DOCTOR OF PHILOSOPHY

Acidophilic sulphate reducing bacteria : candidates for bioremediation of acid mine drainage pollution.

Sen, Anna

Award date:
2001

Awarding institution:
Bangor University

ACIDOPHILIC SULPHATE REDUCING BACTERIA: CANDIDATES FOR BIOREMEDIATION OF ACID MINE DRAINAGE POLLUTION

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

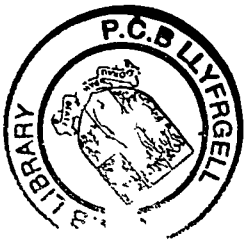
By

Anna Sen
BSc (Hons), MSc

School of Biological Sciences
University of Wales Bangor
Gwynedd
Cymru
LL57 2UW
U.K.

Industrial CASE Studentship
British Biotechnology and Biological Research Council
British Nuclear Fuels Limited
Agreement reference: A747301

February 2001



SUMMARY

Acidophilic/acid-tolerant sulphate reducing bacteria (aSRB) were sampled from three acidic environments in Wales and Montserrat. Generation of alkalinity in mixed cultures containing aSRB was demonstrated in bench-scale bioreactors at pH > 1.7. Experiments at fixed pH were carried out in bioreactor cultures (pH range 2.5 – 5.5 for batch, and 2.5 – 4.8 for continuous cultures). Sulphate reduction rates (SRR) were determined for two batch-mode systems employing immobilised mixed cultures supplied with ethanol. A maximum SRR of 70 µmoles sulphate reduced L^{-1} h^{-1} at pH 4.0 in one system and 58.5 µmoles L^{-1} h^{-1} at pH 5.5 in a second was achieved. Sulphate reduction rates and sulphate reducing activity (SRA) were determined in pH-statted continuous culture experiments using an immobilised mixed culture supplied with glycerol (pH 2.5 – 4.0) or acetate (pH 4.0 and 4.8). A maximum SRR of 0.46 mmoles SO_4^{2-} L^{-1} H^{-1} and an SRA of 23 µmoles sulphate reduced L^{-1} h^{-1} * µmole ATP was achieved using glycerol (or a sulphate conversion rate of 1 g SO_4^{2-} reduced L^{-1} day^{-1}). Samples from bioreactor cultures were used to inoculate solid overlay media at pH 3 - 3.5 to isolate and enumerate aSRB and associated acidophilic/acid-tolerant bacteria. Various culture techniques were tested and developed for growth of aSRB. Growth of pure cultures in liquid media was possible using small batch cultures incubated in anaerobic systems. Solid overlay media were developed to optimise growth of the bacteria, employing the acidophiles *Acidiphilium* SJH and *Acidocella* WJB-3 in a two-phase agarose gel. The role of these bacteria in the solid media was examined. The WJB-3 medium was used for the physiological characterisation of four novel strains of aSRB and three associated novel strains from bioreactor cultures. Phylogenetic analysis showed that the four strains of aSRB were all most closely related to the Gram-positive, spore-forming, incomplete-oxidiser *Desulfosporosinus orientis*.

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ABBREVIATIONS & ACRONYMS

A: Adenine
A_x: Absorbance at x nm
AAS: Atomic absorbance spectroscopy
AMD : Acid mine drainage
aSRB: Acidophilic sulphate reducing bacteria
ATP: Adenosine triphosphate
AW: acid-washed agarose solid medium
bp: base pair
BLAST: Basic Local Alignment Search Tool
BSA: bovine serum albumin
BS: basal salts medium
C: cytosine
CH_3COOH: acetic acid
CH_3COO^-: acetate
CO_2: carbon dioxide
CoA: coenzyme A
°C: degrees Celcius
d: day
DNA: deoxyribonucleic acid
d.p. : decimal places
EDTA: ethylene diamine-tetra-acetic acid
E_h/ $E^{o'}$: redox potential
EtOH: ethanol
Fe^{2+}: ferrous iron
Fe^{3+}: ferric iron
$FeSO_4$: ferrous sulphate
g: gram
g: acceleration due to gravity (in m s^{-1})
G: guanine
h: hours
H^+: hydrogen ions (or protons)
HCl: hydrochloric acid
H_2S: hydrogen sulphide
H_2SO_4: sulphuric acid
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS^-: dissolved sulphide
HRT: hydraulic residence (or retention) time
L: litre
M: molar
min: minute
mM: millimolar
ml: millilitre
mm: millimetre
mmoles: millimoles
µm: micromolar
µmoles: micromoles
mV: millivolts
nm: nanometres
OD_x: optical density at x nanometres
OFN: oxygen free nitrogen
ORP: oxidation/reduction potential
pK_a : dissociation constant of an acid
pK_{sp} : solubility product of a sparingly soluble salt
pH: -log $[H^+]$
PCR: polymerase chain reaction
pers. comm. : personal communication
PHYLIP: Phylogeny Inference Package
ppm: parts per million
RFLP: restriction fragment length polymorphism
RNA: ribonucleic acid
r.p.m. : revolutions per minute
SEM: Scanning electron microscope/microscopy
SRA: sulphate reducing activity
SRB: sulphate reducing bacteria
SRR: sulphate reduction rate
T: thymine
TE: trace elements solution
Tris: tris[hydroxymethyl]aminomethane
TSB: tryptone soya broth
U.S.A. : United States of America
U.K. : United Kingdom
UWB: University of Wales Bangor
UV: ultraviolet light
v: volume
w: weight

WAYE: yeast extract/acid-washed agarose solid medium
YE: yeast extract

ACKNOWLEDGEMENTS

Many, many thanks to my supervisor, Barrie Johnson, for his attentive supervision, inspiration, encouragement and support, his belief in my ability, and also his friendship, which I am sure will continue well beyond my time at the University of Wales Bangor.

I acknowledge the Biotechnology and Biological Sciences Research Council (BBSRC) and British Nuclear Fuels (BNFL) for providing financial support through the Industrial CASE Studentship (Agreement ref. A747301). Thanks to Martin Green and Harry Eccles of BNFL in particular for their input into the project.

A special thank you to my friend and colleague Kevin Hallberg, who has been inspirational, instructive, patient and helpful, especially in the first and second year when I needed him most. I also shared a lot of laughs and good times with him.

Thank you to all the other important people in SBS, Bangor who I worked closely with, and for their help and assistance; especially: Adibah Yahya, Stewart Rolfe, Åsa Kolmert, Paula Bacelar-Nicolau, Marie-Antoinette Dziurla, and Keith Osborne.Thanks to Kelly Milward for helping me with plasmid preps. A big thanks to all the technical and administrative staff at SBS for their help, and for their cheerful smiles.

A big thanks to John Lloyd of the University of Birmingham for helping with DNA sequencing. Thanks also to Frank Roberto of Idaho National Engineering and Environmental Laboratory, Idaho Falls, USA, for help with sequencing, PCR methods etc, and for his enthusiasm.

Thank you to my friends at Paques B.V., (The Netherlands), in particular Johannes Boonstra for many interesting and useful discussions. Thanks to Cees Buisman for frank discussions and constructive criticism.

Thanks to colleagues at the universities of Birmingham, Dundee, Oxford and Imperial College, London, for their useful contributions and discussions at "LINK" meetings with BNFL.

PERSONAL ACKNOWLEDGEMENTS

A very special thank-you to my father, Hindole Kumar Sen, for his continuous love, support and encouragement throughout my education and career. This thesis is dedicated to him.

A big thank you to my mother, Christine, and Antony Vernon-Riley, for their love and support.

Thanks to my sister Sarada and my brother Jonarden, who are my best friends in the world.

I wish to thank William Housley, who for many years told me I could do a Ph.D., and who always had great faith in me.

Thank you to to my special friends in Bangor for their love and companionship, especially Andrew Drake, and Bethan Stallwood. A big thank you to my friends in Menai Bridge for providing friendship, love, support, and distraction (!) during my write-up: in particular, Kirsty Dernie, Ben Lutyens, and Marika Galanidi. A special thank you to Andrew Ford for providing me with smiles, hugs and looking after me whilst finishing my amendments.

Finally, but very importantly, praise to the Lord Krishna, the Supreme Spirit, who has always given me inner strength, courage, and optimism. "*Om Jai Sai Ram, Govinda Hare Hare*".

"Without deviation from the norm, there can be no real "progress" "

Frank Zappa

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"There is nothing beautiful or sweet or great in life that is not mysterious"

F.R. Chateaubriand, 1768

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"All this visible universe comes from my invisible Being....Consider my sacred mystery....I am the source of all beings, I support them all, but I rest not in them....At the end of the night of time all things return to my nature; and when the new day of time begins I bring them again into light."

Bhagavad Gita, Ch.9, v. 4, 5, & 7

1. INTRODUCTION

1.1 Acid Mine Drainage Pollution: a Global Problem

Acid mine drainage (AMD) is a widely recognised phenomenon which is considered to be a critical environmental problem facing the mining industries, environment agencies, and governments. It has been estimated that, in the U.S.A alone, around 2.3 x 10^9 m^3 of effluent are produced annually from acid-generating sites, affecting up to 17,000 km of streams (Herlihy *et al.,* 1987; Lyew *et al.,* 1994). In Canada, potential acid-generating sites from base metal mining total over 15 000 hectares (Feasby *et al.,* 1991). The estimated costs for the prevention of further pollution from all of these sites are in the order of billions of dollars (Feasby *et al.,* 1991). In the U.K., hundreds of kilometres of streams are affected by AMD (Johnson, 1999); the problem has become much more significant since the decline of the coal mining industry, which has resulted in a large number of abandoned mine sites. In some areas of the U.K., drainage from abandoned mines is now the single greatest cause of freshwater pollution (Younger, 1997). Boult *et al.* (1994) sampled the Afon Goch, a stream draining Parys Mountain, Anglesey, North Wales, where copper has been mined since the Bronze Age. Maximum dissolved Fe, Al, Mn, Cu, and Zn concentrations over a 14 month period were 259, 167, 49, 60 and 42 g m^{-3} respectively, and pH was as low as 2.3, making it one of the most metal- and acid contaminated streams in the UK (Boult *et al.,* 1994). However, much more highly contaminated and acidic waters exist elsewhere: mine waters with pH values as low as –3.6 (see Appendix 1.1) and total dissolved metal and sulphate concentrations of 200 and 760 g L^{-1} were encountered in the Richmond Mine at Iron Mountain, California, USA (Nordstrom *et al.,* 2000).

As a result of the global concern regarding the impact of AMD, there are a considerable number of national and international programmes that have

been set up to address the problem. These are often collaborative efforts involving academia, industry and government, and there are several major campaigns that are ongoing in the USA, Canada, Australia, and Sweden (Society for Mining, Metallurgy, and Exploration Inc. (SME), 2000).

1.2 Formation of AMD

AMD is often a highly acidic discharge (pH < 3) containing elevated concentrations of a variety of metal species, and sulphate. It is formed when sulphide ores in mining spoil heaps, tailings or adits undergo chemical and biological oxidation. Mining allows oxygen to be introduced into the deep geological environment where such minerals are normally in a reduced state (Banks *et al.*, 1997). Some mine drainages have a pH of 4.5 and over: these are considered to contain alkalinity, mainly due to dissolved bicarbonate (Johnson, 2000). The acidity of AMD derives from free hydrogen ions ('proton acidity') and the hydrolysis of metal ions ('mineral acidity') (Clarke, 1995), the most important metals being iron (ferrous and ferric), aluminium, and manganese (preceded by oxidation in the case of Fe (II) and Mn (II)), for example:

$$Fe^{3+} + 3H_2O \longrightarrow Fe(OH)_3 + 3H^+ \qquad (1.1)$$

The acidity of AMD can therefore be calculated by measuring the concentrations of these metals; if this exceeds the bicarbonate alkalinity, the drainage waters are net acidic (Johnson, 2000). Another source of acidity is organic acidity, associated with dissolved organic compounds. However, as mine waters generally have a very low content of dissolved organic carbon (DOC), organic acidity is usually insignificant (Clarke, 1995).

In aerobic environments, the abiotic oxidative dissolution of sulphide minerals (such as pyrite, FeS_2) occurs spontaneously but at a relatively

slow rate. The process is greatly accelerated by acidophilic chemolithotrophic bacteria such as *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Johnson and Bridge, 1997; Lyew at al. 1994; Schippers *et al.*, 2000; see Figure 1.1). The bacteria oxidise ferrous ions to ferric ions, which chemically oxidise pyrite (Schippers *et al.*, 2000). At acid pH, the overall microbial pyrite oxidation rate is up to 25 times higher than the chemical rate (Rohwerder *et al.*, 1997), whereas at neutral pH, for example in carbonate buffered mine waste, pyrite oxidation is microbially accelerated by a factor of around 2.5 (Schippers *et al.*, 2000). Metal sulphides are biologically oxidised via two mechanisms: (i) by iron (III) ions (e.g. pyrite), and (ii) by iron (III) ions and protons (sphalerite, galena, chalcopyrite), shown as MS below. These are summarised in equations 1.2 – 1.6 (Schippers and Sand, 1999; Schippers *et al.*, 2000):

(i)

$$FeS_2 + 6Fe^{3+} + 3H_2O \longrightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \quad (1.2)$$

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5H_2O \longrightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \quad (1.3)$$

(ii)

$$MS + Fe^{3+} + H^+ \longrightarrow M^{2+} + 0.5H_2S_n + Fe^{2+} \quad (1.4)$$

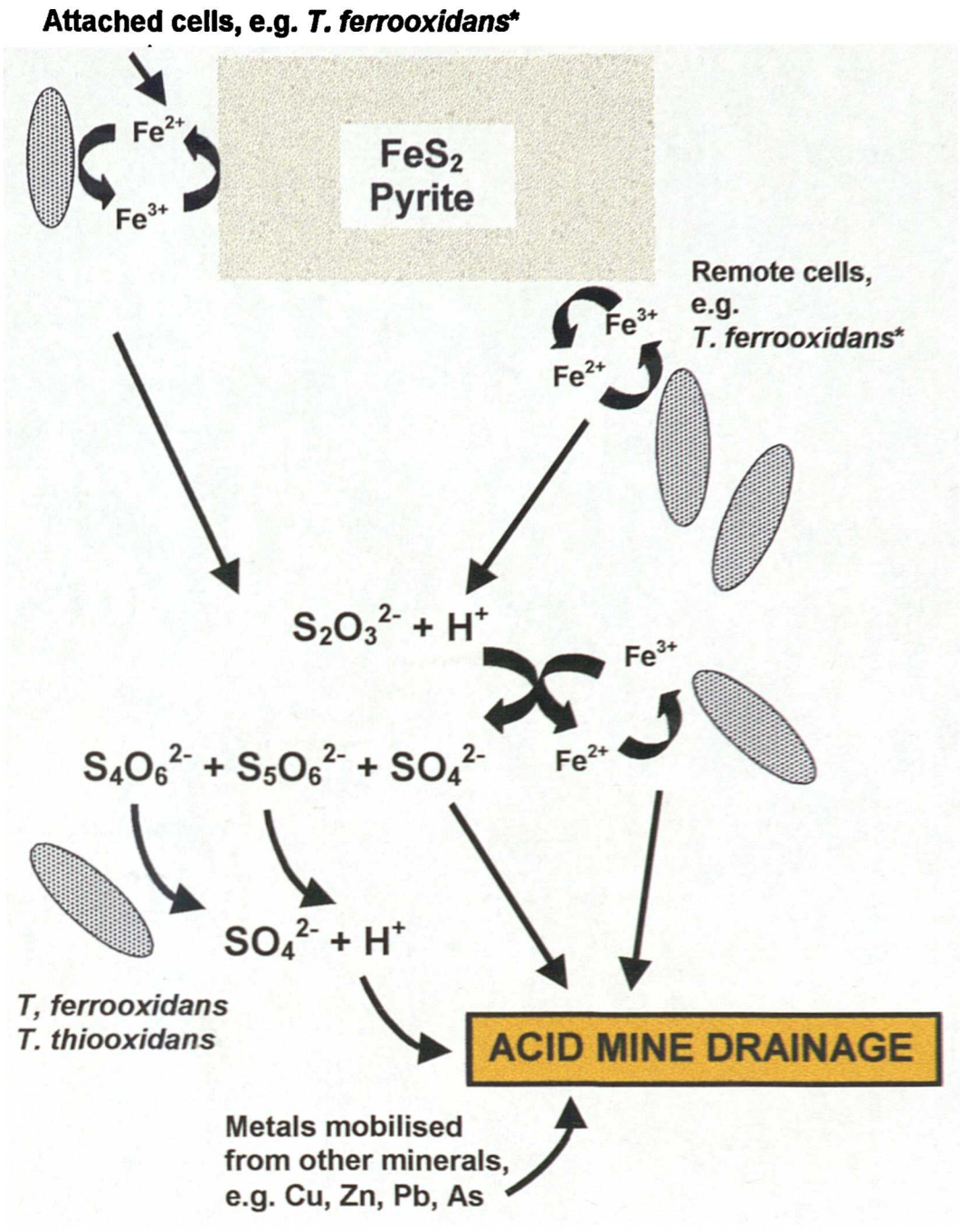
$$0.5H_2S_n + Fe^{3+} \longrightarrow 0.125S_8 + Fe^{2+} + H^+ \quad (1.5)$$

$$0.125S_8 + 1.5O_2 + H_2O \longrightarrow SO_4^{2-} + 2H^+ \quad (1.6)$$

((i) is known as the "thiosulphate" mechanism, and (ii) as the "polysulphide" mechanism; H_2S_n represents a polysulphide.)

The chemical reactions leading to the production of AMD therefore occur in several stages and via a number of mechanisms, but can be summarised simply (in the case of pyrite) as:

$$4FeS_2 + 15O_2 + 14H_2O \longrightarrow 4Fe(OH)_3 + 16\,H^+ + 8\,SO_4^{2-} \quad (1.7)$$



* or *L. ferrooxidans, or* heterotrophic iron-oxidisers

Figure 1.1. Acidophilic microorganisms and their role in the formation of AMD from pyrite (see Johnson, 2000)

1.2.1 Chemical characteristics of AMD

The chemical composition of AMD is variable, but it is typically characterised by high concentrations of iron and sulphate. It may also contain relatively high concentrations of other metals and metalloids (Johnson, 2000). The solubility of most cationic metals is greater in acidic than neutral or alkaline waters, and therefore acidic wastewater streams can be a major cause of metal pollution in areas some distance from their source.

Redox potential is an important index of AMD chemistry. Two major redox systems occur in mine drainage waters (Nordstrom *et al.*, 1979; Boulegue and Michard, 1979). One is the ferrous/ferric iron couple and the other is the sulphide/sulphate couple. Both of these are affected by pH. Below pH 2.5-3, both ferrous and ferric iron are soluble, and the ferrous/ferric couple is +770 mV (see Table 1.3). Above this pH value, the ferrous carbonate/ ferric hydroxide couple is +200 mV (Ehrenreich and Widdel, 1994). For the sulphide / sulphate couple, the E^{o} increases from -221 mV at pH 7 to +149 mV at pH 2 (Johnson, 2000). The large difference in E^{o} between the two couples means that it is easy to determine which couple is dominant in an AMD environment. Because ferric iron will readily oxidise sulphide, the two are mutually exclusive (Johnson, 2000). High redox potentials (e.g. +700 mV) indicate that the ferrous/ferric couple is dominant. This type of AMD usually has lower pH (below 2.5-3) and ferruginous material is often visually evident. Other types of AMD have relatively low redox potentials (e.g. +200 mV), higher pH (around 5), and the discharges may appear less stained with ferric iron (although they may be present downstream if oxidation occurs). The characteristics of a particular discharge can vary a great deal and the type of AMD formed will affect treatment/prevention requirements (see 1.4 – 1.6).

Concentrations of dissolved inorganic and organic carbon in AMD waters tend to be very low, and primary production may be mediated by acid-tolerant phytoplankton and macrophytes or by chemolithotrophic bacteria (Johnson, 2000).

1.3 The Impact of AMD

Streams receiving AMD are often noticeable through being heavily stained with red/orange ferric iron precipitates (see Figure 1.2) and the benthic environment is often clogged with ferruginous material, which is detrimental to most fauna. In addition, the acidity of the leaching solutions adds to the severe deterioration of water quality both directly and through promoting elevated concentrations of other heavy metals, such as Zn, Cu, Pb, Ni, Cd, and Hg. This combination of factors can result in considerable toxicity to terrestrial, aquatic and riparian life (Hellawell, 1986; Kelly, 1988; Lloyd, 1992), causing major reductions in species diversity and biomass. AMD can also enter groundwater, which is often pumped to the surface for human use (Johnson, 1995b), and would therefore require additional treatment.

AMD pollution can produce both acute and chronic pollution. Acute (or catastrophic) effects can occur if an abandoned mine shaft fills with water over time and then releases a large volume of AMD in a single event, often causing immediate impacts on fauna such as fish. An example of an acute pollution incident is the bursting of a plugged adit at Wheal Jane, one of the last deep tin mine sites in the Carnon Valley area of Cornwall, UK, in 1992. Plugs had been installed to prevent the release of AMD following closure of the mine. In a 24 hour period, around 30 000 m^3 of AMD was released into an ecologically sensitive area (Lamb *et al.*, 1998). The quality of water discharging from Wheal Jane following the failure of a plug in the "Nangiles" adit was very poor: pH 2.6-3.1; iron $\leq$ 1900 mg L^{-1} ; zinc $\leq$ 1700 mg L^{-1} ; copper $\leq$ 18 mg L^{-1} ; aluminium $\leq$ 197

Figure 1.2 The Afon Goch at Parys Mountain, Anglesey, U.K. The stream drains the Mona adit, and is the more acidic (pH ~ 2.5) and metal-rich of the two AMD streams at the former mine site.

mg L^{-1} , and a vast red plume was formed in the Fal estuary (Younger, 1997). Since that time, the National Rivers Authority (and subsequently, the Environment Agency) has ensured that the Wheal Jane minewater is being actively treated and have been evaluating the options for environmental management in the future (Younger, 1997; NRA, 1994).

The accidental release of acid mine drainage into one of Europe's premier wildlife sites, Doñana National Park in Southern Spain, was a major pollution incident. On the 25th April 1998, 500 million m^3 of extremely acidic (pH 2) and metalliferrous (Zn, Pb, Cd, Hg; concentrations unknown) waste water entered the Guadiamar river in Southern Spain when a settlement reservoir at a Canadian-owned lead and zinc mine, Los Frailes, ruptured (Wildlife News, 1999). It was thought that the dam had been weakening since around 1996 due to slippage of the foundations. Almost all crops, fish and birds were killed in the contaminated area downstream of the spill, and groundwater and vast areas of farmland (estimated to be at least 10,000 hectares) including orchards, rice paddies and cotton crops were contaminated (Enviromine Technical, 1998). The pH of the water was so low that people involved in rescue operations needed hospital treatment for burns. The Doñana National Park is one of Europe's largest protected areas, where over 6 million migratory birds stop over on the Arctic-Africa route each year. The World Wide Fund for Nature (WWF) initiated a legal inquiry into the spill and previous spills from the mine, and the Secretary General of the WWF was quoted as saying, "The consequences of this environmental disaster are immeasurable" (Wildlife News, 1999).

During the operation of a mine, water is continually pumped to the surface and discharged, and such catastrophic events are generally less likely to occur. Chronic pollution by AMD is common, however, as oxidation processes in mine adits and spoil tips may continue for decades after the closure or abandonment of the mine, as large volumes

of sulphide minerals may have been exposed and are susceptible to accelerated oxidation. In this situation, the release of AMD may also be diffuse, and sources can be difficult to identify and locate, making pollution control more complex.

Younger (1997) suggests that long term acidity generation from abandoned mines has two components: 'vestigial' and 'juvenile'. The former refers to the geochemical trauma which occurs when abandoned mines fill with water for the first time, causing iron salts (intermediate products of pyrite oxidation) to come into solution. The release and dispersal of the acidity depends upon the hydraulic retention time of the mine system, but will usually take place within 40 years (Younger, 1997). Juvenile acidity refers to low pH discharges resulting from on-going pyrite oxidation in the region of the water table in the mine system, and will continue until the supply of pyrite is exhausted, which could take hundreds of years (Younger 1997). Treatment options and methods, and remediation management and planning will therefore depend upon the status and history of the mine site with regards to these and other factors (see 1.4 – 1.6).

Legislation regarding environmental protection has become increasingly restrictive over the last few decades, mainly in developed countries. In the UK, Water Quality Objectives (WQOs) are set by the Environment Agency in accordance with both European Union and national environmental policy. However, until very recently there has been a loophole in the law concerning abandoned mines and polluting discharges. According to the Water Act 1989, it is an offence to cause or knowingly permit poisonous, noxious or polluting matter to enter controlled waters in England and Wales, without a discharge consent issued by the Environment Agency or its predecessor bodies (controlled waters include virtually all freshwaters, groundwaters, tidal and coastal waters). An important exception to the discharging system is that before 31 December 1999 it was not an offence

to permit polluting mine effluent to enter controlled waters from an abandoned mine. However, since this date the Environment Agency can prosecute individuals or companies for allowing polluting mine drainage to enter the aquatic environment. As mentioned below, management of AMD pollution can be difficult and expensive, and some types of treatment may subsequently lead to further pollution control problems. With increasing pressure to comply with legislation, there is now a demand for alternative systems for treating and controlling AMD pollution that are both cost-effective and reliable.

1.4 Treatment and Control of AMD

There are a number of methods currently employed for the management of AMD or acidic waste waters. Different factors determine the most suitable method in each individual case, including: (I) the mineralogical characteristics of the mine site (Ledin and Pedersen, 1996); (ii) the quality and longevity of the effluent, and the type and concentrations of contaminants present; (iii) cost; (iv) availability of space for plant or treatment system; (v) ease and cost-effectiveness of set-up and maintenance; (vi) legislation; (vii) the required final land use of the area; (viii) the status of the receiving waters, and (ix) the ecological sensitivity of the surrounding area. There are therefore a number of types of treatment options (discussed below) and the appropriate choice will depend on these considerations. The options as discussed in this report can be summarised in Figure 1.3. However, there are also effective methods for the *prevention* of AMD, which are outlined separately in 1.4.1.

1.4.1 Physical/chemical prevention of AMD

Acid mine drainage can be ameliorated by preventing or slowing down its production, or by collecting and treating it before damage is caused. Sometimes complete removal of the source of pollution, such as a spoil

tip, is possible, but usually it is prohibitively expensive (Clarke, 1995). An alternative is to prevent water and/or oxygen from contacting the spoil or mine site. However, because of the lower cost, treatment of AMD using *in situ* methods is likely to be preferred over removal and treatment of contaminated material (Clarke, 1995).

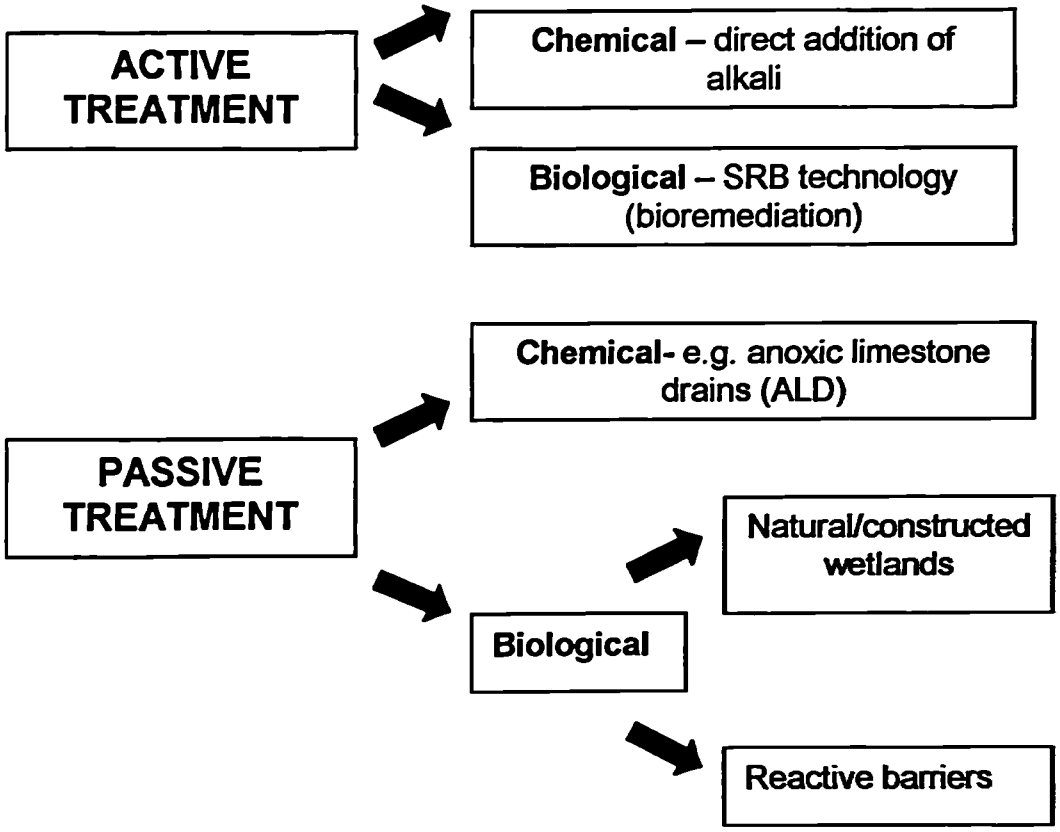


Figure 1.3. Overview of some different treatment methods for AMD pollution.

