy metals. Based on known acidophilic microbial interactions, it is now possible to design microbial consortia for more robust and more efficient bio-oxidation of sulfidic minerals.

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