1.4.1.1. Physical prevention

The physical prevention of effluent migrating from a mine site is one approach to dealing with the problem of treating AMD. Since the

microbiological oxidation of sulphidic minerals requires both water and oxygen to be present, the exclusion of either or both of these should theoretically prevent the production of AMD (Johnson, 1995). Prevention methods based on this principle are known as barrier methods (Clarke, 1995). Examples of such methods include the sealing of mine of shafts by flooding (mine inundation or submergence), which is only effective if mining has occurred below the water table (Clarke, 1995) or clay capping and plastic lining (dry covers) which is only effective if coverage is complete and no water is introduced. In the case of mine tips, tailings or spoils, dry covers can be an effective option (Ledin and Pedersen, 1996). They stabilise the waste and prevent erosion as well as preventing penetration of water and oxygen. The plastic covers used are similar to those used at landfill sites, and a layer of soil is placed above them for protection from physical damage and ultraviolet light. Clay caps can be made from clay materials excavated near to the mine site, or bentonite and other swelling clays have been used (Clarke, 1995). Other covering methods include the use of organic materials such as inert sludges from industry or even sewage sludge, which may enhance the establishment of vegetation and stabilise the area. The feasibility of using any of these approaches depends on the size, hydrology, geology and geochemistry of the site to be treated, and may only provide partial or temporary alleviation.

1.4.1.2 Physico-chemical prevention

Phosphate coating is another method of preventing the release of AMD (Clarke, 1995, Evangelou, 1995). Phosphate ions in solution react with products of pyrite oxidation to form insoluble iron phosphates, which coat the surface of the pyrite, preventing further oxidation by ferric ions. Apatite (calcium phosphate) is a good source of phosphate ions because it is sparingly soluble at neutral pH but becomes more soluble as pH decreases (Clarke, 1995). When oxidation and acid production are

prevented, further dissolution of apatite also stops. Apatite rock is, however, relatively expensive, but low grade phosphate ores or waste rock could provide a cost-effective alternative, and may cost up to ten times less than commercial grade rock. The calcareous components of the waste rock possess acid neutralisation properties, which is an added benefit.

Micro-encapsulation using silicates is a new technology which could be used to control AMD release (Fytas *et al.*, 2000; Mitchell *et al.*, 2000). Pyrite is treated with a solution containing hydrogen peroxide, sodium silicate and a buffering agent. The peroxide oxidises some of the pyrite producing ferric ions, which react with the silicate ions to produce ferric hydroxide-silica (in the buffered solution). This precipitates on the pyrite surface providing a coating, preventing further oxidation (Evangelou and Zhang, 1995), and the pH is stabilised at around 6-7 (Fytas *et al.*, 2000). The silica coating is a good sink for ferric ions, and is more resistant to acid conditions and less polluting to the environment than phosphate coating (Fytas *et al.*, 2000). The exact cost of using this technology in the field is not known, but is estimated to be less than $1 per tonne of tailings (Fytas *et al.*, 2000), and therefore could be a long term solution either alone or combined with another prevention method, according to the situation. Silicate coating may be used on fresh tailings in the processing plant, and may potentially be used on old tailings (Fytas *et al.*, 2000).

1.4.2 Chemical prevention

There are a number of approaches to chemical prevention. Chemicals (bactericides) may be used to inhibit or kill the acidophilic iron oxidising bacteria that accelerate the production of AMD. Anionic surfactants such as sodium dodecyl sulphate are highly toxic to *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Clarke, 1995; Johnson, 1995), and Dugan (1987) showed that the application of this method could be

successful. Dugan and Apel (1983) applied sodium lauryl sulphate and sodium benzoate to high-sulphur coal refuse, causing inhibition of iron- and sulphur-oxidising bacteria. However, application of these chemicals must be controlled in such a way that they give maximum effectiveness in reduction of bacterial populations with minimum pollution to any receiving waters, which could prove difficult in practice (see Sand, 2000; Johnson, 1993; Johnson, 1995b).

Other chemical prevention methods involve neutralisation before AMD can be released to the general water environment. Alkaline materials such as limestone can be added to spoil or underground mines to provide concentrated alkaline recharge (Clarke, 1995). Very strongly alkaline products such as soda ash can be added to trenches and recharge pools to neutralise acid waters formed during surface run-off, or in some cases, acid-generating and acid-consuming rocks can be mixed, providing in situ prevention of acid generation in spoils (Clarke, 1995; Day, 1994).

1.5 Active Treatment

Active treatment systems are those that require continual addition of chemicals or continuous active water treatment (Clarke, 1995). They often require the installation of a plant (for example, including reactors, precipitators, clarifiers), as well as a high demand for reagents, operation and maintenance, and disposal (or additional plant for treatment) of by-products (Gazea *et al.*, 1996). They can be based on chemical and/or biological processes.

1.5.1 Active treatment - chemical

Once AMD has formed, chemical treatment of AMD traditionally involves the addition of an alkaline agent (usually lime, $Ca(OH)_2$ and CaO), which results in an increase in pH, and (with aeration) the oxidation of ferrous

iron (which occurs more quickly at higher pH (Johnson, 1995)), the removal of metals as hydroxides (Lyew *et al.,* 1994) and some of the sulphate as gypsum ($CaSO_4.2H_2O$). An unstable and voluminous sludge (around 2-4% (by weight) of solids (Johnson, 2000)) containing a variety of heavy metals is formed which is difficult to dewater and expensive to dispose of in landfill (Lyew *et al.,* 1994; De Vegt *et al.,* 1997). Large additions of lime can endanger plant growth in the area of the mine site by causing an imbalance in the Ca/Mg ratio and phosphate availability (Ledin and Pedersen, 1996). Active chemical treatment systems usually require the installation of a lime-dosing plant, and incurs high operation and maintenance costs (Johnson, 1995; Gazea *et al.,* 1996). Different strategies are needed for liming, depending on the water body to be treated (e.g. lake or stream, stagnant or fast flowing). More complicated dosing techniques are required to treat lotic systems (streams and rivers); lentic systems (lakes, ponds or reservoirs) are comparatively simple to treat using liming (Olem, 1991). As AMD can cause contamination for a very long time after decommissioning of mines, cost-effective alternatives clearly need to be established.

1.5.2 Active treatment - biological

Bioremediation is the use of microorganisms to degrade organic pollutants or to change the structure of inorganic compounds to form non-toxic degradation products (Rhodes *et al.,* 1994). The microorganisms are used either to partially degrade the contaminant (cometabolism) or to completely degrade the contaminant to innocuous materials (mineralisation) (Rhodes *et al.,* 1994). A successful biological process for pollution control is an engineered system that utilises and accumulates these types of microorganisms to a sufficiently large mass so that the concentration of pollutants are reduced to meet a treatment standard during the time of contact (Rittmann, 1992). There has been much attention given recently to the application of microbial processes to provide

solutions to a variety of pollution problems. The major focus has been the use of bacteria as catalysts or enablers, but a number of other microorganisms and some macroscopic life forms also have potential in remediation processes (Knapp and Faison, 1997). Successful appliance of bioremediation requires understanding of biology, chemistry, geology, soil science and hydrology in each particular situation. A number of organisms have been isolated from natural environments that can degrade oil, solvents and other toxic pollutants in soils or water systems (Brock, 1997). Methods using various microbially mediated transformations of sulphur have been developed to treat waters polluted with AMD (Ledin and Pedersen, 1996).

There are various microbiological processes that mediate the generation of alkalinity which have the potential for the neutralisation of AMD. These are principally reductive processes involving dissimilatory transformations of elements or compounds (Johnson and Bridge, 1997). The high concentrations of ferric iron and sulphate in AMD suggest that there is considerable potential for dissimilatory reduction of both of these species (by iron reducing bacteria and sulphate reducing bacteria respectively). The reductive dissolution of soluble and ferric iron minerals generates net alkalinity (Lovley, 1991; Pronk and Johnson, 1992; Vile and Wieder, 1992; Johnson and Bridge, 1997; Johnson, 2000):

$$4Fe(OH)_3 + CH_2O \longrightarrow 4Fe^{2+} + H_2CO_3 + 2H_2O + 8OH^- \quad (1.8)$$

Dissimilatory sulphate reduction by sulphate reducing bacteria (SRB) occurs predominantly in anaerobic environments that contain significant concentrations of sulphate and readily degradable organic matter (Widdel, 1988). In acid solutions or acidic solutions such as AMD, the process generates net alkalinity (or consumes acidity) in three ways: (i) the consumption of protons by formation of H_2S (a weak acid) from sulphuric acid (a strong acid); (ii) the conversion of carbonic acid to carbon dioxide

and water, and (iii) the formation of pyrite from iron sulphides. In the case of (i), hydrogen sulphide is a weak acid, only partially dissociated at neutral pH, but mainly undissociated at low pH, as its first dissociation constant (pK_{a1}) is 6.88:

$$\underset{}{H_2S} \rightleftharpoons \underset{pK_{a1} = 6.88}{H^+ + HS^-} \rightleftharpoons \underset{pK_{a2} = 14.15}{2H^+ + S^{2-}} \qquad (1.9)$$

H_2S is sparingly soluble in water (about 3.8 g/L; Linke (1958)) and when it is formed at low pH (and therefore mainly undissociated), it is very volatile and is readily released to the atmosphere, with the consumption of protons:

$$2CH_2O + SO_4^{2-} + 2H^+ \longrightarrow H_2S \uparrow + 2H_2CO_3 \qquad (1.10)$$

At circumneutral pH, i.e. when hydrogen sulphide is mainly dissociated into H^+ and HS^-, alkalinity is not generated by sulphate reduction:

$$2CH_2O + SO_4^{2-} \longrightarrow 2HCO_3^- + H_2S \longrightarrow HS^- + H^+ \qquad (1.11)$$

The carbonic acid from (1.10) dissociates into CO_2 and H_2O in acidic solutions, thereby removing another source of acidity, because at low pH (e.g. 3), CO_2 is only sparingly soluble and is lost from solution.

$$CO_2 \uparrow + H_2O \rightleftharpoons \underset{pK_{a1} = 6.4}{H_2CO_3} \rightleftharpoons \underset{pK_{a2} = 10.3}{H^+ + HCO_3^-} \rightleftharpoons 2H^+ + CO_2^{2-} \qquad (1.12)$$

The second dissociation constant of H_2S is above 14 (see (1.9), which means that S^{2-} is unlikely to exist in most aqueous solutions and will immediately deprotonise water to form OH^- and HS^-. Metal ions react with H_2S directly to form metal sulphides, which is, in fact, an acid generating reaction:

$$M^{2+} + H_2S \longrightarrow MS\downarrow + 2H^+ \qquad (1.13)$$

However, in addition, to (I) and (ii) above, the gradual conversion of amorphous iron sulphides (FeS) to pyrite (FeS_2) consumes acid (Castro *et al.*,1999):

$$2FeS + {}^1/_2O_2 + H^+ \longrightarrow FeS_2 + Fe^{2+} + H_2O \qquad (1.14)$$

The metal sulphide precipitate formed in (1.13) is dense; using a bioremediation system such as those outlined below (e.g. Thiopaq®) the sludge volume is around 6 – 10 times lower compared with chemical neutralisation (Boonstra *et al.,* 1999). It is also relatively stable, only becoming remobilised at a comparatively lower pH, and therefore safer and cheaper to dispose of in landfill. In addition, the metals are more easily recovered for re-use by industry, and indeed can be selectively recovered (De Vegt *et al.,* 1997; Boonstra, 1999).

The recognition that SRB play a key role in the neutralisation of acid waters has been documented for some time (Tuttle *et al.,* 1969a; King *et al.,* 1974; Kelly *et al.,* 1982; Schindler and Turner, 1982; Cook *et al.,* 1986; Herlihy *et al.,* 1987), and that the use of such microbially mediated reactions could be used for the abatement of AMD was also proposed some time ago (Tuttle *et al.,* 1969b; Wakao *et al.,* 1979). *In situ* bioremediation of the drainage waters, either by biostimulation or bioaugmentation, is a potential solution. Biostimulation is the injection of growth substrates, co-substrates, and electron acceptors that are limiting the desired bioremediation reaction (Knapp and Faison, 1997). The effectiveness of this approach can be evaluated by the reduced production of the contaminant (e.g. AMD) and utilisation rates of the substrate(s) and electron acceptor. Bioaugmentation is the injection of bacteria to increase the natural subsurface population, with or without addition of growth substrates (Knapp and Faison, 1997). There has been more attention to

the application of biostimulation than bioaugmentation. For example, Castro *et al.* (1999) stimulated populations of sulphate reducing bacteria in a lake occupying a former open-pit mine by the addition of organic waste products, and Kim *et al.* (1999) showed that the generation of acidic, metalliferous leachate from pyritic mine waste piles could be prevented by adding organic compounds to the waste piles to enhance the activity of SRB. This worked directly, by utilising the alkalinity-generating potential of the SRB, and possibly indirectly, by increasing the proportion of anaerobiosis in the waste piles, which is unfavourable for the activity of *Thiobacillus ferrooxidans* and other similar organisms (Kim *et al.,* 1999).

Over the last decade, the process of microbial sulphate reduction has also been used in biological reactors, or bioreactors, through which acidic, metalliferous wastewaters can be pumped and treated. In this situation, SRB are used in an "active" biological treatment process as opposed to their "passive" role in wetland treatment (see section 1.6.2). SRB are inoculated into a suitably designed reactor vessel, where they are usually immobilised onto a solid matrix, and a variety of substrates (simple organic compounds, or in some cases, hydrogen) are introduced, according to cost and availability. The use of SRB in a process for wastewater treatment was studied at the Sittingbourne Research Centre (SRC) of Shell UK from 1987. By 1988/89, a laboratory-scale study had been carried out by the SRC (Scheeren *et al.,* 1993), and subsequently, a pilot plant was installed at the Budelco zinc production plant at Budel-Dorplein in The Netherlands. This site had become highly contaminated by metals and sulphate ions following 100 years of zinc refining (Scheeren *et al.,* 1993). Following the success of the pilot plant, Budelco opted to initiate development of a full-scale treatment plant using SRB in 1990. Paques BV., a Dutch company specialising in anaerobic wastewater treatment, was contracted to undertake the design (Boonstra *et al.,* 1999). The commercial-scale treatment system (Thiopaq®) has been operating at the site since May 1992, treating groundwater at a flow rate of 5000 m^3/day

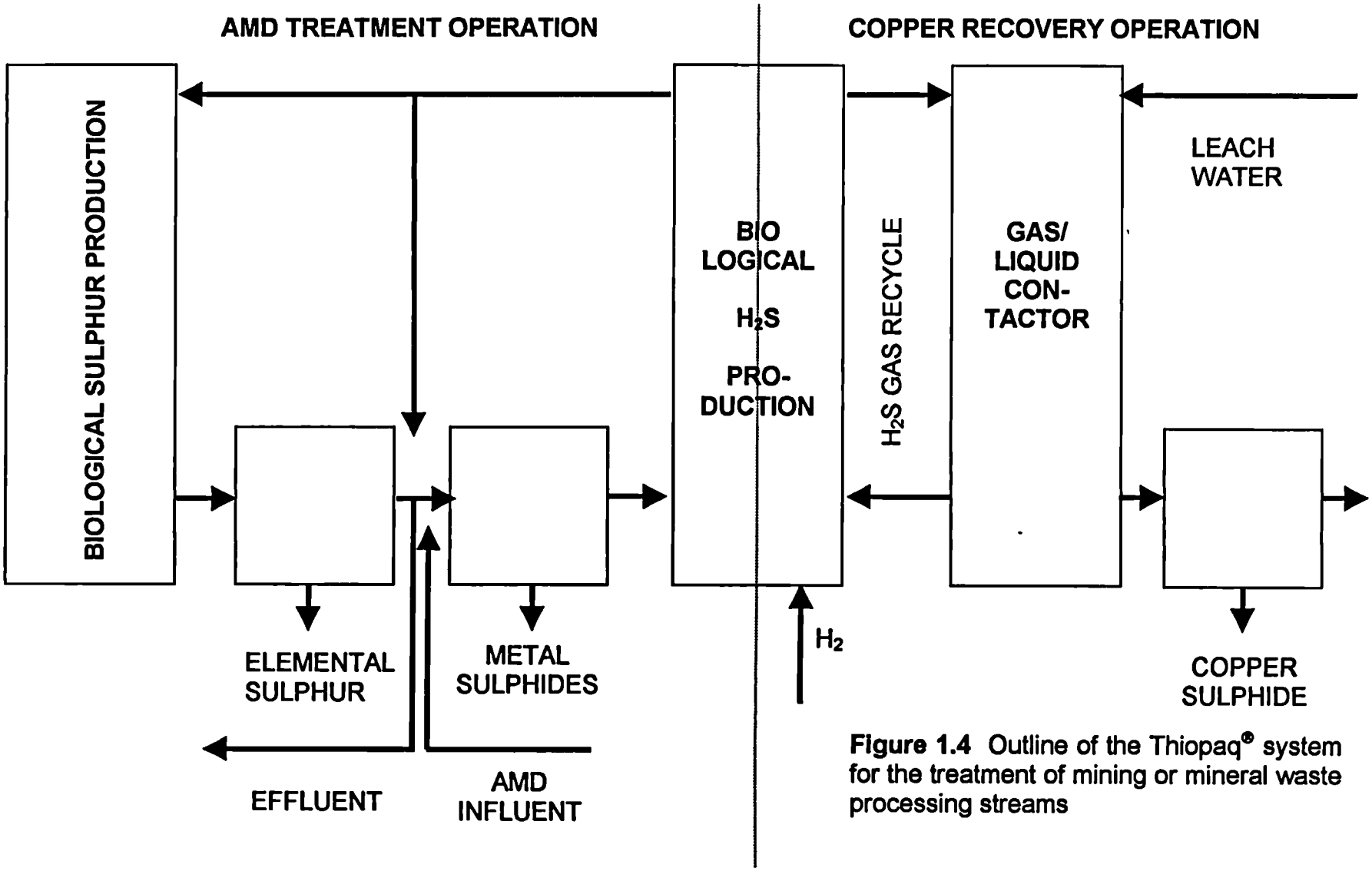


Figure 1.4 Outline of the Thiopaq® system for the treatment of mining or mineral waste processing streams

(De Vegt and Buisman, 1995; De Vegt *et al.,* 1997; Boonstra *et al.,* 1999). This system consists of two main process steps in separate reactors (see Figure 1.4) In the first reactor, an Upflow Anaerobic Sludge Blanket (UASB) reactor (Lettinga *et al.,* 1986), sulphate reducing bacteria convert sulphate in the influent to sulphides, which react with metals which precipitate out in the reactor. In the second bioremediation step, a Submerged Fixed Film (SFF) reactor, sulphide oxidising bacteria convert sulphides to elemental sulphur under aerobic conditions (Buisman *et al.,* 1989 and 1990). If the sulphate load is low, ethanol is used as electron donor and carbon source for the SRB. However, hydrogen gas is more economical to use as electron donor if the sulphate load is high, and carbon dioxide is used as carbon source; the two gases can be produced by cracking methanol (Boonstra *et al.,* 1999). The reactors are in-line (the second reactor directly following and connected with the first), with a tilted plate settler where solids (sulphur, metal sulphides, biomass wash-out) can settle out, and a sand bed filter (DynaSand) which acts as a final polishing step (de Vegt and Buisman, 1995). The metal sulphide sludge that is collected in the process can be sent to a nearby smelter for recovery (Hammack *et al.,* 1994b), and additionally, elemental sulphur can be recovered for other uses. The Paques company alone have actualised over 350 high rate biological treatment plants worldwide, and five plants specially designed for sulphate reduction in particular are currently operated on a continuous basis (Boonstra *et al.,* 1999).

Simple sulphate reduction-based systems for both the generation of H_2S and the precipitation of metal sulphides have generally used a single bioreactor (Hammack *et al.,* 1994b) in an in-line reactor configuration, and this is suitable in situations where all the components of the wastewaters are non-toxic to the bacterial population and where the mixed sludge by-product is acceptable for disposal (Barnes *et al.,* 1991; Dvorak *et al.,* 1992; Scheeren *et al.,* 1992). Such a system was installed to treat metal-contaminated water from a coal mine and a smelting residues dump in

Pennsylvania (Dvorak *et al.,* 1991; Dvorak *et al.,* 1992) using spent mushroom compost as a source of organic carbon (Ledin and Pedersen, 1996). However, mining or mineral processing waste streams (as in the case of Budelco) may contain high concentrations of metals which may be toxic to bacteria, and in addition it may be difficult to recover economically important metals from mixed sludges that also contain biomass and other organic by-products of metabolism. Consequently, designs of some bioreactor systems have been modified to separate the SRB reactor from metal-precipitation reactors in an off-line configuration (see Figure 1.5). H_2S can be sparged from the SRB reactor using an inert carrier gas and transported to the precipitation tank(s) (Hammack *et al.,* 1993). Today, biotechnological processes utilising SRB in a variety of configurations are well known and are in widespread use.

The advantages of using such biological treatment systems are based in their efficiency and potential decreases in environmental impact. The Thiopaq® system has several advantages over lime dosing: effluent metal concentrations that comply with EC directives; a sludge product which is more stable and easier to dispose of; simultaneous removal of sulphate, which is useful in terms of water reuse, and the opportunity for metal recovery (Boonstra *et al.,* 1999). Despite relatively high set-up costs, these advantages are significant, although in this instance, costs of the use of this system compared to lime dosing are more or less comparable (Boonstra *et al.,* 1999). However, operational results of the system when first installed at full scale at Budelco indicated that maintenance and process control requirements were low, and plant start-up after a short shut-down period was easily achievable, with sulphate reduction restarting within 24 hours (de Vegt and Buisman, 1995). In addition, the plant was found to operate without odour emissions. Therefore, it has been shown that performance and cost of these bioreactor systems can be well defined (which is an important advantage over some passive treatment systems, see 1.6.2), and design and bioengineering can be optimised according to

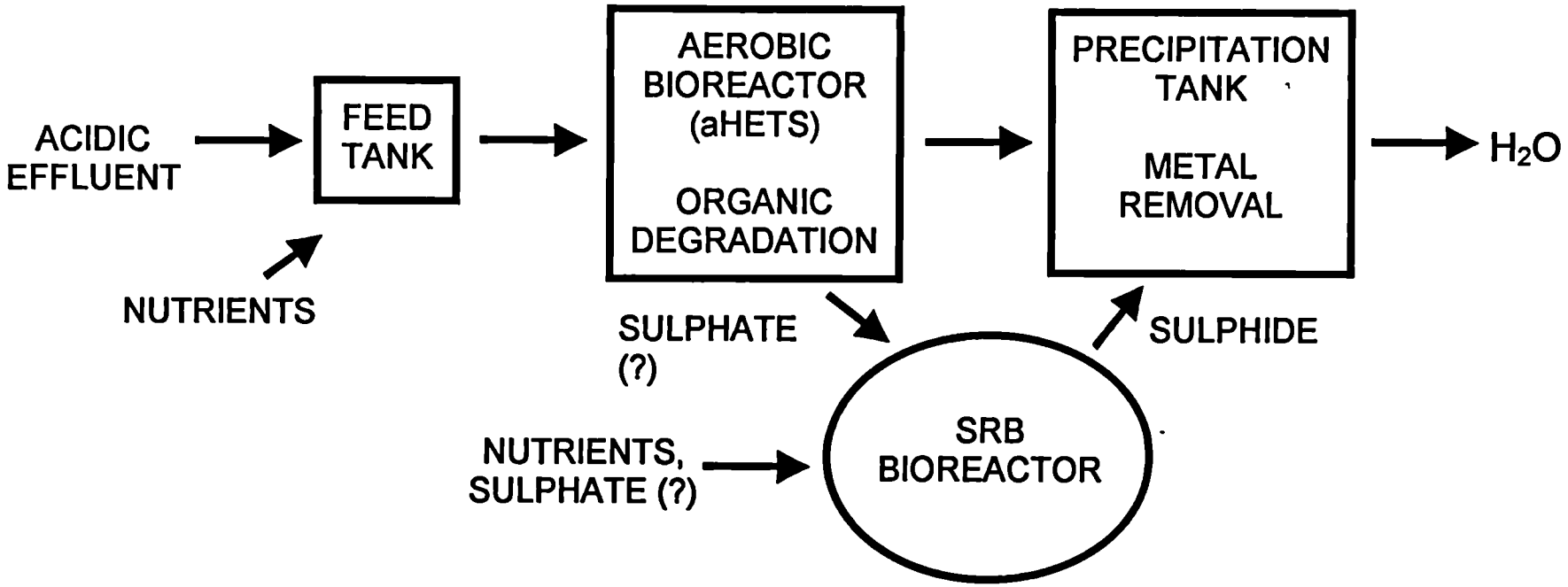


Figure 1.5 An offline configuration for biological treatment systems for acidic wastewaters using acidophilic heterotrophs (aHETS) and sulphate-reducing bacteria. Acidophilic SRB could be used in this process.

the treatment requirements. Because of these obvious benefits, the use of biological treatment technology has significantly increased worldwide.

1.6 Passive Treatment Systems

Passive treatment schemes take advantage of, and maximise, naturally occurring biological and geochemical processes to improve the quality of wastewaters, using simple design and incurring relatively low operation and maintenance costs. In particular, reductive processes such as sulphate- and iron reduction, and possibly ammonification (Kalin *et al.,* 1991) are important for the remediation of AMD.

1.6.1 Passive treatment - chemical

The use of alkaline chemicals for the treatment of AMD is an effective way to increase pH and precipitate metals in wastewaters. One of the alkaline reagents that can be used is limestone. In an oxidising environment, limestone can become coated with reaction products, and it becomes less effective at treating acidic effluents. This problem can be overcome by using anoxic limestone drains (ALDs) or beds (Brodie *et al.,* 1993; Ledin and Pedersen, 1996). This is another method by which alkalinity is generated for treatment of AMD (Gazea *et al.,* 1996). A shallow trench, usually about 1 m wide, filled with high quality (> 90%) limestone is excavated into spoil or buried under several metres of clay and drainage streams diverted through them. They are sealed, sometimes with plastic, to ensure that oxygen inputs are minimised. Anoxic limestone beds of 10 – 20 metres width can be installed in situations where linear ALDs cannot be constructed. The systems incur low costs, but are only suitable for treating waters that are not highly acidic or oxygenated, and with low aluminium and ferric iron concentrations. In many cases, mine waters in drainage adits and pockets contain little dissolved oxygen and ferric iron, and diverting a stream directly from the mine into an anoxic limestone channel

can add up to 400 mg L^{-1} of alkalinity to the water (Gazea *et al.,* 1996). Problems with ALDs occur when cations such as Al and Fe precipitate as hydroxides (e.g. $Fe(OH_3)$ and coat the limestone surfaces (known as armouring), plugging the limestone void space and reducing limestone dissolution and acid neutralisation (Ziemkiewicz *et al.,*1997) However, Ziemkiewicz *et al.* (1994) suggested that open limestone channels could effectively treat AMD if channels were constructed on steep slopes (> 20%) to reduce plugging of limestone pores by metal hydroxides, and by increasing the dimensions of the channels to account for the armouring effect. This could be a more inexpensive and low maintenance form of lime treatment; the author suggests that this system would be useful in land reclamation projects and where water quality standards are not specified (Ziemliewicz *et al.,* 1997).

The combination of using ALDs, i.e. a form of traditional, chemical treatment, with a passive biological sulphate-reduction system has also been described (Kepler and McCleary, 1994). These have been termed successive alkalinity-production systems (SAPS).

1.6.2 Passive treatment – biological

Tuttle *et al.* (1969a) observed that the pH of an acid stream increased, sulphate concentrations decreased, iron sulphide was precipitated and numbers of SRB and other heterotrophic bacteria increased downstream of a wood dust pile at a saw mill, which acted as a porous dam. Using samples taken from downstream of the dam, mixed cultures containing SRB were grown in the laboratory using wood dust as the only nutrient. Sulphate reduction occurred in these cultures at pH 3 (Tuttle *et al.,* 1969a). Following these observations, it was proposed that microbial sulphate reduction could be potentially used as an acid mine water abatement procedure (Tuttle *et al.,* 1969b). It was thought by these authors that saw dust or sewage could provide nutrients and a sufficiently low redox

potential for the natural establishment of SRB, which could thereby dispose of sulphur-containing waste materials from abandoned coal mines (Tuttle *et al.*,1969b). The 'treatment' by the dam at the saw mill is an example of 'passive' biological treatment, and natural and constructed wetlands used for treating AMD today work on similar principles. Organic material is naturally available (or provided) for heterotrophic microorganisms such as SRB and iron reducing bacteria which mediate the neutralisation of acidity and the precipitation of metals.

1.6.2.1 Wetlands

In the early 1980's, observations were made that natural bogs dominated by *Sphagnum* moss were capable of ameliorating acid mine drainage (Huntsman *et al.*,1978; Wieder and Lang, 1982). These observations led to the theory that constructed wetlands could be used for treating acid mine waters. These were the first developments in AMD remediation using passive treatment systems, and in the mid- to late 1980's, this type of treatment system was instituted.

Natural or constructed wetlands are known to be one method of treating mine drainage waters, and since they may provide a low-cost, low maintenance solution, their use for pollution abatement has received considerable attention (Wildeman and Cevaal, 1994; Kalin *et al.*, 1991; Dunbabin and Bowmer, 1992; Vile and Wieder, 1992; Wieder, 1993; Gazea *et al.*, 1996; Ledin and Pedersen, 1996; Reddy and D'Angelo, 1997; Webb *et al.*, 1998, and others). Between 1984 and 1990, over 300 artificial wetlands were constructed in the Eastern United States alone, specifically for the treatment of AMD (Webb *et al.*, 1998). In the UK, wetland treatment systems have not been used as extensively but are considered a treatment option. A pilot plant system is currently in place at the site of the Wheal Jane tin mine in Cornwall (Lamb *et al*, 1998, Gazea *et al.*, 1996; see 1.1). This system consists of a series of shallow lined

cells comprising of pre-treatment with aerobic and anaerobic 'cells' (Gazea *et al.,* 1996, see Figure 1.6). In this situation, wetland systems were chosen as being a cost-effective treatment method (Lamb *et al.,* 1998).

A wetland system, once designed for such a purpose, should be self-generating and be able to buffer fluctuations in water quality, temperature, and flow (Ledin and Pedersen, 1996). Wetland treatment systems can be categorised into three major types: (i) free water surface systems, where water flows slowly over the surface of the soil and through vegetation in shallow basins; (ii) subsurface flow systems, in which the water flows through the soil, and (iii) aquatic plant systems, which have deeper ponds and floating and/or submerged vegetation (Ledin and Pedersen, 1996). Aerobic wetland systems have been effectively employed for the treatment of net alkaline waters (Gazea *et al.,* 1996), and are essentially similar to "natural" wetlands. Anaerobic wetlands consist of basins or channels, with an impermeable bottom to prevent seepage, a medium to support vegetation (such as soil), and shallow water (10-50 cm) flowing over the surface (Gazea *et al.,* 1996). Aerobic processes, such as iron oxidation and the subsequent precipitation of iron oxy-hydroxides are dominant in surface waters of a wetland. In the sub-surface and sediments of a wetland, microaerobic and anaerobic processes (e.g. iron- and sulphate reduction) are usually dominant, and these are seen as being an integral part of the treatment of AMD, in that they generate alkalinity. For example, anaerobic organic substrate systems, or compost wetlands, are one of the systems currently available for the treatment of net acidic mine waters (Gazea *et al.,* 1996). Materials used as substrates in these systems include cheap natural products (such as wood chips, sawdust, peat) and waste products (such as spent mushroom compost or horse/cow manure). Compost substrates with low $CaCO_3$ may sometimes be supplemented with limestone; in this situation, its dissolution promotes additional alkalinity generation. Anaerobic bacterial activity is promoted, resulting in sulphate reduction and the precipitation of metal sulphides and alkalinity

Figure 1.6 A wetland system for mine drainage treatment at the Wheal Jane tin mine site in Cornwall, U.K.

generation (Gazea *et al.,* 1996). The relative importance of aerobic and anaerobic processes in the effective treatment of AMD is not clear (Ledin and Pedersen, 1996), and it varies according to location, geochemistry of the site, flow rates, and the quality of the wastewater to be treated. However, some approaches aim at directing microbial processes according to treatment requirements. For example, attempts have been made to construct wetlands that emphasise sulphate reduction (McIntyre *et al.,* 1990; Tyrell *et al.,* 1997) to mediate pH remediation and metal precipitation. In these studies, metal contaminated water was introduced into cells containing organic substrates. Such attempts, although not completely reliable, demonstrated that the SRB could effectively decrease the concentrations of many of the metals in AMD (Webb *et al.,* 1998) and an increase in pH compared to controls (Tyrell *et al.,* 1997).

As in any treatment system, there are a number of advantages and disadvantages to the use of wetlands for treating AMD. A major advantage is that wetlands can be an inexpensive, "low-technology" option, and long-term costs can be low compared to chemical treatment (Ledin and Pedersen, 1996). If installed correctly, they should operate with low maintenance and low cost over a long period. However, wetlands are land-extensive and space may be limiting, or the location of the site to be treated can mean that construction is impossible or difficult. Ledin and Pedersen (1996) suggest that a disadvantage of using wetlands is that they can be considered to be a "black box", where the individual processes acting on the metals and acid water cannot be specified. This could mean that if the treatment efficiency of a wetland decreases, it would be difficult to establish where action may be taken to improve performance, e.g. by addition of substrates or modifying conditions to encourage proliferation of a particular microbial population. Ledin and Pedersen (1996) suggest that continuous removal and/or addition of organic material may be needed, thereby increasing costs. Furthermore, McGinness *et al.* (1996) suggested that understanding of how wetlands work is not sufficient for the successful

treatment systems for very efficient, cost-effective treatment (Wildeman and Cevaal, 1994). Davison (1993) effectively combined an *in situ* bioremediation system with multi-pond wetlands to treat drainage from coal mines in Pennsylvania, with less treatment space required than with either system alone, and the cost of installation and operation over 2.5 years was comparable to the cost of alkali addition alone (Davison, 1993).

1.6.2.2 Reactive Barriers

Reactive barriers are another recently-developed passive biological treatment system to treat and/or prevent the dispersal of AMD from groundwater on discharge to surface waters. A permeable reactive wall, consisting of a support material mixed with an inexpensive organic material acts as a filter within affected aquifers, where metal precipitation and pH remediation is effected by colonised SRB. Benner *et al.* (1997) described a reactive wall that was installed in an aquifer at the Nickel Rim mine site in Ontario, Canada, in August 1995, and its performance since that time has been monitored (Benner *et al.*, 1997, 2000). It is composed of municipal compost mixed with pea gravel as a support, and was installed to a depth of 3.6 m into the groundwater flow. After nine months of operation, results showed that sulphate reduction and metal precipitation had been occurring (Benner *et al.*, 1997). Sulphate concentrations decreased by up to ten-fold downstream of the wall, pH and alkalinity increased, and iron and nickel concentrations decreased dramatically. However, its performance is primarily limited by organic carbon reactivity, changing groundwater temperature, and variations in residence time within the barrier (Benner *et al.*, 2000). Rates of sulphate and iron removal, whilst still significant, are declining gradually, with peaks in the rate of sulphate removal in autumn. Variations in flow rates through the barrier result in variable residence times, with sulphate and iron removal being higher in slower flow paths (Benner *et al.*, 2000). Despite these limitations, this study has demonstrated that groundwater impacted

by acid mine drainage can be treated using reactive barriers for an extended period of time.

Canty (2000) described in situ treatment of AMD over a four year period in the Lilly/Orphan Boy Mine in Montana, USA, using a similar type of reactive barrier. The flooded subsurface workings of the mine were used as an "*in situ* biological reactor". An organic substrate (animal by-products) was placed in a portal through a shaft approximately 10 metres below the static water level, supported by two platforms. In addition, two injection wells were drilled into the main tunnel of the mine (which is connected to the portal) where more substrate was placed. The AMD flows upward through the substrate and out through the portal horizontally, through the substrate placed there. SRB colonising the substrate barriers effected AMD remediation. The shaft water pH rose and E_h fell, decreasing acid generation within the mine. The pH of the tunnel waters before treatment was around 3; after installation of the reactive barriers, the pH rose to about 7. During normal flow rates the pH in the portal rose to 6. However, the pH in the portal fell to 3.5 during each spring runoff. Sulphate reduction was evident by measured decreases of sulphate and the detection of soluble sulphide in the mine water. High removal efficiencies were observed for Al, Cd, Cu and Zn (70-100%) during the first four years of monitoring (Canty, 2000).

Reactive barriers are a useful method of treating AMD, although their efficiency is limited by a number of factors, including seasonal increases in flow. High flow rates may result in recontamination of water beyond the barriers, and a large flow of oxygenated surface water may cause the dissolution of previously formed metal precipitates and the inactivation of anaerobic SRB.

1.7 Sulphate Reducing Bacteria (SRB)

Sulphate reducing bacteria were described in a classic monograph by Postgate in 1979. At that time, these microorganisms were of minor interest to a small group of specialists, but since then they have become organisms of choice for the study of many key problems in the areas of taxonomy, phylogeny, evolutionary relationships, cell physiology, microbial ecology, wastewater treatment and bioremediation. Therefore they have been unusual in that increased understanding of the group over the last two decades has occurred equally from the results of pure and applied research (Hamilton, 1998). The central importance of the SRB in a major economic and environmental problem, microbially influenced corrosion (MIC) and hydrocarbon reservoir souring, has also prompted a substantial increase in research (Hamilton, 1998; Hao, *et al.*, 1996). In the environment, SRB are not only important to the sulphur cycle, but are central to critical processes in ecosystem functioning. They are important regulators of a variety of processes in wetland soils and aquatic systems, including organic matter turnover and degradation of aromatic compounds in anaerobic sediments (Fauque, 1995; Barton and Tomei, 1995; Castro *et al.*, 2000; Häggblom *et al.*, 2000). Because of the chemical reactions that they mediate, SRB are now employed in a considerable number of biotechnology applications, particularly in the field of bioremediation. Sulphate reducing bacteria are a very diverse, complex physiological bacterial group, and notable advances have recently been made in the understanding of their taxonomy and phylogeny through the development of ribosomal RNA phylogenetic analysis (Castro *et al.*, 2000).

1.7.1 General characteristics

Sulphate reducing bacteria were first described by Beijerinck in 1895. They are an extremely heterogeneous and phylogenetically diverse group of prokaryotes, which share a common metabolic ability to utilise sulphate

as an electron acceptor for the generation of ATP from the oxidative metabolism of organic substrates or molecular hydrogen, H_2 (Widdel, 1988; Devereux and Stahl, 1993). They are widespread in aquatic and terrestrial environments that become anoxic as a result of microbial decomposition processes (Brock, 1997) inhabiting a wide range of ecological niches including oceans, lakes, streams, thermal springs, sediments, soils, oil-bearing porous rock, hydrothermal vents, as well as rumens of animals and human intestines. SRB are also found in sewage works and pipelines; in the latter their presence can cause corrosion in metal structures, which is of major economic importance to the offshore oil and gas industries (Hamilton, 1998; see 1.7.6). They are classed as obligate anaerobes, but some species also have the capacity to tolerate oxic conditions (Dilling and Cypionka, 1990; Marschall *et al.,* 1993). Up to about 15 years ago, there were thought to be only two genera of SRB, but by five years ago, 15 had been well characterised (Fauque, 1995). The most well-known genus is *Desulfovibrio*, which is common in aquatic habitats or waterlogged soils containing sufficient amounts of organic material and sulphate. Species of SRB vary greatly in their morphological characteristics; many forms such as vibrios, rods, spirilloid, cocci and filaments are found among Gram negative SRB; Gram positive spore-formers are also common. Table 1.1 outlines important characteristics of representative genera of SRB.

1.7.2 Taxonomy and phylogeny

The dissimilatory sulphate reducing bacteria comprise seventeen eubacterial genera: D. (*Desulfovibrio*); Da. (*Desulfoarculus*); Dba. (*Desulfobacter*); Dbt. (*Desulfobacterium*); Dbo. (*Desulfobotulus*); Dbu. (*Desulfobulbus*); Dc. (*Desulfococcus*); Dh. (*Desulfohalobium*); Dsm (*Desulfomicrobium*); Dm. (*Desulfomonile*); Dn. (*Desulfonema*); Ds. (*Desulfosarcina*); Dtm. (*Desulfotomaculum*); T. (*Thermodesulfobacterium*); one archaebacterial genus, A. (*Archaeoglobus*), (Fauque *et al.*, 1991;

Widdel, 1992a,b; Widdel and Bak, 1992; and reviewed by Fauque, 1995; Castro *et al.*, 2000); *Desulfocapsa* (Janssen *et al.*, 1996), and more recently, *Desulfosporosinus*, which was recently redescribed as a new genus, previously named *Desulfotomaculum orientis*; (Stackebrandt *et al.*, 1997). Sulphate reducing bacteria are a complex and diverse group. Various properties have been used in their classification, the most important being cell shape and motility, optimal temperature, complete versus incomplete oxidation of acetate (Castro *et al.*, 2000; see 1.7.3, 1.7.4.3 and Tables 1.1 and 1.2), GC content of DNA, and the presence of cytochromes, enzymes and pigments (Postgate, 1984; Widdel, 1988). The testing of growth on different electron donors allows for further classification. Sulphate reduction can be performed by sulphate reducing bacteria and archaea (Widdel and Hansen, 1992). These microorganisms may be divided into four groups based on ribosomal RNA sequence analysis: (I) Gram-negative mesophilic SRB; (ii) Gram-positive spore forming SRB; (iii) thermophilic bacterial SRB, and (iv) thermophilic archaea (Castro *et al.*, 2000; see Table 1.1), and novel groups of sulphate reducers are continually being isolated, e.g. the halophile *Desulfocella halophila* (Brandt *et al.*, 1999), the acetate-degrader *Desulfobacca acetoxidans* (Oude Elferink *et al.*, 1999), and a number of psychrophilic strains including *Desulfofrigus oceanense* (Knoblauch *et al.*, 1999). Assignment of species into groups using rRNA analysis is advantageous in that evolutionary origins of sulphate reduction in distantly related species can be examined, and applications such as ecological studies can be facilitated by the development of probes. By 16S rRNA sequence analysis, the Gram-negative sulphate reducers align most closely with other Gram-negative bacteria in the delta subdivision of the purple (or *Proteobacteria*) bacterial group. However, the variety found within organisms labelled *Desulfovibrio* is so great that Devereux *et al.* (1990) proposed a new taxonomic family, *Desulfovibrionaceae,* to account for the phylogenetic depth and cohesiveness of the organisms (Singleton, 1993). The Gram-positive spore-formers, however, group with the *Clostridium* subdivision of

the Gram-positive bacteria. Members of the genus *Desulfotomaculum* may represent a transition from "strictly fermenting" organisms such as the clostridia (Singleton, 1993). Figure 1.7 outlines the relationships between representative sulphate reducing bacteria in a phylogenetic tree. (The numbers correspond to phylogenetic groupings, and the scale bar is in units of fixed nucleotide substitutions per 16S rRNA nucleotide position.) The non-spore forming Gram negative organisms can be assigned to seven natural groups or lines of descent (Devereux *et al.*, 1989, 1990), and the two Gram-positive organisms *Desulfotomaculum ruminis* and *Desulfosporosinus orientis,* whilst grouped together, share only 83% sequence similarity, and the inferred relationship is one of independent lineages within the Gram-positive bacteria and not with a common origin. This indicates that there is considerable diversity within the Gram positive SRB. Indeed, Stackebrandt *et al.* (1997) published a detailed phylogenetic analysis of the genus *Desulfotomaculum* based on 16S rDNA data and showed that *Desulfotomaculum guttoideum* and *Desulfotomaculum orientis* (as previously named) are phylogenetically separated from the majority of the rest of the species in the genus. These authors demonstrated that the former shares high 16S rDNA similarity with some *Clostridia* spp., and has probably been misidentified, and therefore the latter represents a new species, for which the name *Desulfosporosinus orientis* gen. nov., comb. nov. was proposed (Stackebrandt *et al.*, 1997). *D. orientis* gen. nov. comb. nov. branches most closely to the genus *Desulfitobacterium* (Stackebrandt *et al.*, 1997), an organism which reduces sulphite, thiosulphate and sulphur, but not sulphate, to sulphide (Utkin *et al.*, 1994). Advances in molecular identification techniques have been crucial to the understanding of the sulphate reducers, as it can be seen that they can be morphologically and physiologically similar yet only distantly related, or in some instances, appear to be very different whilst being closely related. Morphology has been found to be an unreliable trait for establishing relationships among the sulphate reducing bacteria (Fox *et*

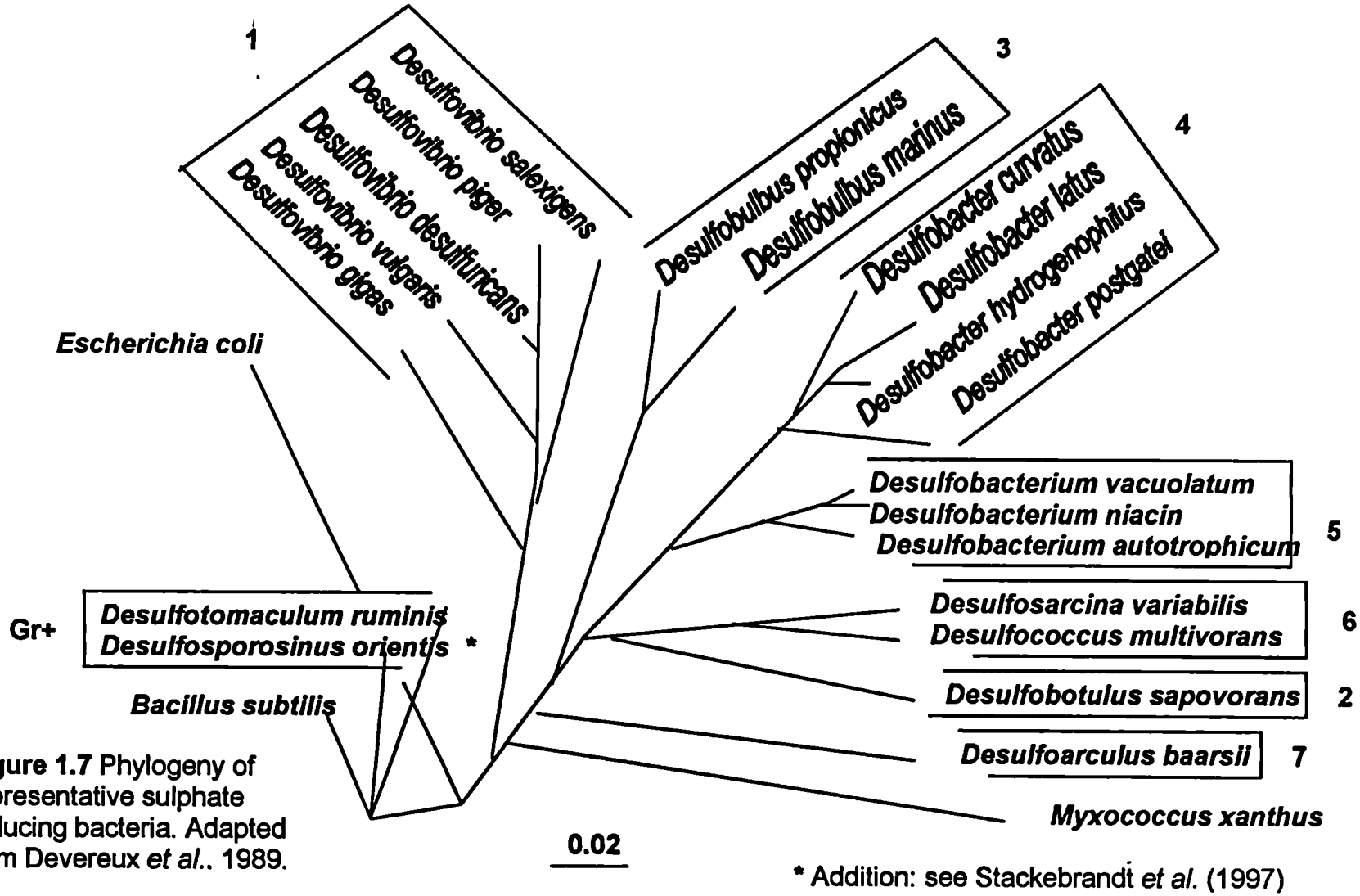


Figure 1.7 Phylogeny of representative sulphate reducing bacteria. Adapted from Devereux *et al.*, 1989.

* Addition: see Stackebrandt *et al.* (1997)

Genus of sulphate-reducer	Shape	Motile/ non-motile	GC content (%)	Acetate oxidation (I/C†)	Growth temp (°C)
Gram-negative mesophilic SRB					
Desulfobulbus	lemon/rod	-/+	59-60	I	25-40
Desulfomicrobium	ovoid/rod	+/-	52-67	I	25-40
Desulfomonas	rod	-	66	I	30-40
Desulfovibrio	spiral/vibrioid	+	49-66	I	25-40
Desulfobacter	oval/rod	+/-	44-46	C	20-33
Desulfobacterium	oval/rod	+/-	41-52	C	20-35
Desulfococcus	sphere/rod	-/+	46-57	C	28-35
Desulfomonile	rod	-	49	C	37
Desulfonema	filaments	gliding	35-42	C	28-32
Desulfosarcina	oval rods/ coccoid/ packages	+/-	51	C	33
Gram-positive spore-forming SRB					
Desulfotomaculum, *Desulfosporosinus**	straight to curved rod	+	48-52	I/C	most 25-40 some 40-65
Thermophilic SRB					
Thermodesulfo-bacterium	vibrioid to rod	-/+	30-38	I	65-70
Thermophilic archaea					
Archaeoglobus	coccoid	+/-	41-46	I	64-92

Table 1.1. Representative genera of sulphate-reducing bacteria and archaea and some important characteristics. Where motility is shown as +/-, they are usually motile; if -/+, sometimes motile. † I = incomplete, C = complete oxidation of acetate. Table adapted from Castro *et al.* (2000), with addition from Stackebrandt *et al.* (1997).

al., 1980; Fowler *et al.*, 1986; Devereux *et al.*, 1989), and their nutritional versatility has led to further problems in classification.

1.7.2.1 Sulphate reducing archaea

Documented archaeal sulphate reducers belong to the genus *Archaeoglobus*, of which two species have been described: *A. fulgidus* and *A. profundus*. Both were isolated from marine hydrothermal systems (Stetter *et al.*, 1987). These archaea exhibit optimal growth temperatures of 83 and 82°C and pH optima of 6.5 and 10 respectively, and both require at least 1% NaCl for growth. It was previously thought that microorganisms that could use sulphate as an external electron acceptor for anaerobic respiration were restricted to the domain Bacteria, but the isolates were found to be able to grow on molecular hydrogen or formate with sulphate as sole energy sources, indicating the coupling of sulphate reduction and energy conservation (Thauer *et al.*, 1977; Thauer and Kunow, 1995). The domain Archaea comprises three kingdoms: the Crenarchaeota, the Korarchaeota and the Euryarchaeota. 16S- and 23S rRNA sequencing shows that the *Archaeoglobus* lineage groups with the *Methanomicrobiales* and extreme halophiles in the kingdom of Euryarchaeota (Woese *et al.*, 1991). In addition to sulphate, *Archaeoglobus* spp. can use sulphite and thiosulphate as terminal electron acceptors. *A. fulgidus* produces small amounts of methane and possesses the required cofactors, whereas *A. profundus* does not produce methane or appear to possess the cofactors. *A. fulgidus* strain VC-16 uses some of the same enzymes of the sulphate reduction pathway found in bacterial sulphate reducers; purified archaeal APS reductase possessed similar structural properties to bacterial reductase (Speich and Trüper, 1988). Observations such as these raise the question of whether sulphate reduction occurred once during early evolution or independently in distantly related groups via lateral gene transfer (Devereux and Stahl, 1993).

1.7.3 Growth characteristics and nutrition

For many years it was thought that there were only a few species of SRB and that these were only able to use lactate or pyruvate to support growth (Barton and Tomei, 1995). However, it is now clear that SRB are capable of using a wide range of compounds for electron donors. The number of substrates which can be oxidised by the various available pure cultures of sulphate reducers amount to over 125 (Fauque *et al.,* 1991; Hansen, 1993), including hydrogen, lactate, formate, benzoate, acetate, malate and ethanol (Stackebrandt *et al.,* 1995). Most of the substrates used are fermentation products and intermediate breakdown products such as some amino acids, glycerol, and fatty acids; direct utilisation of biopolymers is very rare (Hansen, 1994). Sulphate-reducers can be placed into two physiological sub-groups according to whether they are able to carry out a terminal oxidation of their organic substrates, including acetate (Table 1.2). The genera in Group I, including *Desulfovibrio* and *Desulfotomaculum*, can utilise lactate, pyruvate, ethanol, or certain fatty acids as carbon and energy sources, reducing sulphate to hydrogen sulphide (Brock, 1997), but are unable to use acetate. The genera in Group II oxidise fatty acids, particularly acetate, reducing sulphate to sulphide. Most SRB are chemoorganotrophs, but some sulphate reducers can grow chemolithoheterotrophically using molecular hydrogen as an electron donor (e.g. *Desulfovibrio* spp., strains of the genus *Desulfobulbus* and *Desulfosporosinus* (previously *Desulfotomaculum*) *orientis*). In these organisms, a C_2 compound such as acetate is required in addition to CO_2 for cell synthesis (Widdel, 1988). However, Klemps *et al.* (1985) demonstrated autotrophic growth of *Desulfosporosinus* (*Desulfotomaculum*) *orientis* with hydrogen, carbon dioxide and sulphate, and experiments with radioactively labelled carbon dioxide revealed that 91- 99% of cell carbon was derived from carbon dioxide. Other SRB, such as *Desulfosarcina variabilis*, *Desulfonema limicola*, and *Desulfococcus*

niacini can grow autotrophically under these conditions, using CO_2 as the sole source of carbon, although growth may be rather slow.

Group I	*Desulfovibrio, Desulfomicrobium, Desulfotomaculum, Desulfomonile, Archaeoglobus, Desulfobulbus, Thermodesulfobacterium*
Group II	*Desulfobacter, Desulfobacterium, Desulfococcus, Desulfonema, Desulfosarcina,*

Table 1.2. Characterisation of representative sulphate-reducing bacteria/archaea, according to their ability to oxidise acetate. Group I do not oxidise organic compounds, including acetate, to CO_2 whereas Group II organisms can.

In the absence of sulphate, certain strains of SRB can use a single carbon compound as both an electron donor and electron acceptor by a process known as dismutation, for example some *Desulfovibrio* strains can grow with the dismutation of pyruvate, choline, malate, glycerol, and dihydroxyacetone (Barton and Tomei, 1995). Some sulphate reducers can grow through a unique fermentation of inorganic sulphur compounds: disproportionation of sulphite, thiosulphate, or sulphur, in which the sulphur oxyanions serve as both electron donor and acceptor (Bak and Cypionka, 1987; Cypionka, 1995). For example, thiosulphate is disproportionated to equal amounts of sulphate and sulphide:

$$S_2O_3^{2-} + H_2O \longrightarrow SO_4^{2-} + 0.5\,H_2S + 0.5\,HS^- + 0.5\,H^+ \qquad (1.15)$$
$$\Delta G^{o'} = -25 \text{ kJ/mol}$$

Sulphite can be disproportionated to three-quarters sulphate and one-quarter sulphide:

$$2HSO_3^- + 2SO_3^{2-} \longrightarrow 3SO_4^{2-} + 0.5\,H_2S + 0.5\,HS^- + 0.5\,H^+ \quad (1.16)$$
$$\Delta G^{0'} = -235\ kJ/mol$$

The free energy change of thiosulphate disproportionation is low and cannot always be used for growth. Sulphite disproportionation is less widely exhibited, but could be utilised for growth. The disproportionation of elemental sulphur is also possible if the sulphide formed is removed using iron or manganese (Thamdrup *et al.*, 1993). The disproportionation of sulphur at pH 7 is energetically unfavourable but by the scavenge of H^+ at higher pH, the reaction becomes exergonic.

Fermentation of organic substrates is possible in many SRB, in the absence of sulphate or other inorganic electron acceptors (Widdel, 1988). In the simplest case, many SRB belonging to the genera *Desulfovibrio*, *Desulfobulbus*, *Desulfococcus*, *Desulfobacterium* and *Desulfotomaculum* can ferment pyruvate (Widdel, 1988) to acetate, CO_2, and H_2. Energy conservation is achieved by substrate level phosphorylation only (Cypionka, 1995). Two other compounds that are fermented in the absence of sulphate are choline and cysteine (Fauque *et al.*, 1991). Some sulphate reducers such as *Desulfobulbus* can grow by propionate fermentation. Homoacetate fermentation is another important feature of some SRB. *Desulfosporosinus* (previously *Desulfotomaculum*) *orientis* can carry out this type of fermentation. Klemps *et al.* (1985) showed that this SRB was able to grow slowly in sulphate-free medium with formate, methanol, ethanol, lactate, pyruvate, or trimethoxybenzoate. Acetate was excreted, indicating the function of carbon dioxide as electron acceptor in a homoacetogenic process (Klemps *et al.*, 1985). Homoacetate fermentation can be regarded as carbonate respiration, and must be coupled to chemiosmotic energy conservation if electron donors that do not allow substrate level phosphorylation are utilised (Cypionka, 1995).

Some strains of *Desulfovibrio* and *Desulfobulbus* can use nitrate as electron acceptor, forming ammonia as an end product (Cypionka, 1995).

Some are able to fix dinitrogen and use nitrogen from amino acids as an energy source (Barton and Tomei, 1995). A number of SRB are able to reduce elemental sulphur in the absence of other potential electron acceptors such as sulphate, sulphite or nitrate. For example, Widdel and Hansen (1992) cite examples of *Desulfovibrio* and *Desulfomicrobium* spp. that can reduce sulphur, as well as reducing thiosulphate, sulphite, and sulphate. However, elemental sulphur in the presence of some sulphate reducing bacteria may inhibit growth (Fauque *et al.*, 1991). The true sulphur reducing bacteria, such as *Desulforomonas* and *Desulfurella*, meanwhile, cannot reduce sulphate (Widdel, 1988).

Molecular oxygen can be reduced by some SRB (Dilling and Cypionka, 1990; Marschall *et al.*, 1993). Respiration rates comparable to those of aerobic organisms were observed with *Desulfovibrio* species (Cypionka, 1995). However, usually, the cells do not actually grow with O_2 as electron acceptor, and Marschall *et al.* (1993) observed that not more than one doubling occurred in homogenously aerated systems. Various oxygen-reducing systems are present in *Desulfovibrio* species; the respiration capacity appears to have a protective function only (Cypionka, 2000). For example, the non-spore-forming *Desulfovibrio* spp. survive oxic conditions because they possess an oxygen-scavenging system of superoxide dismutase and catalase; Silva *et al.* (1999) found a protein in *Desulfovibrio gigas* with significant superoxide dismutase activity.

It is clear that SRB as a group exhibit broad metabolic capabilities, and interact with and transform many chemicals other than the reduction of sulphate in their environment. In the absence of sulphate and/or preferred electron donors, SRB demonstrate flexibility in the compounds, metabolic pathways and energy conservation mechanisms they can use. Figure 1.8 illustrates some of the oxidation/reduction reactions that can be carried out by various SRB.

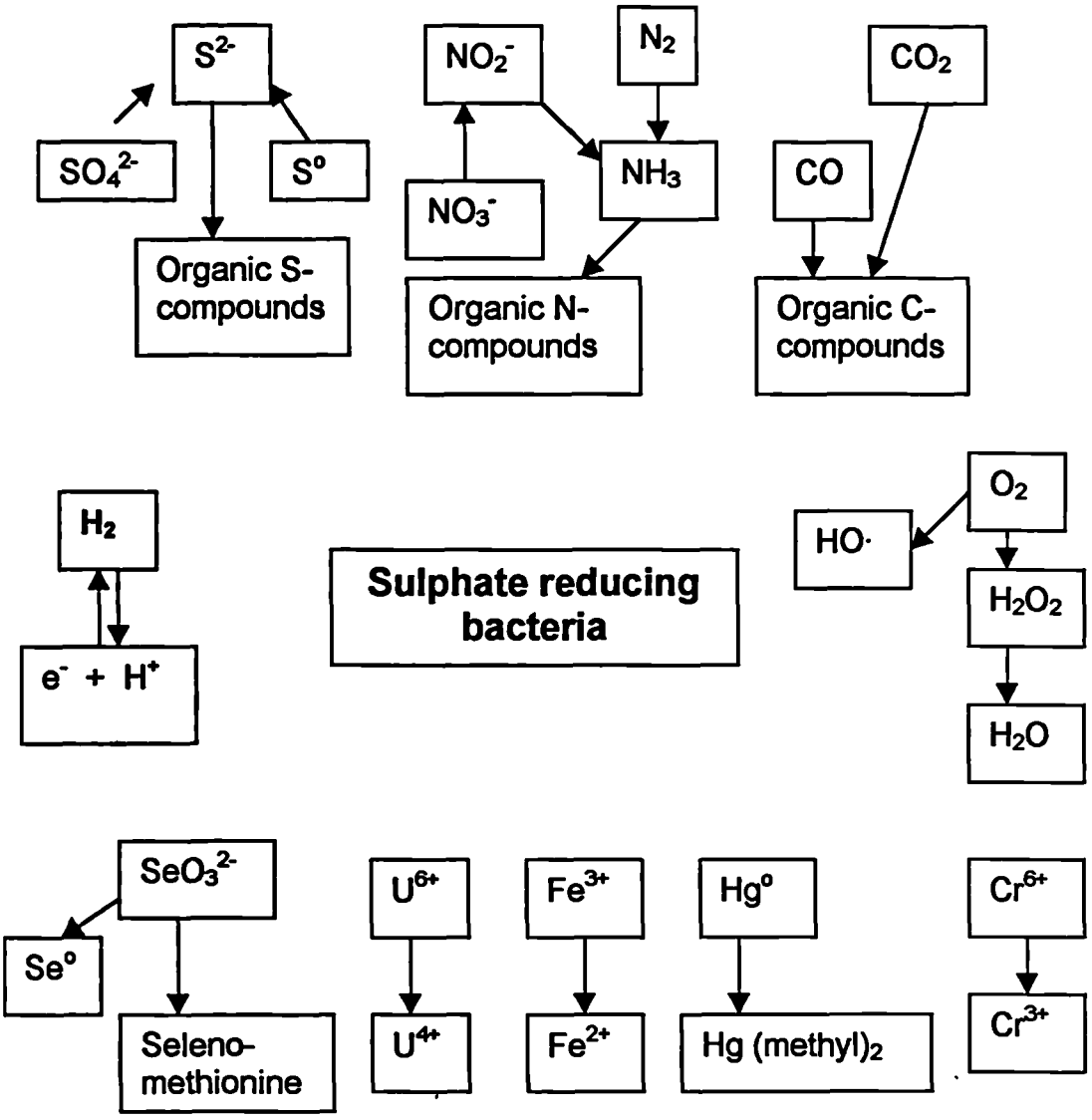


Figure 1.8 Chemical transformations attributed to sulfate reducing bacteria. From Barton and Tomei (1995).

1.7.3.1. Growth requirements

Postgate (1979, 1984) gave details of media for the cultivation of SRB. Medium 'B', 'C', 'D', 'E' and 'F' were all tailored for different growth requirements. Medium 'C' is for general culture of organisms (Postgate, 1984), and consists of the following, in g L^{-1} of distilled water: KH_2PO_4, 0.5; NH_4Cl; 1; Na_2SO_4, 4.5; $CaCl_2$. $6H_2O$, 0.06; $MgSO_4.7H_2O$, 0.06; sodium lactate, 6; yeast extract, 1; $FeSO_4.7H_2O$, 0.004; and sodium citrate dihydrate (or another appropriate electron donor), 0.3. The citrate prevents precipitation of the iron salt, and the medium is adjusted to pH 7.5. The redox potential of the medium must be –100 mV or below (Connell and Patrick, 1968; Postgate, 1984), the reducing conditions being obtained by the addition of a reductant such as sodium sulphide, sodium thioglycolate plus sodium ascorbate, cysteine (Postgate, 1984; Widdel and Bak, 1992; Barton and Tomei, 1995) or titanium (III) citrate (Zehnder and Wuhrmann, 1976). Large inocula in subculture usually produce the reducing conditions required for growth as they contain H_2S. Media used to grow cultures of SRB have been based on similar formulations to Postgate's media, with additions of NaCl or the use of seawater for the growth of marine strains. Addition of agar to media in tubes or petri plates allows for growth on solid media. Cultures should be incubated at the appropriate temperature, according to temperature optima of known strains.

Hydrogen can be provided as an electron donor by the use of a cylinder of compressed gas with a reducing valve to maintain low pressure (Willis, 1969), although this is usually unnecessary as some anaerobic incubation systems produce hydrogen gas, based on the principle by Laidlaw (1915), where platinum or palladium is used to catalyse the combination of oxygen and hydrogen to form water. Oxygen must be excluded by using nitrogen gas over liquid media, by filling tubes or bottles to exclude air, or by using anaerobic systems for incubation. Willis (1969) outlined a number of methods for excluding oxygen for the growth of anaerobic bacteria, such

as the use of palladium catalysts, alkaline solutions of pyrogallol, chromium and sulphuric acid, and the use of aerobic organisms. However, more simple and convenient systems for the generation of anaerobiosis are available today.

All described sulphate reducing bacteria grow better under slightly alkaline conditions over a relatively restricted pH range (7 – 7.8), but tolerate pH values of between 5.5 and 9 (Zobell, 1958; Pfennig, 1981). The pure cultures of SRB isolated from the mixed cultures taken from acid mine waters by Tuttle *et al.* (1969a) did not grow below pH 5.5, and Connell and Patrick (1968) stated that a pH greater than 5.5 was required for SRB activity to occur under laboratory conditions. Consequently it was postulated that SRB in acid environments were found in microniches of higher pH (Fauque, 1995), and growth media used to grow SRB has generally been adjusted to around pH 7.

1.7.4 Metabolism

A variety of organisms can carry out assimilatory sulphate reduction, converting sulphate to organic sulphur (R-SH). However dissimilatory sulphate reduction, in which sulphate only serves as an electron acceptor and is not incorporated into the cell, is only carried out by SRB and archaea. Sulphate is reduced to sulphide, which produces the distinct odour associated with SRB growth, the overall reaction can be expressed as:

$$SO_4^{2-} + 8H^+ + 8e^- \longrightarrow H_2S + 2H_2O + 2OH^- \qquad (1.16)$$

The reduction of SO_4^{2-} to S^{2-} is an eight-electron reduction, which proceeds through a number of intermediate stages. The sulphate ion is very stable and cannot be used without being activated. It is unique in that

it is only capable of acting as terminal electron acceptor once it has been converted to adenosine phosphosulphate (APS) (Hamilton, 1998).

1.7.4.1 Energy balance in SRB

The activation of sulphate to APS and pyrophosphate is a biochemical necessity because of the unfavourable midpoint potential of sulphate/bisulphite (Hansen, 1994; see Table 1.3). The first step of the conversion requires energy: the generation of APS involves reaction with ATP. Thus, SRB are actually unable to reduce sulphate directly (Cypionka, 1995) as it must first be 'activated':

$$SO_4^{2-} + ATP + 2H^+ \xrightarrow{\text{adenylyl sulphatase (ATP sulphurylase)}} APS + PP_i \quad \Delta G^{o'} = +46 \text{ kJ/mol} \quad (1.17)$$

This reaction is endergonic and must be pulled to completion by removal of the end products:

$$PP_i + H_2O \xrightarrow{\text{pyrophosphatase}} 2\,P_i \quad \Delta G^{o'} = \quad \Delta G^{o'} = -22 \text{ kJ/mol} \quad (1.18)$$

This accounts for the loss of an energy-rich phosphate bond. Pyrophosphatase in most strains of SRB has the property of being activated by reducing agents, and is inactivated by the bacteria under aerobic conditions. Thus, the organism can conserve ATP (preventing its conversion to APS) in unfavourable growth conditions (Postgate, 1984; Cypionka, 1995). A second phosphate ester is cleaved in this last reaction, and the energy required for sulphate activation equals that of the hydrolysis of two ATP molecules to ADP and phosphate. The AMP formed can react with a second ATP to give two ADP, catalysed by adenylate kinase, without a change in free energy:

$$AMP + ATP \longrightarrow 2ADP \quad \Delta G^{o'} = 0 \text{ kJ/mol} \quad (1.19)$$

Removal of the APS from the first reaction is the first redox reaction:

$$APS + H_2 \longrightarrow HSO_3^- + AMP + H^+ \quad \Delta G^{o'} = -69 \text{ kJ/mol} \quad (1.20)$$

Equations (3.17) to (3.20) can be summarised as:

$$SO_4^{2-} + H_2 + H^+ + 2ATP + H_2O \longrightarrow HSO_3^- + 2ADP + 2P_i \quad (1.21)$$

$$\Delta G^{o'} = -45 \text{ kJ/mol}$$

Since the reduction of APS is strongly exergonic (equation (3.20)), it would be advantageous if APS reductase was coupled to energy conservation, but in the majority of strains of SRB, the enzyme appears to be located in the cytoplasm (Kremer *et al.*, 1988). The reduction of sulphite to sulphide has to compensate for the investment of sulphate activation and yield additional ATP for growth (Cypionka, 1995):

$$0.5\, HSO_3^- + 0.5\, SO_3^{2-} + 3H_2 + H^+ \longrightarrow 0.5\, HS^- + 0.5\, H_2S + 3H_2O$$

$$\Delta G^{o'} = -174 \text{ kJ/mol} \quad (1.22)$$

Thermodynamically, this allows regeneration of at least 2 ATPs (Cypionka, 1995).

In addition to the energy costs just described for the activation of sulphate, a further, relatively small amount of energy is required for the cellular uptake of sulphate by co-transport with H^+ (in freshwater species) or Na^+ (in marine species; Cypionka, 1995; Hansen, 1994; Hamilton, 1998). The consequence of these energy costs is that anaerobic respiration using sulphate reduction results in a greatly reduced energy yield compared to some other anaerobic respirations and aerobic respiratory processes. This reduced energy yield is related to the negative and low values for the redox potentials of the crucial reductive reactions involved. APS reduction takes place in two steps, characterised by the two redox couples:

$$APS/AMP + HSO_3^- \qquad E^{o\,\prime} \text{ (redox potential)} = -60 \text{ mV}$$

HSO_3^-/HS^- $E^{o\prime}$ (redox potential) = - 116 mV

The energy yield from the oxidation of H_2 or other substrates with APS or bisulphite as terminal electron acceptors ($\Delta E^{o\prime}$ values with H_2 = +354 and +298 mV respectively) is substantially less than is available to aerobic species utilising O_2 ($\Delta E^{o\prime}$ with H_2 = +1232 mV) (Hamilton, 1998). The energy released from the oxidation of an electron donor using oxygen as electron acceptor is higher than if the same compound is oxidised with any of the electron acceptors below oxygen in Table 1.3, which lists the reduction potentials of some important redox pairs. Therefore it is evident that sulphate is a much less favourable electron acceptor than, for example, O_2 or NO_3^-; however, sufficient energy to make ATP is available to the bacteria when an electron donor that yields the electron carriers NADH or FADH is used. Overall, the energy balance in cultures of SRB reflects the conservation of energy in the PP_i bond, ATP production by fermentation, and the energy needed for sulphate activation plus biosynthesis (Fauque *et al.*, 1991). Sulphite and thiosulphate require no activation and can allow for three times the maximum growth yield than that obtained with sulphate in *Desulfovibrio vulgaris* (Thauer, 1989), although smaller growth yield differences were obtained with *Desulfosporosinus* (then *Desulfotomaculum*) *orientis* in similar experiments (Cypionka and Pfennig, 1986). However, sulphite reduction to H_2S is inhibited by uncouplers (which arrest ATP synthesis by increasing membrane permeability, dissipating the proton motive force) and depends on an intact cell structure, indicating an energy-dependent step (Cypionka, 1995).

1.7.4.2. Electron transport

Sulphate reducing bacteria carry out a cytochrome-based electron transport process, the electrons from the energy source being transferred to the sulphate ion in APS and sulphite (Brock, 1997). The cytochrome of the SRB, which is not possessed by organisms using other electron

acceptors, is a very electronegative cytochrome *c*, known as cytochrome c_3, which is the principal cytochrome of *Desulfovibrio* (Postgate, 1984). Other electron carriers in SRB include ferredoxin and flavodoxin. Type II sulphate reducers (species capable of completely oxidising acetate and other fatty acids) also possess a cytochrome of the *b* type (which are not found in species unable to degrade fatty acids).

The mechanism of electron transport in SRB is by the transfer of electrons directly from hydrogen (H_2, from the environment), or generated from certain organic electron donors such as lactate, to the enzyme hydrogenase, which is situated in the intracellular or periplasmic space in association with cytochrome c_3. If a periplasmic hydrogenase passes electrons to a cytochrome and electrons are consumed in the cytoplasm, the process generates a membrane potential and a transmembrane pH gradient without the pumping of protons across the cell membrane. This mechanism is known as vectorial electron transport (Badziong and Thauer, 1980) and is also found in other bacteria and with other substrates (Cypionka, 1995). Because of the way in which the electron transport components are positioned within the cytoplasmic membrane, when the H atoms of H_2 are oxidised, protons (H^+) remain outside the membrane and the electrons are transferred across the membrane (Badziong and Thauer, 1980). A proton motive force is set up that can be used for the synthesis of ATP, and in the cytoplasm, the electrons are used in the reduction of APS and sulphite (Odom and Peck, 1981, Hansen, 1994).

In addition to this, or alternatively, SRB can pump protons across the membrane, performing vectorial proton translocation (Cypionka, 1995).

Redox pair (pH 7)	$E^{o'}$ (mV)
N_2O/N_2	+1136
O_2/H_2O	+820
Fe^{3+}/Fe^{2+} (pH 2)	+770
NO_3^-/NO_2^-	+430
MnO_2/Mn^{2+}	+380
$Fe(OH)_3/FeCO_3$ (pH 7)	+200
HSO_3^-/HS^-	-110
SO_4^{2-}/HS^-	-220
CO_2/CH_4	-240
S^0/HS^-	-270
$S_2O_3^{2-}/HS^- + HSO_3^-$	-400
$2H^+/H_2$	-410
SO_4^{2-}/HSO_3^-	-520

Table 1.3. Reduction potentials of some biochemically important redox pairs (Brock, 1997)

1.7.4.3. Oxidation of acetate

As previously mentioned, some SRB are able to completely oxidise organic substrates (including acetate) to CO_2, whilst others are incomplete oxidisers, with acetate usually being the end product. Incomplete oxidation is due to the absence of a mechanism for acetyl-CoA oxidation (Widdel

and Hansen, 1992). In such cases, a complete oxidation is only possible with one-carbon compounds such as methanol or formate (Klemps *et al.*, 1985) or with two-carbon compounds that are more oxidised than acetate. The presence of a pathway for acetyl-CoA oxidation is usually associated with the ability to use free acetate as a growth substrate (Widdel and Hansen, 1992).

Desulfobacter species use acetate preferentially or even exclusively. Acetate is oxidised completely to CO_2 with reduction of sulphate to sulphide:

$$CH_3COO^- + SO_4^{2-} + 3H^+ \longrightarrow 2CO_2 + H_2S + 2H_2O \qquad (1.23)$$

$$\Delta G^{o'} = -57.5 \text{ kJ/reaction}$$

Although the citric acid cycle is the major means by which acetate is oxidised in most organisms, it does not function in the same way in acetate-oxidising sulphate reducers (Brock, 1997). Instead, sulphate reducing bacteria use either a modified citric acid cycle (CAC) or the acetyl-CoA pathway. Organisms like *Desulfobacter* use the modified CAC for acetate oxidation, which involves most of the enzymes of the CAC. *Desulfobacter* contains an enzyme, succinyl-CoA:acetate-CoA transferase (Widdel and Hansen, 1992) which activates acetate, yielding succinate and acetyl-CoA. The latter then enters the CAC and is oxidised to CO_2. However, the ATP yield from the oxidation of acetate via the CAC is approximately the same as that required to activate sulphate in the first step of sulphate reduction (see above), which could cause a problem to acetate-oxidising sulphate reducers. However, Möller *et al.* (1987) found that condensation of acetyl-CoA and oxaloacetate to citrate in *Desulfobacter postgatei* was energetically favourable. The organism possesses an enzyme, citrate lyase, which allows for net ATP synthesis (and therefore growth) at the expense of acetate. The enzyme couples to ATP synthesis by substrate-level phosphorylation from the conversion of acetyl-CoA, via acetyl-P, to acetate during the production of citrate. The

majority of sulphate reducers which can completely oxidise acetate (such as *Desulfotomaculum acetoxidans*) do not use this modified CAC but employ the acetyl-CoA pathway. These bacteria contain high activities of the enzyme carbon monoxide dehydrogenase, which is absent in *Desulfobacter* species. Through the activity of this enzyme, acetyl-CoA is cleaved into bound carbon monoxide and a bound methyl group, and both one-carbon units are then oxidised to CO_2. This is known as the carbon monoxide/C1 pathway (Widdel and Hansen, 1992). This is a reversible mechanism which is capable of both acetate oxidation and CO_2 fixation, and is also present within other strict anaerobes (Hamilton, 1998). These fundamental differences between the mechanisms for acetate oxidation within the SRB are another demonstration that these bacteria are a very diverse group of organisms, even though their common characteristic of sulphate reduction has led to their being considered a homogenous group.

1.7.5 Ecology of sulphate reducing bacteria

1.7.5.1. Sulphate reducing bacteria in natural habitats

As outlined in 1.7.1., SRB are widely distributed in anaerobic terrestrial and aquatic environments. Zones of active sulphate reduction are easily recognised by blackening of sediments or water due to production of ferrous sulphide, and the characteristic odour of hydrogen sulphide. Although sulphate reduction is commonly found in non-saline environments such as soils, freshwater sediments, and bogs (Postgate, 1984; Widdel, 1986), marine, estuarine and saltmarsh sediments (and those of saline/hypersaline lakes and ponds) are the principal habitats of sulphate reduction due to their high sulphate content (Pfennig *et al.*, 1981, Postgate, 1984; Bass *et al.*, 1996). Sulphate reducers have also been widely found in polluted environments: a number of halogenated aromatic compounds can be degraded and their complete oxidation coupled to sulphate reduction (Häggblom *et al.*, 2000). Eight strains of sulphate reducing bacteria, all most closely related to *Desulfosporosinus orientis*,

Species	Habitat	Growth substrate	N_2 fixation
Desulfovibrio africanus	Well water	Lactate	+
D. alcoholovorans	Purification plant	Glycerol	NR
D. desulfuricans	Soil	Lactate	+
D. furfuralis	Pulp/paper waste	Furfural	NR
D. gigas	Pond water	Lactate	+
D. halophilus	Benthic microbial mat	Lactate	NR
D. piger	Human faeces	Lactate	NR
D. sulfodismutans	Freshwater mud	Thiosulphate dismutation	NR
D. termiditis	Hindgut of termite	Glucose	NR
D. vulgaris	Soil	Lactate	+
Desulfomicrobium apsheronum	Oil deposits	Lactate	NR
Dsm. baculatum	Manganese ore	Lactate	+
Desulfobulbus elongatus	Mesophilic digester	Propionate	+
Dbu. marinus	Marine mud	Propionate	+
Desulfobacter curvatus	Marine mud	Acetate	
Dba. postgatei	Brackish water	Acetate	
Desulfobacterium autotrophicum	Marine mud	Succinate	
Dbt. phenolicum	Marine mud	Phenol	
Desulfosarcina variabilis	Marine mud	Phenylacetate	
Desulfomonile tiedjei	Sewage sludge	Benzoate	
Desulfococcus multivorans	Sewage digestor	Phenylacetate	

Table 1.4. Some characteristic features of representative mesophilic sulphate reducing bacteria, illustrating a variety of habitats and including source of isolation, growth substrates, and N_2 fixation (NR = not reported)

were isolated from an aquifer contaminated with hydrocarbons, and one of the strains was shown to increase the rate of toluene degradation in laboratory experiments (Robertson *et al.*, 2000). Table 1.4 shows a few characteristic features of some SRB.

1.7.5.2. Microbial relationships in anaerobic environments

Dissimilatory sulphate reduction or methanogenesis is the major terminal degradation process in anaerobic environments (Fauque, 1995). Methanogenesis dominates in habitats that are low in sulphate, such as freshwater environments, and sulphate reduction dominates in marine or other sulphate-rich environments (Widdel, 1988). As such, there is competition in nature between methanogenic archaea and SRB for available electron donors. Other trophic groups of bacteria existing in anaerobic environments include fermentative microorganisms, which hydrolyse high molecular weight polymers (for example, proteins, polysaccharides, lipids) and ferment their respective monomers, and acetogenic bacteria, which cleave some of the end-products of these fermentations into acetate, CO_2, and H_2, which may be utilised by methanogens and/or SRB (Fauque, 1995). The methanogens utilise the end-products of these previous processes and produce methane. Sulphate reducing bacteria compete with the methanogens for available substrates. The predominance of methanogenesis or dissimilatory sulphate reduction is mainly dependent on the availability of sulphate. The SRB therefore exist as integral members of mixed species, interacting microbial communities (or consortia) in anaerobic environments, in which the interrelationships between different species are generally based on nutritional provision. This is due to the nature of anaerobic metabolism: coupled redox reactions yield one or more reduced products, which are excreted from the cell. These can then be utilised as electron acceptors by other organisms in the consortium which have different metabolic capabilities. These relationships may almost be described as symbiotic

(Hamilton, 1988). Therefore, whilst individual organisms, including SRB, demonstrate only a relatively restricted spectrum of metabolic activity, the community is capable of a much broader carbon flux and energy yield, within an overall redox balance (Hamilton, 1998).

In an open system such as an aquatic sediment, oxygen is available, and the consortium will contain a range of facultative and aerobic organisms. In these conditions it is possible to follow a nutritional succession, from the initial hydrolytic and fermentative breakdown of primary substrates and intermediate conversions of primary products to acetate and H_2, to the terminal breakdown processes, dissimilatory sulphate reduction and methanogenesis. An essential stage in these successive processes is the interspecies hydrogen transfer linking the acetogens and the SRB or methanogens. Due to thermodynamic reasons, the acetogens can only carry out this conversion, coupled to energy conservation, if H_2 is removed by another bacterium, keeping the reaction equilibrium position such that it becomes exergonic. It is the higher affinity for H_2 and acetate of the SRB that usually determines the outcome of competition with methanogens. If sulphate concentrations are sufficient, H_2 - producing and H_2 - utilising organisms benefit from the interspecies hydrogen transfer, representing an essentially symbiotic relationship (Hamilton, 1988).

The SRB and the methanogenic archaea present many ecological and physiological similarities, e.g. their occupation of similar anaerobic ecosystems, such as sediments, and utilisation of small molecular weight fatty acids and hydrogen as electron donors (Widdel, 1988). Smith (1993) outlined three general relationships between the two types of bacteria: (I) co-existence through utilisation of separate electron donors; (ii) direct competition for electron donors; or (iii) synergism in which species of one group provide an electron donor required by the other group. There has been a considerable focus of attention on the nature of the competition between SRB and methanogens, and a number of studies have been carried out using laboratory reactors to look at populations in anaerobic

carried out using laboratory reactors to look at populations in anaerobic biofilms. On the whole, laboratory studies also indicate that SRB are more competitive than methanogens in high sulphate environments (for reviews see Widdel, 1988, and Smith, 1993). However, Raskin *et al.* (1996) demonstrated that the presence of SRB (especially *Desulfovibrio* and *Desulfobacterium* spp.) was independent of the presence of sulphate in experiments with laboratory biofilm reactors. In addition, these authors observed that some SRB (*Desulfococcus/ Desulfosarcina/Desulfobotulus* group) were more competitive in environments without sulphate; proton-reducing acetogenic and/or fermentation pathways were suggested as the most probable mechanisms allowing the high levels of SRB in the absence of sulphate (Raskin *et al.*, 1996). This evidence once again illustrates that SRB have a considerable capacity to adapt to modifications of biological and chemical factors in their natural habitats.

1.7.5.3. Influence of surfaces on sulphate reducing bacteria

Surfaces are important in microbial habitats because nutrients can adsorb onto them. In the microenvironment of a surface, nutrient levels may be much higher than in the bulk solution (Brock, 1997). This can have effects on the rate of microbial metabolism. Sediments and muds are not homogenous as are media used for culture in the laboratory; substrate concentrations inside and at the surfaces of decaying organic particles tend to be higher than in interstitial situations (Widdel, 1986). Bacteria can be free-living (planktonic), but studies of microbial colonisation of surfaces in natural ecosystems have shown that most microorganisms grow on surfaces enclosed in biofilms (Beveridge *et al.*, 1997, and references therein). A biofilm is distinguished from other microbial aggregates in that it forms at a phase boundary, most commonly a liquid:solid interface (Wimpenny, 2000). Bacterial biofilms are, in broad terms, communities of bacterial cells attached to a surface by extracellular polymeric substances

(EPS), which are excreted by the cells. Once a microbial community is established, the cells and the EPS create a protective organic matrix, within which there may be nutrient fluxes and pH and redox gradients. In the resulting biofilm, the matrix of EPS provides cohesiveness for immobilised microorganisms (Flemming *et al.*, 2000; Wingender and Flemming, 1999; Wingender *et al.*, 1999), and it is considered the basic requisite for the formation and maintenance of the biofilm (Wingender and Fleming, 1999). EPS are thought to play an essential role in wastewater treatment processes, e.g. they are involved in floc formation of activated sludge and performance of fixed biofilm reactors with attached microbial biomass (Flemming *et al.*, 2000). The EPS create a gel-like matrix in which the microorganisms can be fixed, in close proximity to each other, with a long retention time (Flemming *et al.*, 2000); this is a prerequisite for the formation of stable consortia, which are advantageous in a bioremediation system.

Biofilms effectively trap nutrients for growth by charge-charge or hydrophobic interactions (Beveridge *et al.*, 1997; Mayer *et al*, 1999; Flemming et al., 2000) and provide vital protection against detachment of cells on surfaces in flowing systems (Brock, 1997). They may develop on solid, inanimate interfaces, such as rocks in aquatic environments and pipelines, or softer, animate surfaces such as plant roots or animal intestines (Beveridge *et al.*, 1997). Microniches with more favourable conditions than in the bulk phase may be found within biofilms on a surface. As complex amalgams of bacteria may live in close proximity to one another in a biofilm, metabolic processes can be shared between bacterial types, which can result in co-dependence (Beveridge *et al.*, 1997). For example, anaerobes may interact with oxygen-consuming species, which reduce the environmental levels of oxygen sufficiently for the anaerobes to utilise protective enzyme systems (see below) for detoxification (Marquis, 1995; Marsh and Bowden, 2000).

Bacteria that inhabit the subsurface (such as sediments and soils) tend to grow more successfully in association with surfaces (Lawrence *et al.*, 1995). Sulphate reducing bacteria are anaerobes, and are therefore frequently found in the subsurface. In nature, SRB are found in soils and sediments where they congregate on surfaces of particles (Lyew and Sheppard, 1997). Aggregation of SRB in cell-clumps associated with particles or surfaces may provide protection against unfavourable changes in environmental conditions such as pH and redox potential, or nutrient deprivation (Widdel, 1986; Bass *et al.*, 1996; Beveridge *et al.*, 1997). Fukui and Takii (1990a) found that the rate of sulphate reduction by free-living *Desulfovibrio desulfuricans* Beijerinck was higher than in those associated with FeS particles. Association of SRB with sediment particles in nature may provide a physical diffusion barrier to oxygen from outside (Fukui and Takii, 1996). Spore-forming sulphate reducers such as *Desulfotomaculum* spp. can tolerate exposure to oxic conditions for months or even years (Widdel, 1992; Cypionka, 2000). *Desulfovibrio desulfuricans* was found to survive prolonged aeration in association with particles whilst free-living ones died rapidly (Fukui and Takii, 1990b). Pfennig *et al.* (1981) found that pronounced growth of *Desulfosarcina variabilis* in cell-packets was associated with the attachment of the organism to available surfaces.

One of the possible advantages of surface growth or clump formation is the capacity for more effective substrate utilisation under nutrient-limiting conditions. The gliding filamentous sulphate reducers of the genus *Desulfonema* combine motility with attachment to surfaces. The gliding motility allows movement between sediment particles and viscous organic matter; the motion may be directed with chemical gradients, enabling the filaments to access more favourable growth conditions (Widdel, 1988). Laanbroek and Geerligs (1983) observed that the presence of illite (clay) particles had a positive effect on conservation of the oxidative capacity of cultures of *Desulfobacter postgatei*, *Desulfovibrio baculatus* and *Desulfobulbus propionicus*, with the greatest influence observed in the

latter strain. Bass *et al.* (1996) isolated mixed cultures of thermophilic sulphate reducers from chalk formations in North sea oil reservoirs, and offered surfaces in varying amounts to planktonic cultures. The amount of sulphide produced from cultures in contact with a surface was significantly more than in cultures without a surfaces, and in one culture, viability was maintained for 7 months when attached to oil-bearing chalk rock chips without nutrients. Starved cells from the same culture were metabolically active for 8 weeks but did not recommence sulphide production after this time following nutrient provision (Bass *et al.*, 1996). This implied that attachment to a surface conferred a selective advantage on the starved cell population (Bass *et al.*, 1998).

The North Sea oil reservoirs off the east coast of the UK provide a combination of extreme environments, including high temperature and pressure, and in some zones, low nutrient status, low pH and anoxic conditions (Bass *et al.*, 1996). Sulphate reducing bacteria are known to inhabit oil-bearing porous rock formations; this porous matrix seems to present a physically stable environment, and probably offers protection against varying environmental conditions. Thermophilic sulphate reducers readily attach to rock surfaces, assisted by the production of large amounts of exopolysaccharide (Coutinho *et al.*, 1994; Beech *et al.*, 1996). Here, they can survive adverse conditions by the formation of dormant cells. The reduced size of the dormant cells means that they can pass easily through rock spaces and inhabit the deep reservoir (Bass *et al.*, 1996). Biofilms containing thermophilic SRB can develop within the porous matrix of oil-bearing rocks, as well as on metal surfaces in the oil production system (where they can initiate corrosion; see 1.7.6.). In this environment, they may be able to withstand periods of nutrient deprivation (Bass *et al.*, 1996).

In laboratory bioreactor experimental systems, such as those used to investigate bioremediation or biodegradation, surfaces are often provided for the colonisation of SRB and the establishment of a biofilm. Biofilm

formation by heterogenous communities in these systems is a very important mechanism to improve interactions, both among the microorganisms as well as between microorganisms and their physical and chemical environment (Bryers, 2000; Evans, 2000; Wolfaardt *et al.*, 2000). Lyew *et al.* (1994) considered the use of a gravel bed, for the establishment of a biofilm of SRB, to be of primary importance in the design of a bioreactor for the treatment of AMD. Lyew and Sheppard (1997) investigated the effects of physical parameters of limestone and granite gravel beds on SRB activity in column reactors, and concluded that the larger the available surface area, the greater the potential populations of active SRB as well as the sulphate removal. There was no apparent advantage in using the more alkaline limestone gravel over granite (Lyew and Sheppard, 1997). Elliott *et al.* (1998) incorporated sand into an upflow anaerobic bioreactor for the growth of SRB under acidic conditions, as a treatment system for AMD. In this system, viable SRB were recovered from the column after 21 days of operation at pH 3 (Elliott *et al.*, 1998). Christensen *et al.* (1996), in a bench-scale experiment for the treatment of acid mine water using SRB, used cylinder reactors with a layer of crushed stone over a layer of quartz sand. They observed blackening from ferrous sulphide precipitates at the interface between the two layers, as well as extensive biofilm formation on microscope slides placed on top of the crushed stone layer during the experiment. They concluded that sediments and solid surfaces are colonised first by sulphate reducers, and a large fraction of the bacteria remained attached to them. The authors suggested that growth conditions in the stone and sand were more favourable than in the free water phase (Christensen *et al.*, 1996). Maree and Strydom (1985) demonstrated that in an upflow packed bed reactor for biological removal of sulphate, crushed dolomite was a better surface than quartz sand or plastic. However, Somlev and Tishkov (1994) compared reactors containing metallic iron chips and crushed dolomite, and found that the reactors with iron chips gave a conversion efficiency for sulphate of 75% compared with 60% for dolomite. They confirmed that iron chips were

more effective as biofilm carriers in the sulphate reducing process (which is important to note in the context of SRB-induced corrosion, see 1.7.6.), and concluded that packed bed bioreactors are more suitable for industrial treatment of sulphate-containing wastewaters than fully mixed reactors. Also in this investigation, it was possible to effectively treat influent waters with a pH of 4.1 (Somlev and Tishkov, 1994), suggesting again that the provision of a surface offers an advantageous habitat to the usually acid-sensitive SRB.

Some bacteria are able to modulate their local pH, particularly in biofilms, by up-regulating genes involved in acid or base production, even at pH values below or above which the bacteria can grow (Marsh and Bowden, 2000). Individual bacteria in the biofilm may also possess specific molecular strategies that allow them to adapt to rapid changes in pH (Bowden and Hamilton, 1998). Therefore, it is possible that biofilms allow SRB to establish microenvironments that enable their survival, growth and proliferation.

1.7.6 Economic and medical importance of SRB

Sulphate reducing bacteria appear to be indigenous inhabitants of the waters of oil-bearing shales and strata, and their activities in this context include: (i) biocorrosion of pumping machinery, storage tanks and pipelines; (ii) generation of H_2S, which contaminates natural gas associated with oil deposits, and (iii) growth in injection waters and potential for clogging systems (Postgate, 1984). Although difficult to accurately assess, in 1985 it was estimated that the costs to UK industry of biofouling was about £300-500 million per annum (Hamilton, 1985). The sulphate reducers are the single most common causative organism in microbially influenced corrosion (MIC), which may constitute 50% of all instances of corrosion (Booth, 1964).

The formation of a biofilm on a number of metal surfaces can result in biocorrosion. Bacteria, including SRB readily colonise such surfaces, as shown by Somlev and Tishkov, 1994 (see 1.7.5.3). Beech *et al.* (1991) further indicated that the excretion of polysaccharides (or, more generally, extracellular polymeric substances; EPS) by a pure culture of SRB was stimulated by the presence of a steel surface, leading to steel corrosion. Corrosion is a blanket term covering a number of specific electrochemical processes (such as pitting, graphitisation, stress corrosion cracking), which can affect iron, mild steel, stainless steels, as well as aluminium, copper, nickel and cobalt alloys (Hamilton and Lee, 1995). In most cases, SRB-induced MIC occurs in cast iron and mild steel (Hamilton, 1994). In buried cast iron pipes, MIC can result in complete removal of the iron, leaving behind an apparently unaltered structure composed solely of graphite, therefore retaining virtually none of its strength (Hamilton, 1985; Hamilton, 1994). In mild steels, which are used in virtually all construction work, MIC can cause what is known as pitting corrosion (Hamilton, 1994). The worst cases of corrosion involving SRB are linked to environments in which the necessary reducing conditions in a biofilm are in close proximity to an aerobic phase (Hamilton, 1994; Hamilton and Lee, 1995; Hamilton, 1998); i.e. oxygen has a direct role in stimulating SRB-induced corrosion (Hamilton and Lee, 1995). This is explained by the fact that SRB are component members of mixed microbial consortia that contain aerobic and facultative organisms which supply nutrients for SRB and reducing conditions are generated. In base region of a biofilm on a metal (or other) surface, anoxic conditions can readily be generated at biofilm thicknesses in excess of 20 μm (Hamilton and Lee, 1995). Figure 1.9 illustrates a model for anaerobic microbial corrosion.

The understanding and control of biocorrosion has been of considerable importance over the last few decades. Hundreds of millions of dollars are spent annually to minimise economic and environment effects attributed to corrosion (Jack and Westlake, 1995). A large research effort has been

ongoing to understand the mechanisms of SRB-related corrosion in sufficient detail (Videla, 2000). The importance of microorganisms in corrosion has been recognised for more than half a century, and since that time, the oil and gas industries have invested considerable time and energy into researching techniques for the detection, enumeration and control of SRB (Jack and Westlake, 1995).

Sulphate reducing bacteria have also been implicated in the cause and maintenance of inflammatory bowel diseases (IBD's), i.e. Crohn's disease and ulcerative colitis (Gibson *et al.*, 1991; Pitcher *et al.*, 2000). It is possible that SRB associated with gut mucosa result in an increased systemic and local immune response (Matsuda *et al.*, 2000), or indeed that the persistence of fast-growing, actively metabolising, H_2S-generating SRB strains could result in continuous irritation of the colon (Zinkevich and Beech, 2000). Research is on-going to understand more about SRB community structures in diseased and healthy human bowels (e.g. Pitcher *et al.*, 2000; Zinkevich and Beech, 2000).

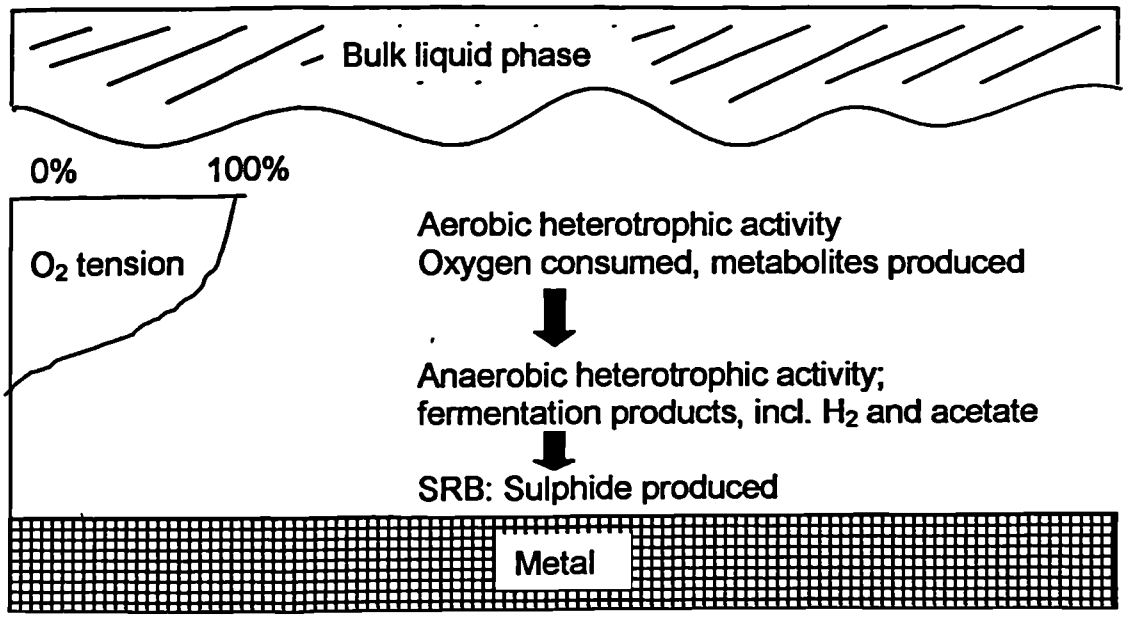


Figure 1.9. A model for anaerobic microbial corrosion (Hamilton, 1994), illustrating the importance of the mixed microbial consortium in the process.

1.8 Acidophilic microorganisms: characteristics and diversity

Each organism has a pH range in which growth is possible, and usually a pH optimum. Natural environments generally have pH values that are circumneutral, i.e. between 5 and 8, and organisms with growth optima in this range are most common. Microorganisms generally cannot tolerate extreme pH values, because in these conditions, microbial cell components may be hydrolysed or enzymes may be denatured (Atlas and Bartha, 1993). Relatively few species can grow at pH values below 2 or higher than 10; such organisms, along with others that grow optimally at extremes of temperature, pressure, or salinity are collectively known as 'extremophiles'. Organisms that live at low pH are known as acidophiles. Several species of bacteria, archaea, fungi, microalgae and protozoa grow at low pH, and some are obligate acidophiles, i.e. they cannot grow at pH values which approach neutrality (Johnson, 1998). Many bacteria that accelerate the production of AMD, such as *Thiobacillus* spp., are obligately acidophilic, as are several genera of archaea, such as *Sulfolobus* and *Thermoplasma*. Other species may be acid-tolerant and can grow in a broader range of low to neutral pH, but have pH optima close to neutrality.

Acidophiles may be found in a number of different environments, from hot springs to volcanic streams, or in acidic soils, bogs and sediments. A number of different acidophilic prokaryotes and eukaryotes exist within these environments (Table 1.5). Most extremely acidic environments contain low concentrations of dissolved organic carbon, and primary production in sub-surface environments such as mines is based on chemolitho-autotrophy; in illuminated environments, primary production may be mediated by phototrophic acidophiles such as microalgae and diatoms (Johnson, 1998; Gyure *et al.*, 1987). Research into acidophilic microbiology has focused mainly on iron-oxidising chemolithotrophs, in particular the iron/sulphur-oxidising bacterium *Thiobacillus ferrooxidans*, as it was the first iron-oxidising acidophilic organism to be isolated and

described, is relatively easy to grow and purify in the laboratory, and is ubiquitous in pyritic environments. These bacteria are known to have a central role in the production of AMD. A variety of heterotrophic microorganisms have been isolated from extremely acidic environments. Acidophilic microorganisms indigenous to acidic, metalliferous environments are very diverse, and often exist in consortia in structural entities such as flocs, or, where there is a surface available for attachment, biofilms (Hamilton, 1985, 1998). For example, Figure 1.10 shows acidophilic bacteria existing in consortia in "acid streamers" (Johnson *et al.,* 1979) in a disused pyrite mine, "Cae Coch", Conwy valley, North Wales, UK. In this site, the main drainage stream passing through the mine is filled with streamer growths up to 0.5 m deep (Jenkins and Johnson, 1993). Lackey (1938) first reported the presence of such growth forms in a mine in Indiana, describing them as 'bacteria in a zoogloeal jelly'. 'Pipes', or stalactite-like bacterial slime growths formed by water dripping from the upper surface of a mine shafts in the U.S.A. were observed by Temple and Koehler (1954) and described by Jenkins and Johnson (1993) as occurring in the Cae Coch mine. Streamer growths consist of a fibrillar polymer network (a polysaccharide matrix) produced by predominantly aerobic bacteria (Dugan *et al.,* 1970a,b), and Johnson *et al.* (1979) found the bacterial components to be a mixture of heterotrophic and autotrophic bacteria. Some microorganisms within the AMD environment are directly involved in AMD genesis, whereas some of the heterotrophic acidophiles play a more passive role in AMD production, metabolising organic materials that can be toxic to autotrophic iron-oxidisers (Ingledew, 1982; Pronk, 1991; Norris and Ingledew, 1992; Johnson, 1995; see sections 4.1 and 5.4). Other isolates can reduce soluble or solid phase ferric iron (Johnson and McGinness, 1991), promoting metal mobilisation by a reductive route, as opposed to the oxidative dissolution by *T. ferrooxidans* and similar organisms.

Physiological Group	Example
(a) bacteria	
Fe^{2+}- oxidising autotrophs	*Leptospirillum ferrooxidans*
Fe^{2+}/S-oxidising autotrophs	*Acidithiobacillus ferrooxidans*
S-oxidising autotrophs	*Acidithiobacillus thiooxidans*
Fe^{2+}/S-oxidising mixotrophs	*'Sulfobacillus montserratensis'*
Fe^{2+}-oxidising heterotrophs	*'Ferrimicrobium acidiphilum'*
Fe^{3+}-reducing heterotrophs	*Acidiphilium* spp
(b) archaea	
Fe^{2+}-oxidising autotroph	*Ferroplasma acidophilum*
(c) eukarya	
Fungi/ yeasts	*Rhodotorula* spp
Algae	*Euglena mutabilis*
Protozoa	*Eutreptia, Cinetochilium, Vahlkampfia*

Table 1.5 The diversity of acidophilic, mesophilic microorganisms (Norris and Johnson, 1998).

Figure 1.10 Acid streamers—consortia of acidophilic microorganisms, in an AMD environment.

At the surface, acid streamer bacteria are predominantly aerobic and require oxic conditions, but these aerobic species are also responsible for creating the anoxic conditions necessary for the growth of microaerobic or anaerobic species (such as sulphate reducers) deeper within or below the streamer or biofilm structure (Hamilton, 1985, 1998). Johnson *et al.* (1993) incubated acid streamer material (from pH 2.4 AMD) at 30°C in the laboratory and found that zones of sulphide accumulation developed, indicating the presence of SRB in the streamer material. An isolate from these zones was found to be a sulphate reducing bacterial strain which had a wide pH range for growth but which could grow in glycerol/yeast extract medium initially poised at pH 2.9 (Johnson, *et al.*, 1993). Species diversity can therefore be observed in acidic environments; Figure 1.11 outlines microbial transformations of carbon, sulphur and iron in surface and sediment waters of the Afon Goch, Parys Mountain, Anglesey, a historic site of metal mining where waters emerging from the site have a pH as low as 2.3 (Boult *et al.*, 1994). In acidic geothermal environments such as solfatara (acidic sulphur-rich environments), water temperatures may vary from almost boiling (at the source of the thermal spring) to much lower temperatures downstream. In these environments, therefore, a variety of acidophiles with differing temperature optima may be found, from extremely thermophilic archaea, moderately thermophilic archaea and eubacteria, to mesophilic bacteria (Johnson, 1999; Table 1.5).

Once an understanding about acidophiles and their ecological niches had developed, they became the focus of considerable research interest due to their potential role in biotechnology and its commercial applications. Johnson (1999) highlighted the importance of understanding how acidophilic microorganisms interact with each other, suggesting that this has a major bearing on mineral technologies, as microbial consortia (or mixed cultures) rather than single organisms are generally used in commercial-scale bioprocesses. The use of acidophilic microorganisms in microbial leaching (also known as 'bioleaching' and 'biomining') has

developed into one of the most successful areas of biotechnology, with an estimated global value of around $10 billion (Johnson, 1998; Johnson, 1999). In this situation, metal mobilisation and acid production by acidophilic bacteria have a significant commercial benefit in mining. If concentrations of metals in sulphide ores are low, it may not be cost-effective to recover metals by conventional processes. In these situations, particularly for copper ores (as copper sulphate is very water-soluble), microbial leaching is highly appropriate; in 1986, about 18% of copper production in the USA was accounted for by bioleaching processes (Torma, 1986). Bacteria such as *Leptospirillum ferrooxidans* are able to catalyse a rapid rate of oxidation of sulphide minerals, enabling solubilisation of metals, mediated by direct or indirect mechanisms (Figure 1.1).

Novel acidophilic microorganisms may be of great value in remediation of a number of contaminated media. For example, Hallberg *et al.* (1999) isolated a variety of acidophilic bacteria from acidic environments that were able to use several aromatic compounds as growth substrates, of which two isolates were able to grow in higher concentrations of some aromatic and aliphatic organic acids than other acidophiles. This apparent tolerance to organic acids is a trait shared with two species of the acidophile *Acidocella* (as opposed to the sensitivity exhibited by another genus of acidophiles, *Acidiphilium*). In the same study, an acidophilic sulphate reducing culture also obtained from an acidic environment was shown to grow on benzoic acid and benzyl alcohol (1mM). This study demonstrated the potential for use of the novel organisms in biological treatment of acidic wastewaters containing organic and inorganic pollutants.

It is clear then, that naturally occurring acidic environments, as well as acidic ecosystems created by human activities, may contain a considerable range of indigenous microorganisms. Geothermal

environments such as solfatara are often ancient, whereas many mine sites are relatively recent. However, in older mine sites such as Parys Mountain (Wales), acidophilic communities may in fact be quite complex and diverse (Johnson, 1999; Jenkins and Johnson, 1993). All of these sites could therefore be important to the field of biotechnology as they may be the source of many novel organisms that could be used in bioprocesses, including bioremediation.

1.9 Molecular perspectives in microbial ecology

The understanding of microbial ecology has been advanced by the development of molecular biological methodologies over the last decade. Therefore, information regarding the biodiversity of acidophilic microorganisms (and indeed all microorganisms) has rapidly expanded. Due to the insufficiency of using morphology to identify and classify microorganisms, (as it is not specific enough to be reliable), until very recently, microbial identification required the isolation of pure cultures, or defined cocultures, followed by a number of tests on physiological and biochemical traits (Amann *et al.,* 1995). However, due to the advances in molecular techniques, the total necessity to cultivate and obtain pure cultures of an organism has been removed. Now, nucleic acids can be extracted directly from an environment, and DNA can be selectively amplified using the polymerase chain reaction (PCR). For example, the fraction of the total extracted DNA which is usually amplified for analysis is the part of the genome coding for the small sub-unit (16S) ribosomal RNA. The 16S rRNA gene of bacteria is very suited to amplification using PCR as it has highly conserved regions of DNA at the beginning and end of the gene that are suitable for use as primers. The regions are conserved among all prokaryotes and therefore "universal 16S rRNA gene primers" have been developed. This eliminates the need to know the gene sequence of each individual microorganism. Once a gene from an organism has been amplified and sequenced, primers specific for the

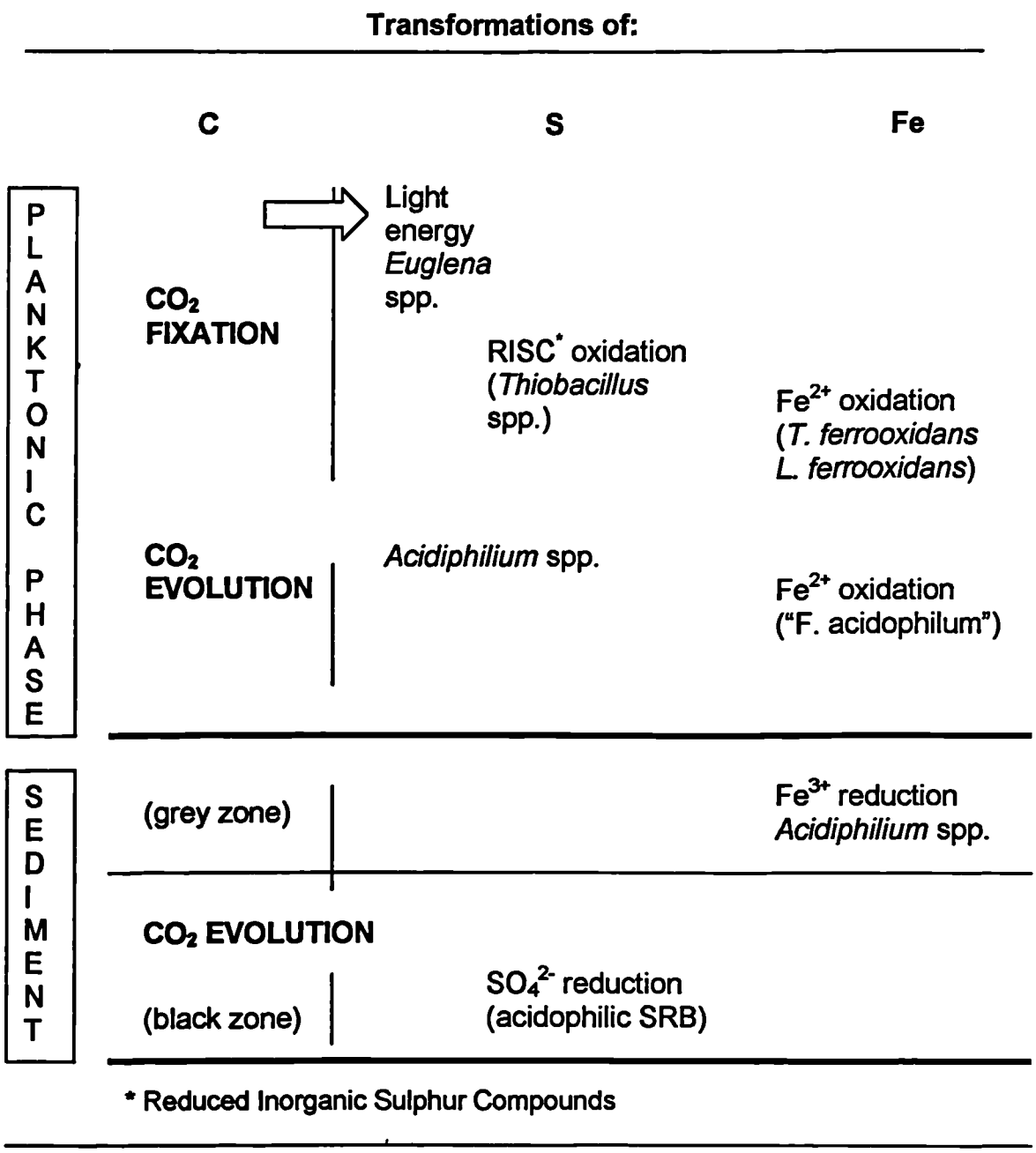


Figure 1.11 Microbial transformations of carbon, sulphur and iron in surface and sediment waters in an AMD environment (Johnson, 2000)

organism can be made and used for the detection of the specific microorganism. Crucially, the 16S rRNA genes from unknown organisms can be amplified using universal primers, and these can then be analysed using other molecular techniques to identify the relationship between unknown organisms or with known organisms. Again, the universality of the primers means that no prior specific sequence data needs to be known. The impact of these technical developments on the ability to discover and identify novel microorganisms has therefore been great, and indeed, organisms which are deemed "unculturable" in the laboratory, or organisms which have been successfully grown in the laboratory, but whose DNA has not been previously sequenced, can be detected and identified (Amann *et al.,* 1995; Head *et al.*, 1998).

Restriction Fragment Length Polymorphism (RFLP) is a useful technique that can be used to determine the level of similarity of genes among a group of strains. Restriction enzymes, or endonucleases, are proteins that cut double stranded DNA at very specific locations ("restriction enzyme sites") within the molecule. The enzymes are isolated from various species of bacteria; the first enzyme to be purified and studied is *Eco*RI (from *E. coli*). Each one recognises a highly specific DNA sequence before cleaving the DNA. The enzymes can be used to determine the presence or absence of the specific sequence recognised by the enzyme, the total number of these specific sequences, and the relative location of the sequences in a DNA molecule. The 16S rRNA genes of bacteria are very frequently compared to determine the relationship of one strain to another. The rRNA approach is today routinely applied for phylogenetic analysis and *in situ* identification of uncultured microorganisms (Amman *et al.*, 1995; Amman *et al.*, 1997)

A powerful approach to the identification and quantification of microorganisms in nature is the use of nucleic acid probes. A nucleic acid probe is a short piece of DNA or RNA complementary in base sequence to

part of a gene to which the probe can hybridise. Often, 16S rRNA sequences are employed as tools for differentiating microorganisms in natural environments. It is possible to identify different species in an environmental sample using a collection of such probes. The probes are labelled using radioisotopes or fluorescent dyes. The biodiversity of microbial communities can be investigated using ribosomal RNA probes. The bulk DNA or RNA samples are extracted from representatives of the entire microbial community, and using specific nucleic acid probes, ribosomal RNA clones are obtained, e.g. by PCR amplification of ribosomal RNA genes. Following sequencing of the ribosomal RNA gene, phylogenetic trees showing species composition of the microbial community can be constructed (Brock, 1997). Probe technology can also be used for identifying particular genes; for example, those that code for proteins involved in biodegradation of pollutants or specific enzymes, such as APS reductase (section 1.7.4.1). It can also be used for investigating the effects of environmental modifications or perturbations on microbial communities (e.g., changes in pH). The advantages of using this technology are many, especially when it comes to investigating novel or "unculturable" organisms (Amann *et al.*, 1995).

The fluorescence *in situ* hybridisation (FISH) method has great potential for improving determinations of active microbial populations (Amann *et al.*, 1995; Christensen *et al.*, 1999). FISH is a powerful tool that can provide taxonomic as well as positional data on organisms in a population (Kolenbrander *et al.*, 2000). Oligonucleotide probes are designed to recognise specific regions of 16S rRNA; these are then labelled with different fluorescent dyes. Whole families of probes can be generated to recognise different microbial groups, from a broad to a narrow range, e.g. a probe that recognises prokaryotes, right through to those designed to target individual species. The method is currently used to investigate microbial populations in a wide range of situations. Okabe *et al.* (1999) used FISH and conventional methods to investigate the distribution of SRB

in microaerophilic wastewater biofilms, alongside microelectrodes to measure chemical concentration profiles (O_2, S^{2-}, NO_3^-, and pH) within the biofilms. Power *et al.* (1999) used FISH, as well as PCR for the dissimilatory sulphite reductase gene, to monitor SRB in heterotrophic biofilms grown on stainless steel under aerobic conditions. Similarly, Santegoeds *et al.* (1998) combined the use of microsensors and molecular techniques (FISH and DGGE (see below)) to investigate the development of sulphate reduction and SRB populations in an aerobic biofilm. Bond and Banfield (2001) designed and tested oligonucleotide probes for the detection of microorganisms inhabiting an AMD site in California. More specifically, Hristova *et al.* (2000) developed *Desulfotomaculum* genus- and subgenus-specific 16S rRNA hybridisation probes to determine the abundance of *Desulfotomaculum* species in thermophilic anaerobic digesters, and Manz *et al.* (1998) investigated abundance and spatial organisation of various Gram-negative SRB in activated sludge flocs using *in situ* probing with specific 16S rRNA targeted oligonucleotides.

Denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman, 1979) is a method by which fragments of the same length but different sequence can be resolved electrophoretically (Head *et al.*, 1998). Individual bands on a gel can be challenged with a range of oligonucleotide probes to investigate the composition and diversity of a microbial community. When the identity of an organism associated with a band has been established, fluctuations in components of the population in relation to environmental changes can be assessed. This method has numerous applications; for example, it could be applied to studying changes in populations of SRB in biotechnological processes (such as wastewater treatment), or in the natural environment following exposure to acid mine drainage or oxygen.

1.10 Bioremediation of AMD using acidophilic SRB (aSRB)

Environmental biotechnology uses a biological process to remove polluting substances from contaminated waste waters and other media. It can be considered to be an engineering application of microbial ecology (Rittman, 1992). A biological process is an engineered system in which biomass of desired microorganisms is accumulated so that the concentration of pollutants in the medium are lowered to meet treatment requirements during contact with the biomass. Process controls are used to select the strains and biomass of different microorganisms in the process, creating and optimising an ecological system that accomplishes the treatment goal.

The role of acidophilic communities and microbial dissimilatory reduction processes in the bioremediation of metal-rich, acidic drainage waters has been described by Johnson (1995b) and Johnson and Bridge (1997). Johnson (1995b) suggested that there is considerable potential in using microbial populations which are indigenous to extremely acidic metalliferous environments in bioremediation schemes, as such populations are unlikely to be rendered inactive or killed on exposure to AMD. Recent studies have shown that dissimilatory reduction of ferric iron can be carried out by a variety of acidophilic bacteria which can be isolated from AMD and impacted water courses (Johnson and Bridge, 1997). However, truly acidophilic sulphate reducing bacteria have not been isolated and characterised; indeed the study of aSRB has not been documented at the time of writing, but there is evidence to show that sulphate reduction occurs in acidic environments (Tuttle *et al.*, 1969a,b; Satake, 1977; Herlihy and Mills, 1985; Spratt *et al.*, 1987; Gyure *et al.*, 1990) and under acidic conditions in the laboratory (Christensen *et al.*, 1996; Hard *et al.*, 1997; Elliot *et al.*, 1998; Lyew *et al.*, 1994; Lyew and Sheppard, 1997, and others).

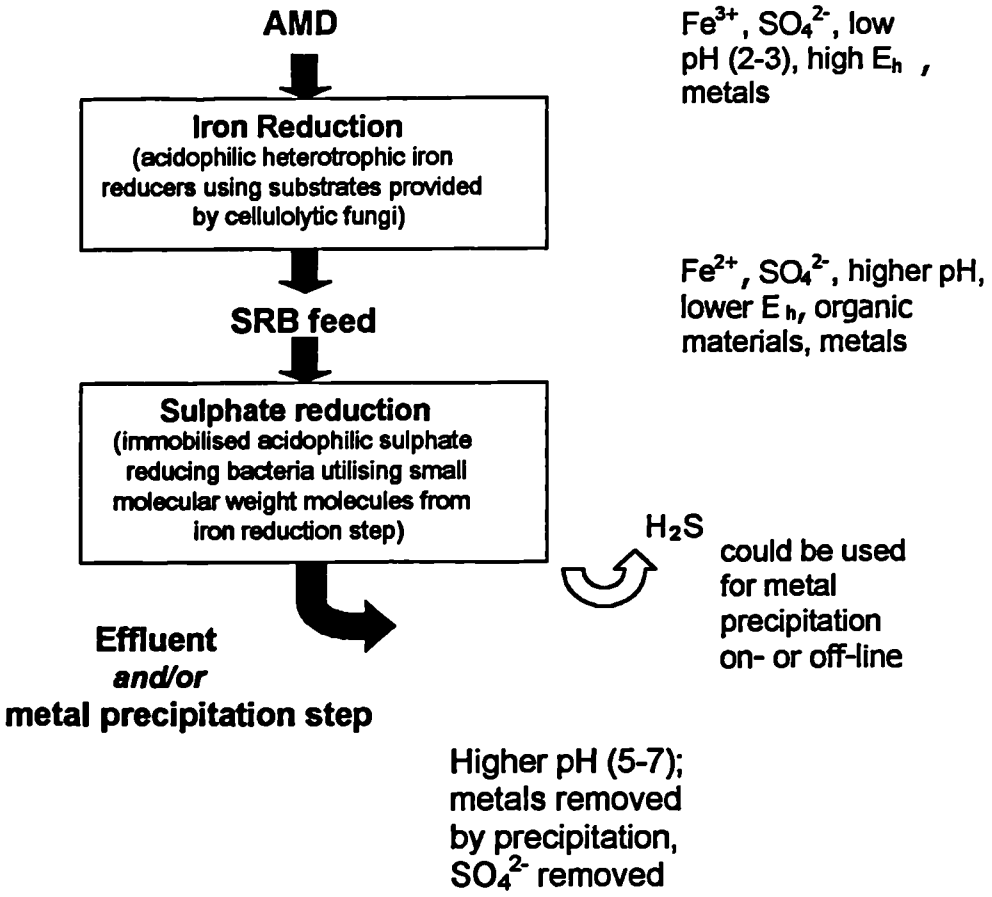


Figure 1.12 Two-step bioremediation of AMD: possible configuration (Johnson, 1995b)

In the light of this evidence, Johnson (1995b) proposed a two-stage microbial treatment of AMD comprising ferric iron reduction followed by sulphate reduction, utilising acidophilic/acid tolerant bacteria in both stages (Figure 1.12) as well as acidophilic fungi. In theory, this treatment could effectively reverse the reactions of pyrite oxidation that are central to the production of AMD. Ferric iron reduction causes a decrease in redox potential of AMD waters, creating more reduced conditions which are required for sulphate reduction by SRB. Thus, an in-line dual bioreactor system (Johnson, 1995) could potentially be an extremely effective treatment for certain types of AMD. If significant amounts of soluble iron are present, these would be first reduced to ferrous iron, and in the SRB reactor, simultaneous removal of sulphate and the generation of alkalinity could further treat the effluent from the first reactor.

With increasing pressure to find cost-effective, "environmentally friendly" solutions to the problem of AMD, much attention has been given to alternative treatments. Biotechnology provides an alternative to traditional chemical treatment, which may be detrimental to the environment. Organisms which are indigenous to acidic environments may have the greatest potential for bioremediation of acidic wastewaters, as they are likely to possess genetic components which make them able to not only survive but thrive in this extreme environment. Acidophilic SRB have the potential to offer a novel solution to the problem of AMD, but this would first require their isolation in the laboratory, the determination of their growth requirements, and an assessment of their performance at bench-scale. Acidophilic SRB, with a growth optimum pH of around 4.5 or lower could, in theory, be a preferred candidate for bioremediation of AMD in such a system. In addition, no pre-treatment to raise the pH prior to the SRB reactor would be required, thus eliminating one costly step in the bioremediation process.

To date, putative aSRB indigenous to AMD environments have not been investigated for such purposes, because there is no proof of their existence. While considerable numbers of proposed and actual bioprocesses have been described that utilise SRB (e.g. Thiopaq®, see 1.5.2), these strains or cultures are not acidophilic and have not been isolated from acidic, metal-rich environments, although some have been used for the biological treatment of wastewaters with low pH and elevated concentrations of metals (Maree and Strydom, 1985; Maree *et al.,* 1987; Barnes *et al.,* 1991; Scheeren *et al.,* 1991; Dvorak *et al.,* 1992; Scheeren *et al.,* 1993; Lyew *et al.,* 1994; van Houten and Lettinga, 1985; and van Houten *et al.,* 1995; Moriwaki *et al.*, 1997). In many cases, circumneutral conditions are provided for the SRB by pre-treatment with alkali (e.g. Dvorak *et al.,* 1992). In addition, SRB are often used in bioprocesses for the precipitation of metals as they can provide biogenic H_2S (Hammack *et al.,* 1993; Hammack *et al.,* 1994 a,b). Therefore, the use of 'SRB technology' is well-established, although the possibility of using acidophilic strains has not been seriously explored other than in some preliminary investigations (Sen and Johnson, 1999; Kolmert, 1999).

1.11 Aims and scope of the current research project

This research programme focused on acquiring fundamental information on acidophilic and/or acid tolerant SRB indigenous to naturally acidic and AMD sites, and to undertake preliminary investigations to assess the suitability of these novel isolates for use in bioremediation of acidic metalliferous waste-waters through the use of bench-scale bioreactor systems. Figure 1.13 summarises the approach to and the direction of the research.

In summary, the overall objectives of the research were:

- Verification of the existence of acidophilic/acid-tolerant sulphate reducing bacteria (aSRB);

- Isolation and identification of aSRB from samples taken from acidic derelict mine sites;
- Development of suitable methods for reproducible laboratory culture of aSRB, i.e. to develop and optimise culture media, liquid and solid;
- Evaluation of mixed communities of aSRB for the bioremediation of acidic, metal-rich waters using bench-scale batch and continuous bioreactor systems;
- Investigation of the microbial ecology of the bacteria in mixed communities;
- Purification and characterisation of isolates, and comparison of biodiversity, using biomolecular and traditional techniques.

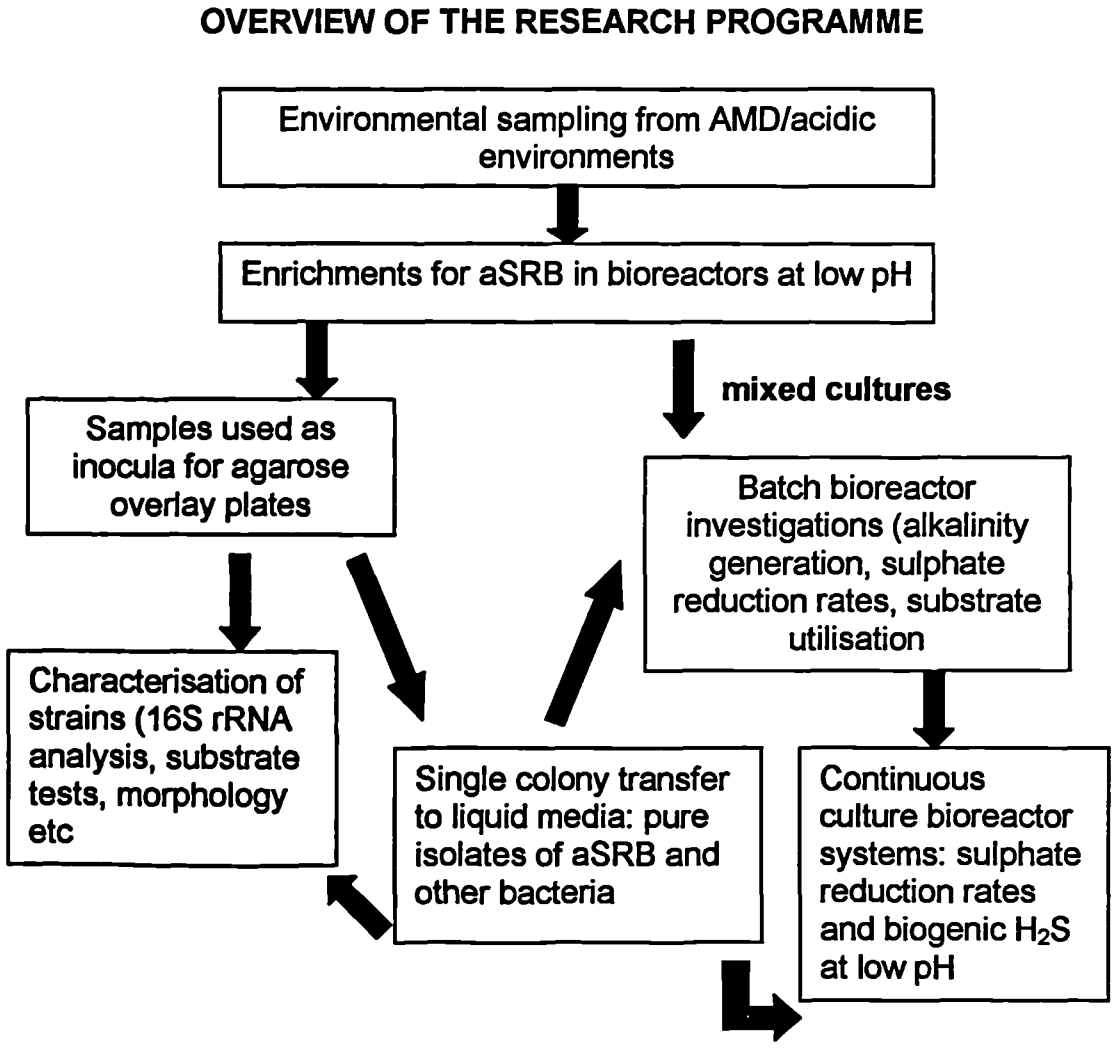


Figure 1.13. An overview of the proposed research proposal.

2. MATERIALS AND METHODS

The following methods were those used routinely throughout the research programme. Other, more specific methodologies and modifications to the standard techniques are described in later chapters. All chemicals used were supplied by BDH-Merck (UK) except where indicated, and were of 'Analar' grade when these were available from the suppliers.

2.1 Microbiological Techniques

2.1.1 Culture techniques

2.1.1.1 Liquid Media

Liquid media for the routine growth of aSRB cultures were prepared using a basal salts mixture containing the following (in g L^{-1} of distilled water):

$(NH_4)_2SO_4$: 0.45
KCl: 0.05
$MgSO_4 \cdot 7H_2O$: 0.5
KH_2PO_4: 0.05
$Ca(NO_3)_2 \cdot 4H_2O$: 0.014

A trace elements solution was added at 1 ml L^{-1} of medium; the stock solution contained (in g L^{-1} of 0.01 M H_2SO_4):

$ZnSO_4 \cdot 7H_20$: 10
$CuSO_4 \cdot 5H_2O$: 1
$MnSO_4 \cdot 4H_2O$: 1
$CoSO_4 \cdot 7H_2O$: 1
H_3BO_3: 0.6
$NiSO_4 \cdot 6H_2O$: 1
$Na_2WO_4 \cdot 2H_2O$:
$Cr_2(SO_4)_3 \cdot 15H_2O$: 0.5
$Na_2MoO4 \cdot 2H_2O$: 0.5
$NaVO_3$: 0.1
$Na_2Se_4O_4 \cdot 10H_2O$: 1

Organic amendments were made by adding yeast extract (usually 0.02% w/v), and glycerol, ethanol, and organic acids were added from stock solutions to the appropriate concentrations.

2.1.1.1.1 Standard Liquid Medium for aSRB

A standard liquid medium for culturing aSRB was prepared containing basal salts and trace elements (as above) together with 5 mM K_2SO_4, 5 mM $FeSO_4$; 0.5 mM Na_2S and 0.02% yeast extract (all expressed as final concentrations). Basal salts, potassium sulphate and iron sulphate solutions, together with yeast extract were combined, and the pH adjusted to ~ 3.2 using 1M H_2SO_4. The solution was bubbled with oxygen-free nitrogen (ORN; BOC gases, UK) until dissolved oxygen levels were below 20% of maximum. The medium was stirred gently and the sodium sulphide added slowly (from a 100 mM stock solution). A black precipitate of ferrous sulphide temporarily formed, but this dissolved after about 30 minutes of stirring. The pH was adjusted to 3.5 and the solution was stirred again for ~ 15 minutes until clear. The medium was then filter sterilised into sterile bottles using 0.2μm cellulose nitrate filters (Millipore Ltd.).

2.1.1.1.2 Modified Liquid Media

The standard liquid medium was amended with various small molecular weight organic compounds (glycerol, ethanol, lactic and acetic acids, added from sterile stock solutions) to 1-10 mM final concentration.

The standard liquid medium was poorly buffered, and the pH tended to increase markedly as a result of sulphate reduction, particularly in organic substrate-amended media. Attempts to improve the buffering capacity of the medium involved adding either aluminium sulphate (final concentration 5 mM) or zinc sulphate (final concentration 10 mM) to the standard medium, ahead of filter sterilisation. Both systems were used in an attempt to generate protons (consume hydroxyl ions) as:

(a) $Al(OH)^{2+} + OH^- \rightarrow Al(OH)_2^+$ (2.1)

(b) $H_2S + Zn^{2+} \rightarrow ZnS + 2H^+$ (2.2)

2.1.1.2 Bioreactor Cultures

Enrichment and experimental cultures of mixed populations of aSRB were grown in two bench-scale bioreactor systems (LH Fermentation Series 502, and Electrolab P350). Environmental samples (section 3.1.1 and 3.2.1) were used to inoculate 2L bench-scale bioreactors to enrich for aSRB. Enrichments were set up using basal salts/trace elements medium (as described in 2.1.1.1), with addition of glycerol (10 mM final concentration), $FeSO_4$ (also to 10 mM) and 0.02% yeast extract. The pH of the medium was adjusted to ~ 2.5 with 1M H_2SO_4 before autoclaving and was adjusted, as required, using 1M each of potassium hydroxide and sulphuric acid. The ferrous sulphate and glycerol solutions were added from sterile stocks (1M) after autoclaving. Bioreactors were filled to a working volume of 1.8 L, and the cultures were sparged with OFN to create and maintain anaerobic conditions. The pH of the enrichment cultures was set at a maximum value of 4.0, and the temperature maintained at 25°C. The cultures were not agitated (i.e. no stirring, except before measuring/adjusting pH or taking samples), sparged daily with OFN to remove hydrogen sulphide, and kept under darkened conditions by loosely shrouding the vessel with a black plastic sheet.

2.1.1.3 Use of 'Poraver' Beads

In some liquid cultures, and in bioreactor experiments, glass beads were incorporated to provide surfaces for colonisation by aSRB. In early experiments, solid glass beads of 0.2 and/or 1.5-2mm diameter glass balls (BDH, UK) were used. These were acid-washed with ~ 1M HCl before autoclaving with growth media. In later work, porous (>80% pore space) beads (manufactured by Dennert Poraver GmbH, Germany) of varying

size (2-4, 5-6 or >8 mm) were used in culture and bioreactor vessels. Several acid washes with 1M HCl was required to remove any traces of residual basic minerals (mainly soda glass). It was also necessary to remove air from within the porous beads, which was most readily achieved by by autoclaving them (at 121°C for 20 minutes), which resulted in air displacement, causing the beads to sink during cooling. The pH of the liquor was measured, the surplus liquor decanted and replaced with fresh acidic (pH ~ 3) medium/basal salts solution before re-autoclaving. This was repeated until the pH of the liquor did not change after autoclaving and air had been displaced from most of the porous beads.

2.1.1.4 Solid Media

Table 2.1 summarises the various solid media employed in the research programme for culture of aSRB. Sections 2.1.1.4.1 and 2.1.1.4.2 detail methods and materials for their preparation, as well as those of additional media designed for culture of other acidophilic microorganisms.

2.1.1.4.1 Overlay Media for aSRB

Samples from the bioreactor enrichment cultures were used to inoculate modified agarose 'overlay' plates (based on the ferrous iron overlay formulation; Johnson, 1995a), adjusted to pH 3 or 3.5, to allow selection of aSRB. Acidic overlay media have been shown to promote colony growth of heterotrophic as well as autotrophic acidophilic microorganisms (Johnson, 1995a). The general principle is that, by incorporating an acidiphilic heterotrophic bacterium to metabolise small molecular weight materials that are inhibitory to many acidophilic bacteria, agar(ose)-gelled media can be used to support the growth of both autotrophic and heterotrophic acidophiles (Johnson, 1995a; Johnson, 1999). Two variations on the overlay plate technique were used to develop media for aSRB:

(a) *Acidiphilium* SJH Overlay Plates:

These were used routinely throughout the research programme. The solid medium was prepared by combining three separately-sterilised solutions: (i) basal salts/trace elements (section 2.1.1.1)/0.027% yeast extract/6.7 mM potassium sulphate, adjusted to pH 5.0; (iii) 2% (w/v) agarose (Sigma Type I); (iii) 1 M ferrous sulphate (pH 2). Solutions (i) and (ii) were heat-sterilised (121°C, 20 mins.) and solution (iii) filter-sterilised, and these were combined in the ratio 149:50:1 (giving a 0.02% yeast extract/5 mM K_2SO_4/5 mM $FeSO_4$ solid medium). The final pH of the combined medium was 3.5. A double layer gel was poured. An acidophilic heterotrophic bacterium (*Acidiphilium* SJH; Johnson, 1995a), grown in a 10 mM galactose/0.025% (w/v) tryptone soya broth/basal salts medium (pH 2.5), was added (5 ml of culture/L) to the combined medium (held at ~45°C) and poured as a thin layer into sterile petri plates (~15 ml/plate). When set, this was covered with an overlayer (~20 ml/plate) of uninoculated combined medium. Prepared plates were left to stand at room temperature for a minimum of 2 days to facilitate detoxification of the medium, and then placed in an anaerobic chamber (section 2.1.2) for 1-2 days to remove any residual oxygen.

(b) *Acidocella* 'WJB-3' Overlay Plates:

These were prepared in the same way to the above, except that *Acidocella* WJB3 was used in place of the *Acidiphilium* culture. *Acidocella* WJB3, unlike *Acidiphilium* SJH, does not grow on yeast extract, though it does metabolise pyruvic acid (Hallberg *et al.,* 1999), which is considered to be an important inhibitory compound in acidic agar(ose)-gelled media (Johnson, 1995a). For this purpose, *Acidocella* WJB-3 was grown in a liquid medium containing 5 mM pyruvic acid/5 mM aluminium sulphate (for buffering at pH 3.5), basal salts and trace elements, pH 3.5, and the cells harvested at stationary phase. These were washed with acidified (pH 3.5) basal salts solution to remove traces of pyruvic acid, and 3 ml was added to the medium used for the underlayer gel.

There were some variations to the overlay media used for aSRB. This included the use of non-overlay yeast extract plates, and overlay plates to which different potential organic substrates (such as glycerol) were added (generally at ~10 mM). To avoid metabolism of the added substrates by the *Acidiphilium* SJH present in the underlayer, these were generally added after plate maturation/detoxification by spreading aliquots of stock solutions evenly over the surface of the gelled media. These plates were then inoculated promptly with aSRB, and incubated anaerobically (section 2.1.2) under which conditions *Acidiphilium* SJH is inactive (D. B. Johnson, personal communication).

2.1.1.4.2 Other Solid Media

Three other solid media were used on occasions, ferrous sulphate (FeO) and ferrous sulphate/potassium tetrathionate (Fe-tet) overlay media (Johnson, 1995a)., and washed agarose yeast extract plates (WAYE; non-overlay). These had been formulated to promote the growth of acidophilic iron- and sulphur oxidising bacteria, though some heterotrophic acidophiles could also grow on them.

For 10-12 plates of ferrous sulphate overlay medium, the following solutions were prepared: (a) 40 ml basal salts solution (g L^{-1} : $(NH_4)_2SO_4$, 12.5; $MgSO_4.7H_2O$, 5.), 0.1g tryptone soya broth, 245 ml distilled water, adjusting the pH to 2.5 with H_2SO_4; (b) 2 g agarose (Sigma Type 1) in 100 ml distilled water, and (c) 1M ferrous sulphate, pH 2.0. Solutions (a) and (b) were heat-sterilised separately (by autoclaving at 121°C for 20 minutes) and cooled to ~ 45°C. They were mixed and 10 ml of solution (c) was added. The combined molten medium was split ~ 50 : 50 in the containers used for sterilisation and one was inoculated with 5 ml of an active culture of *Acidiphilium* SJH, mixed and poured immediately as a thin (15-20 ml) under layer in standard petri plates. These were allowed to gel then covered with approximately the same volume of sterile medium (kept

Solid medium	Constituents*	Organic amendments**	Experimental use(s)	Section reference(s)
SJH overlay (two-phase)	BS/YE/TE/K_2SO_4/$FeSO_4$/ agarose/*Acidiphilium* SJH (pH 3.5)	YE only/GLY/GAL/LA	Routine culture/ enumeration of aSRB	3.5.1; 4.3.1; 4.3.2; 4.3.3; 4.3.5; 5.2.1.1.5; 5.2.1.2.6
WJB-3 overlay (two phase)	BS/YE/TE/K_2SO_4/$FeSO_4$/ agarose/*Acidocella* WJB-3 (pH 3.5)	YE only/LA/AC/PY/MAN/GLY /ETH/MET/GLU/GAL/RIB/ GLN/	Enumeration/substrate utilisation/routine culture	4.3.5; 5.3.2.1; 6.3
YE (single phase)	BS/YE/TE/K_2SO_4/$FeSO_4$/ agarose (pH 3.5)	YE occasionally replaced with TSB	Growth requirements of aSRB/screening for other microorganisms	3.5.2
WAYE/AW (single phase)	BS/$FeSO_4$/acid washed agarose (+/- YE) (pH 3.5)	YE only	Growth requirements of aSRB	4.3.3

Table 2.1. Summary of solid media used for the growth of aSRB. *BS= basal salts; YE= yeast extract; TE= trace elements, TSB= tryptone soya broth. **GLY= glycerol; GAL= galactose; LA= lactic acid; AC = acetic acid; PY= pyruvic acid; MAN= mannitol; ETH= ethanol; MET= methanol; GLU= glucose; RIB= ribose; GLN= glycine.

molten in a water bath at ~45°C) to form an overlayer. Plates were used immediately or stored at room temperature for up to a week. FeTSB medium was the same medium without an underlayer that included *Acidiphilium* SJH. Overlay variants were made using this medium but by incorporating a layer with the addition of SJH as described above.

Washed agarose yeast extract (WAYE) non-overlay medium was made by stirring 2.5 g of agarose with 10 mM H_2SO_4 for about 15 minutes, to remove acid-soluble constituents. The suspension was then neutralised using 1 M KOH, the liquor removed and the agarose rinsed briefly with distilled water. The agarose was then autoclaved with 100 ml of distilled water; in a separate vessel, 40 ml basal salts solution (g L^{-1} : $(NH_4)_2SO_4$, 12.5; $MgSO_4.7H_2O$, 5) was combined with 260 ml of distilled water, and also autoclaved, after adjusting the pH to 3.5 with 1M H_2SO_4. After autoclaving and cooling as described above, the solutions were combined, ferrous sulphate was added to a final concentration of 500 μM, and the medium poured into petri plates.

Yeast extract plates were used in some experiments. These had exactly the same constituents as the yeast extract overlay plates but without using a layer containing SJH or WJB-3., i.e. a single layer.

2.1.2 Anaerobic systems

Solid media and test tubes containing liquid media were placed into anaerobic jars or sleeves. Two types of anaerobic system were used (Imhof and Heinzer, 1996; Brazier and Hall, 1994). In the first, the Oxoid Anaerobic System (Oxoid Unipath Ltd., UK), anaerobiosis was generated in an air-tight, sealed 3.5 litre jar unit (HP011A) from the addition of 10 ml of water to tablets containing sodium borohydride, sodium bicarbonate and tartaric acid. An activated (by pre-heating to 160°C) palladium catalyst was fixed in the lid of the jar (to catalyse the reaction $H_2 + 0.5O_2 \rightarrow H_2O$) and

an anaerobic indicator strip (Oxoid, BR55) containing resazurin was included to confirm the anaerobic status of the internal atmosphere. Hydrogen and carbon dioxide are generated in this system in a ratio of approximately 5:1. In the second system (Oxoid AnaeroGen™) anaerobic conditions were achieved in 2.5 L sealed jars through the use of sachets containing ascorbic acid which are activated by exposure to air. A carbon dioxide atmosphere of 9-13% is generated in this system but no hydrogen is produced. In a similar, smaller scale system (AnaeroGen Compact), plastic sleeves were used which could hold up to 4 petri plates. A controlled atmosphere chamber (Plas Labs, Lansing, MI, U.S.A) was used for plating experiments and for storage of some media.

In one experiment, microaerobic conditions (oxygen ≤ 5 %) were required. The CampyGen™ system (Oxoid) is designed in a similar way to AnaeroGen™ through the use of a sachet placed in a sealed jar. No indicator strips were required.

2.1.3 Gram staining

A crystal violet dye solution was made by mixing 2 g of crystal violet with 20 ml of 95% (w/v) ethanol (solution A) with 0.8 g of ammonium oxalate and 80 ml distilled water (solution B). Solutions A and B were mixed and allowed to stand for 48 hours before use (the solution is stable for 2-3 years). Lugol's solution was made by mixing 1 g of crystalline iodine and 2 g potassium iodide in a mortar and dissolved in 300 ml distilled water. A 0.5% (w/v) of safranin (the counter-stain) was made with distilled water, and the decolourising agent was 95% (v/v) ethanol. Liquid cultures or colonies of bacteria were used in the test. Where colonies were used, about 10 µl of potassium chloride (8.5 g L^{-1}) was pipetted onto a clean microscope slide and a loop of bacteria from a well-isolated colony was spread into the drop and over the surface of the slide. The smear was dried by gently warming the underside of the slide. This was stained for

one minute with the crystal violet solution and then rinsed briefly under running water. The smear was then treated with with Lugol's solution for 1 minute and rinsed again with water. Decolourisation was carried out by running the decolourising agent over the slide until no more dye ran from the smear, and was then immediately rinsed with water. The smear was then counter-stained for 10 seconds. Finally the smear was rinsed with water, blotted dry and examined under the microscope (magnification 1000x). Gram-negative cells appeared red-pink and Gram-positive cells appeared blue-violet.

2.1.4 Freezing and storing of bacterial cultures

Long term storage of cultures was carried out by addition of dimethylsulphoxide (DMSO; 7%, final concentration) to bacterial suspensions and freezing in cryo-vials at -70°C. To recover cultures, tubes were removed from storage, and frozen cell stocks put into an ice-bath without allowing them to thaw. A small amount of the frozen culture was used to inoculate fresh sterile growth medium by gently scraping in some of the frozen material using a sterile loop; the minimum amount was therefore used so as not to transfer large quantities of DMSO to new cultures.

2.2 Analytical Techniques

2.2.1 Measurement of pH, redox potential and oxygen

pH and redox potentials were measured *in situ* in bioreactors, using either Ingold or Mettler Toledo InPro combination pH or, redox electrodes (MC805). The pH of liquid samples was measured *ex situ* with a pHase rapid renew glass electrode (BDH-Merck), while those of gelled media were measured using a combined pH/reference cell with a flat surface membrane (model CFW711, Russell pH Ltd., U.K.) Redox potential was measured *ex situ* with a combined platinum/reference redox cell (Russell). The pH and E_h electrodes were employed (*ex situ*) in association with an

Accumet® pH meter 50 (which was also used for measuring redox); or a DR 359 Tx Series 3 pH meter (EDT Instruments). Both were calibrated using appropriate buffer solutions (Whatman, UK) between pH 2 and 7. The performance of the redox electrode was tested periodically by immersing it in a standard ferrocyanide/ferricyanide solution.

Dissolved oxygen (DO) was measured *in situ* with a Mettler Toledo polarographic oxygen electrode. However, their use was limited as the ferrous sulphide formed in most aSRB cultures coated the membrane, disabling the electrode. *Ex situ* measurements of DO were made with a polarographic DO electrode connected to a Jenway 9015 dissolved oxygen meter (Philip Harris Scientific, UK), and with a portable dissolved oxygen meter (Whatman International Ltd., England; DO 400).

2.2.2 Sulphate determination

Concentrations of sulphate were analysed using the turbidimetric method described by Rand *et al.* (1975). This is based on the principle that sulphate ions are precipitated in a hydrochloric acid medium with barium chloride to form uniform barium sulphate crystals. Ten ml of sample was placed in a 20 ml beaker, 0.5 ml of a 'conditioning reagent' (a mixture of 25 ml glycerol, 15 ml concentrated HCl, 150 ml distilled water, 50 ml of 95% ethanol, and 37.5 g NaCl) added, followed by about 0.75 g of barium chloride dihydrate crystals (Lancaster, UK). The suspension was stirred for 1 minute, and the absorbance at 420 nm measured after a further 2 minutes (when maximum turbidity was most likely to occur) using a Cecil CE 1011 1000 Series Digital UV spectrophotometer. Sulphate ion concentrations were determined by comparison of the reading with a polynomial standard curve in the range 0 – 40 mg L^{-1} ; samples were diluted as necessary (usually 50x) before determinations were made. Standard solutions were made using sodium sulphate decahydrate. Corrections for the colour and turbidity present in samples were made by

running blanks from which the barium chloride was withheld. All measurements were carried out in triplicate, and means calculated. A standard curve was prepared before each batch of samples was analysed. A typical standard curve, with an equation for the line, is shown in Appendix 2.1(a). The minimum detectable concentration of sulphate using this method is approximately 1 mg L^{-1}.

Later in the research programme, sulphate measurements were made using a modified version of the turbidimetric method. This was developed by Kolmert *et al.* (2000) and allowed for more rapid determination of sulphate, as the stirring period was reduced to 30 seconds, and the absorbance could be measured immediately. 1ml of conditioning reagent was added to 1 ml of a diluted sample (10x) during vortexing in test tubes, and barium chloride (approximately 100 mg) was added. A detailed comparison of the two methods was carried out, and no significant differences in results obtained from measurements were observed.

2.2.3 Sulphide determination

Dissolved sulphide was determined according to the method of Cord-Ruwisch (1985). In a slightly modified method, a sulphide-containing solution was added to a copper sulphate solution. A dark brownish colloidal CuS solution, which remains stable for 20-40s, was formed. The copper sulphate reagent was made with 5 mM $CuSO_4$ in 50 mM HCl. To 0.1 ml of sample, 1.9 ml of copper reagent was added during stirring. Immediately following stirring for 5 seconds, the reagents were placed in an acryl cuvette and the absorbance read at 480 nm against a blank of 1.9 ml of distilled water and 0.1 ml of sample, using a Cecil CE 1011 1000 Series Digital UV spectrophotometer. Standards were prepared using sodium sulphide over the expected experimental conditions (e.g. 1-15 mM), in anoxic distilled water. A 50 ml stock of 100 mM sodium sulphide was made in water that had been bubbled with OFN for 15 minutes, and the stock solution was kept in a serum bottle with a thick rubber stopper.

Standard solutions were prepared in anoxic water in serum bottles using additions from the stock solution. The standards were stable as long as they remain anoxic. All measurements were carried out in triplicate, and means calculated. A standard curve was prepared before each batch of samples was analysed. A typical standard curve, with an equation for the line, is shown in Appendix 2.1(b). The minimum detectable concentration of sulphide using this method is approximately 0.1 mM.

2.2.4 Ferrous iron determination

Ferrous iron was measured by the colorimetric method using ferrozine (Stookey, 1970; Lovley and Phillips, 1987). The assay is based on the chelation 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) to form a stable magenta-coloured complex ferrous iron-ferrozine, which exhibits a single sharp peak with maximum absorbance at 562 nm. Ferrozine reagent was prepared by dissolving 1 g of ferrozine (Sigma Ltd.) in 1 litre of 50 mM HEPES buffer (pH 7.0; Sigma Ltd.). A 0.1 ml aliquot of filtered culture was added to 1.9 ml of ferrozine reagent, mixed thoroughly and absorbance read at 562 nm in acryl cuvettes, against a water blank, using a Cecil CE292 spectrophotometer. Standards were prepared with ferrous sulphate ($FeSO_4$. $7H_2O$) over the range 0-1 mM (the range at which the method is most accurate), and thereafter samples were diluted as necessary (10-50x) in 10 mM H_2SO_4 for comparison with the standard curve. All measurements were carried out in triplicate, and means calculated. A standard curve was prepared before each batch of samples was analysed. A typical standard curve, with an equation for the line, is shown in Appendix 2.1(c).

2.2.5 Glycerol determination

Glycerol concentrations in cultures were measured using three different methods according to the available resources, the number of samples to be analysed, and convenience: the colorimetric assay described by Pesez and Bartos (1974); high pressure liquid chromatography (HPLC); and by an enzyme coupled assay (UV method) with a test-combination kit (Boehringer Mannheim GmbH, Biochemicals).

In the colorimetric analysis for 1,2-diols (Pesez and Bartos,1974), the principle is that oxidation with periodate ion and development of the formaldehyde formed with phenylhydrazine and ferricyanide ion gives a red colour (formazan dye). The reagents used were: (a) a 6.6% aqueous solution of anhydrous sodium metaperiodate; (b) a saturated solution of potassium nitrate; (c) a 1% aqueous solution of phenylhydrazine hydrochloride, freshly prepared; (d) a 2% aqueous solution of potassium ferricyanide, freshly prepared; (e) concentrated hydrochloric acid, and (f) ethanol. To 0.5 ml of aqueous solution of the filtered sample (0.2 μm cellulose nitrate, Millipore), 2.5 ml of reagent (a) was added and allowed to stand at room temperature for 20 min. This was chilled in an ice bath to 0°C, and the excess sodium periodate was precipitated out with 0.5 ml of solution (b) and left for 10 min at 0°C. To a 1 ml aliquot of the clear supernatant, 2 ml of reagent (c) and 1 ml reagent (d) were added and left to stand at 0° C for 20 min. Five ml of concentrated hydrochloric acid and 5 ml of ethanol was slowly added with stirring, and the mixture left to stand at room temperature for 15 min. The absorbance of the final reaction mixture was read at 520 nm. Samples were read against a blank in which MilliQ water had been taken through the assay procedure (a reagent blank), with a Cecil CE 1011 1000 Series Digital UV spectrophotometer. In the original protocol, glycerol concentrations were calculated from the basis that for A = 0.3 (1cm cell), glycerol = 25 μg. Measurements were carried out in duplicate, and means calculated. A standard curve was

prepared before each batch of samples was analysed. A typical standard curve, with an equation for the line, is shown in Appendix 2.1(d).

Glycerol was also measured using a Kontron HPLC fitted with an Aminex column and a UV detector. HPLC was carried out using a Kontron system, with an Aminex HPX-87H Organic Analysis Column (300 x 7.8 mm) (BIORAD), an HPLC 560 autosampler, an HPLC 535 detector, and a System 525 injector/pump (pump 525C and injector SA 560). Kromasystem 2000 software was used to process data. The water jacket surrounding the column was heated to 35°C. Samples to be analysed were filtered using 0.2µm cellulose nitrate filters to remove cells and precipitates that could block the column. Samples were eluted from the column using 0.02 N H_2SO_4 at a flow rate of 0.6 ml/min. Peaks were detected by measuring absorbance at 195 nm, and were compared with those obtained by running known standards between 0.5 and 10 mM.

For routine measurement of glycerol concentrations using the enzymatic bioanalysis, filtered samples were analysed by adding a series of reagents to give the series of reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{L-glycerol-3-phosphate} + \text{ADP} \qquad (2.3)$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{phosphokinase}} \text{ATP} + \text{pyruvate} \qquad (2.4)$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+ \qquad (2.5)$$

(LDH = lactate dehydrogenase)

The amount of NADH oxidised in the above reaction is stoichiometric to the amount of glycerol; NADH was determined by reading absorbance at 340 nm using a Cecil CE 1011, 1000 Series Digital UV spectrophotometer.

With this method, the glycerol content present in the cuvette has to be between 1 and 40 μg; the minimum detectable concentration is effectively approximately 0.48 mM, when taking this into account. All samples were analysed in duplicate and means calculated.

2.2.6 Ethanol determination

Ethanol concentrations were measured using an enzyme-coupled assay in a test-combination kit by Boehringer Mannheim (Boehringer Mannheim GmbH, Biochemicals), based on the following reactions:

$$\text{Ethanol} + NAD^- \xrightleftharpoons{ADH} \text{acetaldehyde} + NADH + H^+ \quad (2.6)$$

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{Al\text{-}DH} \text{acetic acid} + NADH + H^+ \quad (2.7)$$

(ADH = alcohol dehydrogenase; Al-DH = aldehyde dehydrogenase)

All samples were analysed in duplicate with a Cecil CE 1011, 1000 Series Digital UV spectrophotometer at 340 nm, and means calculated. The amount of ethanol present in the cuvette must be between 0.3 and 6 μg at this wavelength; the minimum detectable concentration is effectively approximately 0.22 mM, when taking this into account.

2.2.7 Acetic acid determination

Acetic acid concentrations were measured using enzymatic bioanalysis with a test-combination kit, as for glycerol and ethanol (Boehringer Mannheim). The test utilised the following reactions:

$$CH_3COO^- + ATP + CoA \xrightarrow{\text{acetyl CoA synthetase}} \text{acetyl CoA} + AMP + \text{pyrophosphate} \quad (2.6)$$

$$\text{Acetyl CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\text{citrate synthase}} \text{citrate} + CoA \quad (2.7)$$

$$\text{L-Malate} + NAD^+ \xleftrightarrow{\text{L-malate dehydrogenase}} \text{oxaloacetate} + NADH + H^+ \quad (2.8)$$

The determination is based on the formation of NADH measured by the increase in light absorbance at 340 nm. All samples were analysed in duplicate, with a Cecil CE 1011, 1000 Series Digital UV spectrophotometer, and means calculated. The amount of acetic acid present in the cuvette must be between 0.3 and 15 μg at this wavelength; the minimum detectable concentration is effectively approximately 0.5 mM, when taking this into account.

2.2.8 Protein determination

Protein concentrations were measured by the Folin-Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as a standard, scaled to measure protein concentrations of 10 - 200 μg ml^{-1} (the determination of as little as 0.2 μg is possible with this method). Due to the presence of ferrous sulphide in aSRB cultures, cells were first washed 2-3 times in 10 mM H_2SO_4 (with centrifugation at 9300 x *g* for 4 minutes using a bench-top Biofuge 15 centrifuge (Heraeus, SEPATECH) followed by 1 wash with distilled water. Pellets were resuspended in 0.1 M NaOH and left for 15 minutes at room temperature, for harvesting of cell protein by lysis. The following reagents were required: (a) 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide; (b) 1% (w/v) copper sulphate and 2% (w/v) sodium tartrate, mixed 1:1; and (c) 50 ml of (a) with 1 ml of (b) mixed immediately before use. Solutions (a) and the components of (b) were kept as stock solutions at room temperature. The indicator, Folin-Ciocalteau's reagent, was diluted 1:2 with distilled water before use. BSA was mixed with distilled water to make standards. To 0.1 ml of sample, 1 ml of reagent (c) was added and the solution left to incubate at room temperature for 10 minutes. 0.1 ml of the diluted Folin-Ciocalteau's reagent was added and

the solution incubated as before for another 10 minutes. Absorbance was then read at 750 nm, using the reagent mixture plus 0.1 ml of water as a blank. A standard curve was required for each test run as reagent (c) was always made up directly before use. All analyses were carried out in duplicate, with a Cecil CE 1011, 1000 Series Digital UV spectrophotometer, and means calculated. A typical standard curve, with an equation for the line, is shown in Appendix 2.1(e).

2.2.9 ATP determination

Concentrations of ATP in samples taken from bioreactors were determined to give a measure of bacterial biomass. Analyses were carried out using the systemSURE™ hygiene monitoring kit, which is a bioluminescence test system designed for use in the food and manufacturing industries. The system allows detection of ATP concentrations as low as 10^{-15} M. It consists of an enzyme reagent (luciferase), an enzyme buffer, and a swabbing (lysis) solution, luminometer tubes, a pipette for dispensing 400 µl and pipette tips. The enzyme was reconstituted using the enzyme buffer and allowed to warm to room temperature before use. To measure ATP in the bacterial samples, the sample was mixed well and 100 µl placed into a clean plastic luminometer tube. Swabbing (lysing) solution (100 µl) was added to release ATP from biomass and mixed by gentle swirling. After waiting for about 15 seconds, 400 µl of the enzyme reagent was added, the tube capped and placed directly into the luminometer. A reading of relative light units (RLU) was given for each sample. Analyses were performed in triplicate for each sample and compared with a blank using sterile MilliQ water; this was subtracted from the average value obtained for the sample. Over the range 10-100,000 RLUs, there is an approximate relationship of 10 RLU = 1 femtomole (*f*mol; 10^{-15} M) ATP.

2.2.10 Atomic Absorption Spectroscopy (AAS)

Atomic absorption spectroscopy was used to determine concentrations of copper in bioreactor experiments (Chapter 5). Standards of 0, 1, 2, 4, 8 and 10 ppm copper (II) were prepared using a standard solution of copper nitrate, and a standard curve was produced for each set of samples to be analysed. The sensitivity of the spectrophotometer allowed for a minimum detection limit of 0.035 mg L^{-1} of copper. Therefore, samples were diluted 100-fold with double-distilled water before measurements were made. Determinations were made using an atomic absorption spectrophotometer (Pye Unicam SP2900), with a hollow cathode copper lamp and a gas control unit (Pye Unicam, SP9-10). Samples were combusted in an air/acetylene flame (air 4.5L/min, acetylene 11L/min), and their absorbance measured at 324.8 nm. All analyses were carried out in triplicate and means calculated. A typical standard curve, with an equation for the line is shown in Appendix 2.1(e).

2.3 Microscopic Techniques

2.3.1 Stereomicroscopy

Colonies of aSRB grown on solid media were viewed or counted using a stereomicroscope (Leitz-Wild M32, Switzerland) with a magnification range from x 50 to x 400, which was convenient for viewing a wide range of colony sizes. A camera (Olympus OM –2) was fitted to the microscope to photograph colonies.

2.3.2 Phase contrast microscopy

For viewing cells or for using the Thoma counting chamber to determine cell numbers (section 2.3.2.1), a Leitz "Labolux" phase contrast microscope fitted with a Zernike condenser and objective was used (magnification x 250 to x 400). Estimations of cell sizes, culture density,

description of cell morphology and absence/presence of spores were routinely made. Bacterial spores were simple to observe; being highly refractile they can be observed as bright circular bodies.

Cell counts of planktonic bacteria were made using a Thoma counting chamber, consisting of a grid set within a microscope slide. Cells were dispersed in the sample as thoroughly as possible, and a small drop (around 7 µl) was placed into the centre of the slide. The sample was then viewed using phase contrast microscopy (magnification x 400). Bacterial cells were counted in the small squares. If cells were highly motile, formaldehyde was added at 50 µl (of 37% solution) per ml of sample. At least 50-100 bacteria were counted to get a good estimation of cell numbers. The number of small squares that were used was noted. The calculation used was as follows:

Distance between each of the parallel lines on the chamber = 50 µm
∴ Area of each square = 50 x 50 = 2500 μm^2
Depth of sample with cover slip in place = 20 µm
∴ Volume of the sample in each small square = 50 000 μm^3 (5 x 10^4 μm^3)

The average number of bacteria per ml was calculated by multiplying the average number of bacteria per small square by 2 x 10^7 (i.e. 10^{12}/5 x 10^4).

Cells that had been Gram stained (section 2.1.3) were observed on the prepared slides using a Jenalumar SH50 microscope (Zeiss) with a magnification of x 1000.

2.3.3 Scanning electron microscopy (SEM)

Bacterial cultures were prepared for SEM by drying thoroughly without damaging the cell structure. This required a number of detailed procedures before viewing and photography was carried out:

(1) dehydrating and fixing cells; (2) critical–point drying; (3) sputter coating with gold particles.

2.3.3.1 Dehydration and Fixation

In stage (1), the following reagents and materials were used: basal salts solution, pH 3; 0.1 M H_2SO_4; or "MilliQ" water; ethanol solutions: 30%, 60%, 90%, 100% (in MilliQ water, v/v); 4% gluteraldehyde; Swinnex filter units fitted with polycarbonate membranes (0.2 µm pore size, 12 mm diameter; Nuclepore Inc. USA); 5 ml syringes; clamps, and a punctured 1.5 ml eppendorf tube for each sample to be prepared (made by piercing the tube in several places with a hot wire). All liquid samples were pre-filtered (0.2 µm cellulose nitrate filters) before use. The cell density of each culture was determined using the Thoma counting chamber to assess how much of each was required. The filter unit was assembled and the membrane pre-wetted by flushing with water, the culture suspension filtered through the fitted membrane and washed by flushing with (in sequence) 1ml of H_2SO_4, 3ml of basal salts, and 3ml of MilliQ water. The cells were then fixed using gluteraldehyde; 2 ml aliquots were dispensed into the syringe and about 0.5 ml pushed through. This was held for 10-15 minutes, ensuring that a reservoir of gluteraldehyde remained above the membrane. The remaining gluteraldehyde was then flushed through the membrane. Immediately following this step, the sample was dehydrated with ethanol, in sequence: 30%, 60%, 90%, 100% (twice). As with the previous step, 3 ml of each were put into the syringe, 1ml flushed through and the last 2 ml held in the syringe for 10-15 minutes. After the final flush with 100% ethanol, the filter unit was dismantled and the membrane carefully removed, noting on which surface the cells were attached and placed into a punctured eppendorf tube. The tube was placed in a sealed vessel containing 100% ethanol.

2.3.3.2 Critical-Point Drying

The eppendorf tubes were placed into the critical point drying chamber (Polaron) and the ethanol replaced with liquid CO_2 by allowing the gas to enter to 800 lb/in^2 , venting into the load chamber, and flushing every 15 minutes for 2½ hours. At the final flush, the temperature was allowed to rise to about 37°C (the point at which liquid and gas are in equilibrium) and the pressure to rise to about 1400 lb/in^2. At this point the gas was evacuated very slowly, ensuring that the temperature stayed between 34 and 40°C. At 0 lb/in^2, the sample was removed, and was totally dry, with no cell destruction. The sample was at this point ready for sputter coating. The sample was removed from the tube, placed on a SEM stub and placed in the sputter coating chamber (Polaron E5000) to be sputtered with gold. Following this, the stub was placed in the SEM (Hitachi S-520) for viewing and photography. Images were taken using Ilford PAN F film and developed in AGFA Rodinal.

2.4 Biomolecular Techniques

2.4.1 Chromosomal DNA isolation and purification

The protocol was based on a method published by Wilson (1987), and the reagents used were: TE buffer (10mM Tris (tris (hydroxymethyl) methylamine) HCl + 1mM EDTA (pH 8); 20% SDS (20% sodium dodecyl sulphate in deionised H_2O); proteinase K (20 mg ml^{-1} deionised H_2O), stored at –20°C; CTAB/NaCl solution (warmed slightly above room temperature to make it less viscous); NaCl (5M); sodium acetate (408.1 g sodium acetate·3H_2O was dissolved in 800 ml deionised H_2O, the pH adjusted to 5.2 using glacial acetic acid, and made up to 1L in a volumetric flask with deionised H_2O); phenol/chloroform (25 parts phenol: 24 parts chloroform: 1 part isoamyl alcohol; chloroform (24 parts chloroform: 1 part isoamyl alcohol); isopropanol, and 70% ethanol. The TE buffer, the sodium acetate and the NaCl solutions were autoclaved before use to

completely inactivate any DNAses. As the aSRB cultures did not have a very high cell density, 200 ml volumes of each of the cultures were weighed out into 250 ml centrifuge bottles (within 1 g of each other). The cultures were centrifuged at 10 000 x *g* and 4°C for 20 minutes, in a Beckman JA-14 fixed angle rotor. The pellet that formed was scraped and pipetted out, using a Gilson pipette, into sterile eppendorf tubes and diluted with a little of the respective liquid cultures. These were centrifuged at 10 000 x *g* for 30 seconds, the supernatant removed, and the pellet resuspended in deionised H_2O (pH 1.8), then centrifuged for 5 minutes at 2300 x *g*. The pellet was washed twice at this low speed to attempt to remove the iron sulphide precipitates from the pellet. Following the washing, the pellet was resuspended in 564 µl of TE buffer in 1.5 ml eppendorf tubes, and 15 µl of 20% SDS and 6 µl of proteinase K added, mixed thoroughly, and incubated at 37°C for 1 hour. NaCl (5 M, 100 µl) was added carefully and tubes were inverted several times to mix (not vortexed as DNA would shear). CTAB (hexadecyltrimethyl ammonium bromide)/NaCl (80 µl) was added, followed by incubation at 65°C for 10 minutes. Chloroform/isoamyl alcohol solution (600 µl) was added and gently but thoroughly mixed, followed by centrifugation at 10 000 x *g* for 5 minutes. The extraction removed CTAB-protein/polysaccharide complexes. A white interface was visible following centrifugation; this was left in the tube and the supernatant decanted into a fresh microcentrifuge tube. To this, 1 volume of phenol/chloroform/isoamyl alcohol was added, mixed and the tubes centrifuged again for 5 minutes. The supernatent was transferred to a fresh tube and 1 volume of isopropanol added (to precipitate nucleic acids; no salt was required as the NaCl concentration was already high). The tubes were shaken back and forth until a stringy white DNA precipitate was clearly visible. The tubes were placed on ice for 10 minutes then briefly centrifuged (at approximately 10 seconds at 6000 x *g*) to pellet the DNA. The DNA was then washed with 70% ethanol to remove residual CTAB and spun for 1 minute at 2000 x *g*) to repellet it. The supernatent was carefully removed by pipetting and the pellet was air-

dried. The pellet was redissolved in 100 µl TE buffer for PCR, cloning and sequencing (section 2.4.2; Chapter 6). All steps were carried out at ~20°C.

2.4.2 Amplification of eubacterial 16S rRNA genes using the polymerase chain reaction (PCR)

The 16S rRNA genes of selected bacteria were amplified from colonies of the bacteria using universal primers "27f" (forward) and "1492r" (reverse) (EUB338; Snaidr *et al.,* 1997). The forward primer is complimentary to positions 8 to 27 of *Escherichia coli* 16S rRNA and has the sequence 5'-AGAGTTTGATCCTGGCTC-3'. The reverse primer compliments positions 1510 to 1492 (Lane, 1991) and has the sequence 5' TACGGYTACCTTGTTACGACTT-3'. The reagents that were not supplied were made with sterilised deionised water ("MilliQ"). Reagents required were: *Taq* polymerase, supplied as a 5u μl^{-1} stock (Promega Corporation, Madison, WI, USA); 10x reaction buffer supplied with enzyme; 25 mM $MgCl_2$ supplied with enzyme; dNTP stock containing 2 mM each of dATP, dCTP, dTTP and dGTP; forward primer stock (100 ng μl^{-1}); reverse primer stock (also 100 ng μl^{-1}); lysis solution: 0.05 M NaOH + 0.25% sodium dodecyl sulphate (SDS); and sodium acetate solution (3M sodium acetate adjusted to pH 5.0 with glacial acetic acid and autoclaved. A 5x solution of TBE buffer was prepared using 54 g Tris, 27.5 g boric acid, 20 ml of 0.5 M EDTA (ethylenediaminetetra-acetic acid disodium salt) stock, and MilliQ water added to 1000 ml before autoclaving. EDTA stock was made with 36.53 g EDTA (adjusting pH to 8 with 10 M NaOH), and adding MilliQ water to 250 ml before autoclaving. The DNA was prepared by placing fresh colonies of aSRB (a small number of large colonies or a large number (~ 50) of small colonies into 20 µl of lysis solution in 0.5 ml eppendorf tubes; these were placed into the thermocycler (Progene, Techne) at 95°C for 10 mins for cell lysis. Following this, the DNA preparations were diluted tenfold with MilliQ water, to 'dilute out' any inhibitory substances (such as nucleases) that might be present; the use

of undiluted samples resulted in failure of PCR. These were kept on ice, along with all the reagents that were taken from storage at –20°C and allowed to thaw. A "master mix" for X reactions (X being the number of individual reactions) was then prepared (leaving the addition of DNA and *Taq* polymerase until last); this consisted of 5X µl of 10X reaction buffer; 5X µl of 25 mM $MgCl_2$; 5X µl of dNTPs; 1X µl of forward primer; 1X µl of reverse primer; 31.5X µl MilliQ water. 48.5 µl of the mastermix was aliquoted into fresh, sterile 0.5 ml eppendorf tubes (clearly marked), and 1 µl of the cell lysate added, followed by 0.5 µl of *Taq* polymerase. The tubes were placed into the thermocycler for PCR, with the following cycles: 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing), and 72°C for 2 minutes (elongation); this was followed by a final 10 minute incubation at 72°C (a final elongation step). A "cold-hold" at 4°C was used for up to 48 hours if immediate analysis was not possible. Confirmation that the 16S rRNA gene had been successfully amplified was accomplished by analysing 5 µl of the PCR reaction(s) using 1 µl of 6X loading buffer (30% (v/v) glycerol, 0.1% (w/v) bromophenol blue, and 60 mM EDTA (from a 0.5M stock solution) with a GIBCO BRL 1kb ladder (250 ng), on a 0.7% agarose tris-borate-EDTA (TBE) gel, made with 0.5x TBE buffer (from a 5x stock consisting of 54 g Tris (typically 0.21 g of agarose was heated in the microwave with 30 ml TBE to give a 30 ml gel, which was suitable for the mini-gel apparatus, supplied by BioRad). The gel was run at about 50 volts for around 45 minutes (or until the blue dye was observed to have migrated about ¾ of the way down the gel) using an electrophoresis power supply SL-3656 (Scotlab Bioscience, UK). Gels were carefully removed and placed in a bath of ethidium bromide (approximately 10 µg ml^{-1}) for around 15 minutes to stain the DNA. The DNA patterns were observed using a short-wave UV transilluminator. If the PCR was successful, DNA was precipitated by adding $^1/_{10}$ volume of sodium acetate solution and 2.5 X volume of absolute ethanol stored at –20°C. DNA was collected by centrifugation at 4°C and 9300 x *g* for 15 minutes. The pellet was washed

with 250 µl of 70% ethanol at 4°C and centrifuged again as previously. The ethanol was then evaporated off and the DNA pellet resuspended in 9 µl of MilliQ water. The DNA was then stored at –20°C.

2.4.3 Cloning of 16S rRNA genes

The PCR products obtained for the various bacterial strains using the above procedure were joined to a cloning vector (pCR 2.1-TOPO Invitrogen, vector length 3.9 kb) using a TOPO TA® cloning kit. One µl of the PCR product was mixed gently with 3 µl of autoclaved MilliQ water and 1 µl of the pCR-TOPO vector and incubated for exactly 5 minutes at room temperature, in 0.5 ml eppendorf tubes. The tubes were put on ice before proceeding to the transformation using TOPO10 'One Shot' competent cells. Two µl of each cloning reaction were added to vials containing the thawed competent cells, mixed, and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C using a heating block for 30 seconds, and transferred immediately back to ice. SOC medium (consisting of 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added (250 µl) and the tubes placed into incubation at 37°C with horizontal shaking for at least 30 minutes. The tubes were placed back on ice and 50 µl from each transformation was spread on LB-agar plates, containing 100 µg ml^{-1} ampicillin or 50 µg ml^{-1} of kanamycin, that were warmed at 37°C for 30 minutes and spread with 20 µl of 4% X-gal in DMF (dimethylformamide) before use. LB (Luria-Bertani) medium was prepared by adding 5 g tryptone, 2.5 g yeast extract and 5 g NaCl to 475 ml distilled water, adjusting to pH 7.0 with 5 N NaOH and autoclaving. Agar (7.5 g) was added to this before autoclaving to make plates. Plates were placed into incubation at 37°C and left overnight. Blue and white colonies grew: white colonies were usually positive clones which were checked by PCR screening and confirmed as positive by RFLP analysis alongside the original PCR product used in the cloning.

In later work, the cloning vector pGEM®-T Easy (Promega, Madison, WI, USA) was used in a similar protocol, prior to transformation into *E. coli* DH5α cells. Ligations were set up using 5 µl of 2X Rapid Ligation Buffer, T4 DNA Ligase, 1 µl of pGEM®-T Easy Vector (50 ng), 3 µl of PCR reaction, and 1 µl of T4 DNA Ligase (3 Weiss units/µl). The ligation reaction was allowed to proceed over a minimum of 1 hour at room temperature, or preferably overnight at 16°C. A very similar protocol to the one used in earlier work using the TOPO cloning kit was followed for the transformation. The only modifications from the Promega protocol were that the heat-shock at 42°C in the heating block was extended to 2 minutes to give enough time for total heat transfer through the tubes, and that 50 µl of transformation reaction was used to inoculate LB-ampicillin plates only.

2.4.4 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. It was carried out using primers specific for the cloning vector. The cloning procedure was efficient enough that generally only 4 white colonies needed to be screened. Reagents (kept on ice) and materials were essentially as in section 2.4.2, except for the primers: standard "M13" forward and reverse primers in stocks of 100 ng/µl, with sequences 5'- GTA AAA CGA CGG CCA G-3' (forward) and 5'- CAG GAA ACA GCT ATG AC-3' (reverse). A master mix was made for 4 reactions by adding together the following and aliquoting 19.5 µl into separate sterile 0.5 ml eppendorf tubes: 8 µl of 10x reaction buffer, 8 µl of 25 mM $MgCl_2$, 8 µl of dNTPs, 2 µl each of M13 forward and reverse primers, and 12.5 µl of MilliQ water. To each tube, a large, well-separated white colony from the transformations was added using sterile toothpicks, and the position of the colony on the plate noted by marking the petri plate. *Taq* polymerase (0.5 µl) was added to each tube and immediately placed into the thermocycler for PCR, which ran with the following cycles:

95°C for 10 minutes before 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, with a 10 minute incubation at 72°C at the end (and a 48 hour "cold-hold" at 4°C if required). On completion, confirmation that the cloned DNA was the 16S rRNA gene of interest was checked using RFLP.

2.4.5 Restriction fragment length polymorphism (RFLP) analysis of cloned 16S rRNA genes

RFLP patterns of the cloned inserts were compared with the original authentic 16S rRNA genes from the first PCR reactions. Two restriction enzymes, *Msp*I and *Eco*RI were used. If 4 colonies were picked for screening, 5 eppendorf tubes (0.5 ml) were placed on ice and a master mix of the following made: 5 µl of 10 X 'Buffer B'; 2.5 µl of *Msp*I; 2.5 µl of *Eco*RI; 15 µl of sterile MilliQ water. An aliquot of 5 µl was placed into each tube, and then 5 µl of PCR product from each cloning reaction added to each of four tubes, leaving one tube for addition of 5 µl of the original PCR product. The tubes were then incubated at 37°C, without shaking, for 1 hour. The digests were then run on a 3 % Sigma Type 3 "AMRESCO" high resolution agarose horizontal gel, using 1 X TBE buffer. For a gel 3-5mm thick, for example, 0.9 g of agarose was heated with 30 ml of 1 X TBE in a microwave, cooled slightly, and poured into the gel tank. (An option when making the gel was to add ethidium bromide to the cooled molten agarose, saving time later as staining was not required. When this was carried out, 1.4 µl of ethidium bromide (1 mg ml^{-1}) was added and swirled into the molten agarose). Two µl of 6x loading buffer was added to each 10 µl and the whole volume pipetted into wells. The gel was run at 60 mV for about 1 hour, or until the dye migrated around ¾ of way down the gel. The gel was either stained with ethidium bromide for about 15 minutes, or if it was added to the agarose previously, was viewed immediately using a UV transilluminator. The cloned insert(s) with a pattern that matched exactly with the original PCR product was noted.

2.4.6 Plasmid preparation and purification

Returning to the plate(s) from which the colonies were taken, the plate with the colony/colonies of interest was left to incubate, either at room temperature or at 37°C overnight, depending on the number of colonies on the plate (crowding might mean that the colony would not be well-separated after re-growth), the amount of material left of the colony, and the temperature of the laboratory. Once the colony had re-grown, some of the colony was used to inoculate LB-ampicillin medium (usually 5 ml in a test-tube; the volume used depended upon the chosen method of plasmid preparation following growth), with a final concentration of ampicillin of 100 μg ml^{-1}. These were incubated at 37°C, with shaking, overnight. This culture was then ready to be used either in the plasmid mini-prep method described by Saunders and Burke (1990), or by using a variety of mini- or midi-prep kits, the most frequently used being the Qiagen Mini-prep. Other kits used included the Wizard® Plus SV Minipreps DNA Purification system (Promega, USA), and the CONCERT™ Rapid Plasmid Miniprep System (GibcoBRL®); these are all based on the same principles. For simplicity, the method used by Saunders and Burke (1990) is described. A number of solutions and reagents were prepared: (i) 'solution 1' (0.9008 g glucose, 0.3029 g Tris, 2 ml of EDTA stock, and distilled water to 100 ml); (ii) 'solution 2' (0.9 g NaOH, 5 ml SDS stock, and distilled water to 100 ml); (iii) 'solution 3' (29.45 g potassium acetate, 11.438 ml glacial acetic acid, and distilled water to 100 ml); (iv) isopropanol and 70% ethanol, both kept at –20°C; (v) 0.5 M EDTA (made with 46.53 g EDTA, adjusted to pH 8 with 10M NaOH and distilled water added to 250 ml); (vi) 20% SDS (20 g of EDTA in 100 ml of distilled water) stock solutions.

For each plasmid prep, 1.5 – 5 ml of the *E. coli* culture grown overnight was spun for 4 minutes at 9300 x *g* in sterile 1.5 ml eppendorf tubes. The pellet was resuspended in 100 μl of solution 1. Solution 2 (200 μl) was added and gently mixed for cell lysis. Solution 3 (150 μl) was added and

immediately mixed by gentle inversion. The tubes were then centrifuged for 4 minutes at 9300 x *g*. A volume (380 µl) of the supernatant was transferred into clean sterile eppendorf tubes and 720 µl of isopropanol added. A further 5 minutes of centrifugation at 9300 x *g* were required, before removing the supernatent. The pellet was washed with 1 ml of 70% ethanol, with brief (~ 1 minute at 6000 x *g*) centrifugation. The supernatent was removed very carefully by pipetting and the pellet left to air dry. Once dried, the pellet was resuspended in 40 µl of MilliQ water and 10 µl of RNAse added. This was left to incubate for 1 hour at room temperature. Purification and elution of plasmid DNA was then completed by using QIAprep spin columns (QIAprep spin manual), following the supplied protocol.

2.4.7 Determination of DNA yield

The yield of DNA obtained was quantified by spectrophotometry. Five µl of the DNA was diluted with 495 µl MilliQ water in a quartz cuvette, and the absorbance read at 260 nm (with use of the deuterium lamp) on a Cecil CE 292 Series 2 UV spectrophotometer. The yield was calculated on the basis of the relationship that an A_{260} of 1 = 50 µg DNA/ml.

The appropriate amount of DNA required for sequencing was then precipitated by adding 0.1x volume of sodium acetate and 2.5x volume of absolute ethanol, mixing and centrifuging for 15 minutes at 9300 x *g*. The supernatent was carefully removed and the pellet left to air dry overnight, covering the tubes with a tissue. The DNA was sent to MWG Biotech (Milton Keynes, UK) for SEQ-3-XL double strand sequencing after performing a restriction digest to check that the insert (which should be about 1500 base pairs) was definitely present in the plasmid.

The yield of DNA was also occasionally estimated by running DNA samples on a 0.7% agarose gel containing ethidium bromide against a

Lambda Hind III (λH) molecular weight marker in the first lane. One µl of λH with 19 µl of MilliQ water and 4 µl 6x loading buffer was mixed and pipetted into the first well. One µl of DNA, with the same volumes of water and loading buffer were pulse spun at 6000 x *g* for about 1 second then loaded into parallel wells. The gel was run at about 50 mV for around 30 minutes, and then the third band to separate from the DNA samples was compared in intensity with the first band (23 kb) of the λH. The 23 kb band has approximately 50% of the total λH DNA (= 175 ng μl^{-1}, so 50% = 87.5 ng μl^{-1}), and this was used to give a visual estimate of sample DNA.

2.4.8 Restriction enzyme digests

To check that the desired insert was definitely present in the plasmid before sequencing, restriction digests using the enzyme *Eco*RI were set up. Volumes were sometimes altered for optimisation but generally the following was used for each DNA sample: 1 µl digestion buffer, 1 µl *Eco*RI, 1 µl DNA, and 7 µl of MilliQ water (a total of 10 µl). These were incubated at 37°C with shaking for about 1 hour. Two µl of 6x loading buffer was added to each digest and the whole volume pipetted into wells of a 3% Sigma Type 3 "AMRESCO" high resolution agarose horizontal gel, using 1x TBE buffer. Into the first well, 12 µl of 1 kb ladder was added, and the gel was run at about 60 mV until the blue dye had migrated ¾ of the way down its length. The presence of the 1.5 kb insert was looked for by viewing the gel under a UV transilluminator. A photograph of the gel was included with the DNA samples sent for sequencing.

2.4.9 Sequence and phylogenetic analysis

After removal of primer sequences, the 16S rDNA sequences were entered into the latest version of GenBank at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). BLAST searches (Altschul *et al.*, 1997) were carried out to find the closest

known relatives for each isolate, by comparison with previously deposited 16S rRNA gene sequences. Using sequences retrieved from the NCBI database 'Entrez', multi-sequence alignments were carried out using the CLUSTALW program (Thompson *et al.*, 1994) and phylogenetic trees were constructed with the latest version of the Phylogeny Inference Package (PHYLIP) (Felsenstein, 1993). Phylogenetic inferences were carried out using DNA Distance and trees were constructed using Neighbour Joining (Saitou and Nei, 1987).

3. ENRICHMENT AND ISOLATION OF SULPHATE REDUCING BACTERIA FROM ACIDIC ENVIRONMENTS

At the outset of the research programme, methods for culture and isolation of aSRB had not been developed, and it was possible either that these bacteria did not exist, or that attempts to isolate them would be unsuccessful. A number of preliminary experiments were therefore carried out in an attempt to obtain aSRB from environmental samples, and to maintain them in the laboratory. The methods used at this stage were subject to development throughout the course of the research programme and formed the basis for later work.

3.1 Environmental Sampling

Environmental samples that were expected to contain the target microorganisms were required, and therefore appropriate sites impacted by mining and/or low pH and metals were sought. Samples from three sites were used as inocula for enrichment cultures.

(i) ***Parys Mountain mine, Anglesey, North Wales*** (Walton and Johnson, 1992; Boult *et al.*, 1994). This area has a long history of metal mining (evidence of Bronze age and Roman mining has been found). One of the two streams draining the now abandoned mine site (the *Afon Goch*; 'red river') has a pH of around 2.5-3 in its uppermost stretches and contains elevated concentrations of iron, sulphate, zinc and copper (Walton and Johnson, 1993). Sediment cores (30 x 5 cm) were taken from the stream bed of the Afon Goch using a stainless steel core sampler. The pH of the surface water was measured on site using a portable water tester (Whatman Ltd.). The pH 'profile' of a typical sediment core (shown in Figure 3.1) was recorded using a flat-bed pH electrode (Russell Ltd.), moving the surface of the electrode from the top of the core to the bottom 1 cm

at a time (Table 3.1). A small amount of a blackened zone of the core was scooped out from the core with a spatula; a few drops of sulphuric acid (1 M) was added to it and resulted in the evolution of hydrogen sulphide gas, confirming that the black coloration was due to the presence of (ferrous) sulphide. A sediment sample consisting of the black sulphidic zone of a core, together with sediment some 5 mm above and below this layer was used to inoculate a bioreactor ('A'; section 3.2).

(ii) ***Cae Coch pyrite mine, Conwy Valley, North Wales*** (Johnson *et al.*, 1979; McGinness and Johnson, 1993). This abandoned mine hosts a drainage stream (pH ~2.5) which contains extensive growths of "acid streamers" (section 1.8). These consist of morphologically- and physiologically-distinct acidophilic microorganisms (predominantly bacteria) many of which have not been characterised (Johnson *et al.*, 1979; Johnson and Kelso, 1981; D.B. Johnson, personal communication). A mixture of surface and subsurface streamers (taken from around 40 cm depth and characterised by black zones, in contrast to the uniformly cream-coloured surface streamers) were collected in sterile beakers (300 ml) from the Cae Coch site and used as inocula for bioreactors ('B' and 'C').

(iii) ***White River, Montserrat, West Indies***. Sediment samples from this river, which drained the main geothermal ('*soufriere*') area in the south of the island, prior to the catastrophic volcanic events of 1996, were sampled as part of the 1996 BBSRC-sponsored microbiological expedition (Atkinson *et al.*, 2000). This material was used to inoculate a fourth bioreactor ('M').

Figure 3.1 A sediment core taken from the Afon Goch stream bed, Parys Mountain, Anglesey, U.K. pH across the core ranged between 3.0 and 5.5. The black zones are deposits of ferrous sulphide, indicating sulphate reduction..

Distance from sediment surface (cm)	Colour of sediment	pH (units)
4 (top of core)	Brown	3.0
4.5	Black	4.8
6.0	Brown	4.0
7.0	Brown	3.75
8.0	Brown	3.75
9.0	Brown/black	4.9
10.0	Black	5.15
11.0	Brown	4.8
12.0	Brown	4.48
13.0	Black	4.95
14.0	Brown/black	4.15
15.0	Brown	4.42
19.0	Brown	4.45

Table 3.1 Profile of sediment core taken from Afon Goch stream bed, Parys Mountain, Anglesey. Between 15 and 19 cm, the pH was virtually unchanged. A sample from the core consisting of material from zones 9 – 11 cm was used as inoculum for bioreactor 'A'.

3.2 Enrichment Cultures

3.2.1 Methodology

Initially, enrichments were made in bench-scale bioreactors (section 2.1.1.2) to select for mesophilic aSRB by introducing environmental samples into growth medium containing glycerol as an electron donor, which had previously been successful in isolating an SRB under acidic conditions (Johnson *et al.*, 1993). A number of initial investigations were subsequently made to assess the nature of the aSRB that had been selected from the environmental samples.

Environmental samples were added directly to bioreactors once the vessels and associated apparatus had been assembled and autoclaved with liquid medium (section 2.1.1.2). Prior to inoculation, the medium was bubbled with OFN in the bioreactor for around 30 minutes (or until O_2 levels were registered as $\geq$ 1%); ferrous sulphate (from a filter-sterilised 1M, pH 2 stock solution) was added to the medium after autoclaving to prevent iron oxidation. The pH of the culture was adjusted to around 3.5, and was observed visually and monitored frequently (at least daily) for changes in pH and redox potential. To maintain the appropriate selection pressure, the following parameters were controlled: (a) pH was kept low (2.5 - 4) by manual addition of 1M sulphuric acid or sodium/potassium hydroxide; (b) cultures were maintained without stirring, other than before taking measurements and withdrawing samples; (c) cultures were sparged regularly (at least twice daily for 15 minutes) with OFN to remove hydrogen sulphide and/or any traces of oxygen. Light was excluded by covering bioreactor vessels with black plastic sheets, and the temperature was maintained at 25°C.

3.2.2 Results

Acidophilic SRB cultures were obtained in all but one of the bioreactors, though the time taken for sulphate reduction to become evident in the systems varied considerably. Evidence of SRB activity was: (i) blackening of the medium, due to the formation of ferrous sulphide precipitates); (ii) an increase in pH; (iii) the production of hydrogen sulphide gas, readily detected when the bioreactors were sparged with OFN. Enrichment for aSRB in Bioreactor B (data not shown), using a mixed streamer/sub-surface streamer sample from Cae Coch, was not successful, i.e. there was no blackening or increase in pH despite prolonged (approximately three months) incubation. However, samples taken from this bioreactor in universal tubes and placed in storage at 4°C blackened some weeks after the enrichment culture was abandoned, indicating that sulphate reduction had eventually taken place in the culture. These were retained for the inoculation of solid media. In contrast, sulphate reduction was evident in the Parys mine-inoculated culture (Bioreactor 'A') after only 15 days, when pH rose from 3.6 to 4.7 over a period of about 36 hours. At this point alkali-generating potential by the culture was investigated (Figure 3.2). Bioreactor 'C', the second culture using an inoculum from Cae Coch, took a long time to establish, with little changes in pH and no evolution of hydrogen sulphide gas until after around 65 days, when the bioreactor vessel blackened, and hydrogen sulphide gas was produced by the culture.

3.3 Demonstration of alkali generation by mixed aSRB cultures

3.3.1 Methodology

Sulphidogenesis in acidic liquors is an acid-consuming (alkali-generating) reaction (section 1.5.2). The ability of the undefined (though assumed to be mixed) cultures of aSRB in the bioreactors to generate alkalinity following exposure to episodes of acidification was investigated. This was

achieved by periodic addition of sterile sulphuric acid (1M) to bioreactor cultures. pH control of the reactors was disabled, and the response of the cultures, in terms of changing pH, E_h and hydrogen sulphide production, was monitored. If there was evidence of SRB activity, bioreactor pH was lowered further and further observations made. This process was continued until the point at which SRB activity was inhibited by acidity was established. Samples (in 30 ml sterile universal bottles) were taken daily from the bioreactor, and the microorganisms examined with phase contrast microscopy to observe the morphology of the various cell types present. pH was measured *in situ* but was also regularly measured with an independent electrode to check the accuracy of the bioreactor measurement, as electrodes were often found to be 'poisoned' with a thick coating of ferrous sulphide and biomass following establishment of a biofilm within the reactor vessel. Samples were used to inoculate solid media (glycerol/yeast extract/ferrous sulphate overlay plates (sections 2.1.1.4.1 and 3.5).

3.3.2 Results

Figure 3.2 shows alkali generation in response to culture acidification in Bioreactor 'A' (Parys culture) over a 65 day period. Simultaneous reduction in redox potential was also observed, providing further evidence of sulphate reduction. Net alkali production was observed with the Parys enrichment culture in medium adjusted to pH values as low as 1.73, though was notably less pronounced when acidified to pH <2.0. Alkali generation was also observed initially in Bioreactor 'M' (Figure 3.3a), although this culture was not as robust as in 'A', and was not able to rapidly generate alkalinity following a large input of acid (day 10). However, sulphate reduction activity resumed following two additions of alkali to raise the pH above 2.5 (Figure 3.3a). Until Day 81, the culture exhibited alkalinity generation following batch replacements and small reductions (approximately 0.5 units) in pH. Between day 65 and 81, the pH

in the culture had been gradually reduced in this way from 4.8 to 2.5. However, beyond this point, the culture did not demonstrate further alkalinity generation. In fact, from this point onwards, it appeared that the pH in the culture was decreasing, and that sulphate reduction had slowed significantly or stopped. In an attempt to re-establish sulphate reduction, alkali was added to the culture repeatedly, but the culture continued to generate acidity, until Day 93, when a small increase in pH and the odour of hydrogen sulphide was observed. However, at this point, when the culture was challenged with a reduction of approximately 0.75 pH unit, the culture immediately returned to acid generation. Subsequently, between Day 95 and Day 120 (data not shown), the culture continued to generate acidity despite repeated addition of alkali and the experiment was discontinued. A 'low pH threshold' for this culture was therefore not obtained, although it was firmly estimated from the data to be around 2.5 – 3. Bioreactor 'C', the second Cae Coch culture, took a long time to establish; after around 65 days, the bioreactor blackened, and subsequently alkalinity generation was again demonstrated in the batch culture (Figure 3.4). The 'low pH threshold' in this case was 2.4. In all enrichment cultures, redox potentials recorded were higher than those generally observed in cultures of neutrophilic SRB; a probable reason for this is discussed below (section 3.8.1).

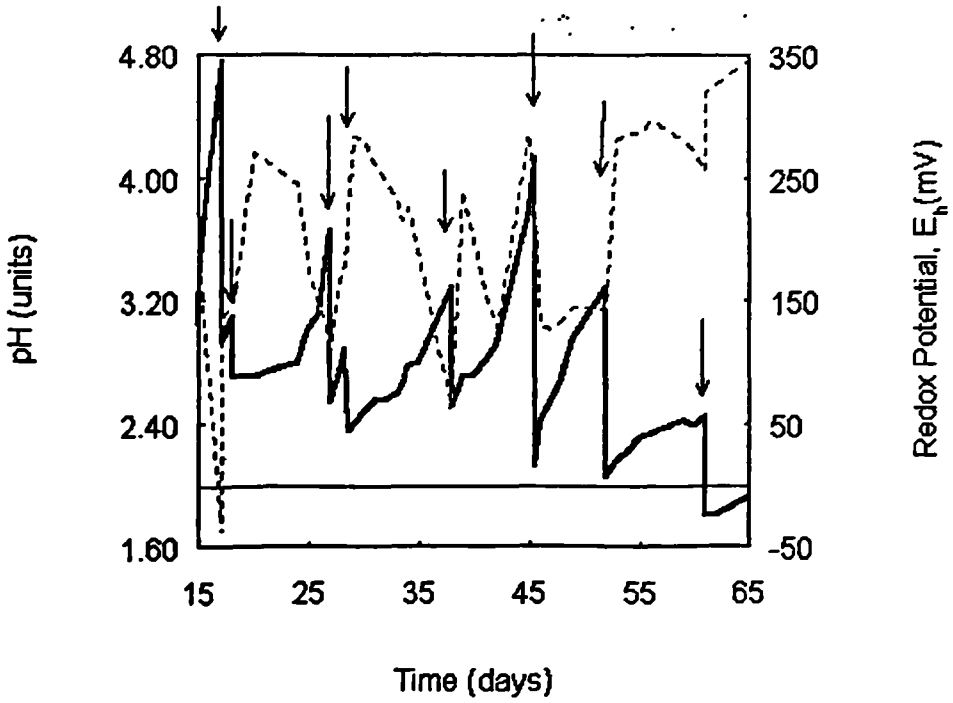


Figure 3.2. Alkali generation in Bioreactor 'A' (Parys enrichment) in a bench-scale bioreactor run in batch mode, following acidification with sulphuric acid. Changes in pH (——) and redox potential (——) are shown, with downward arrows indicating acidification events.

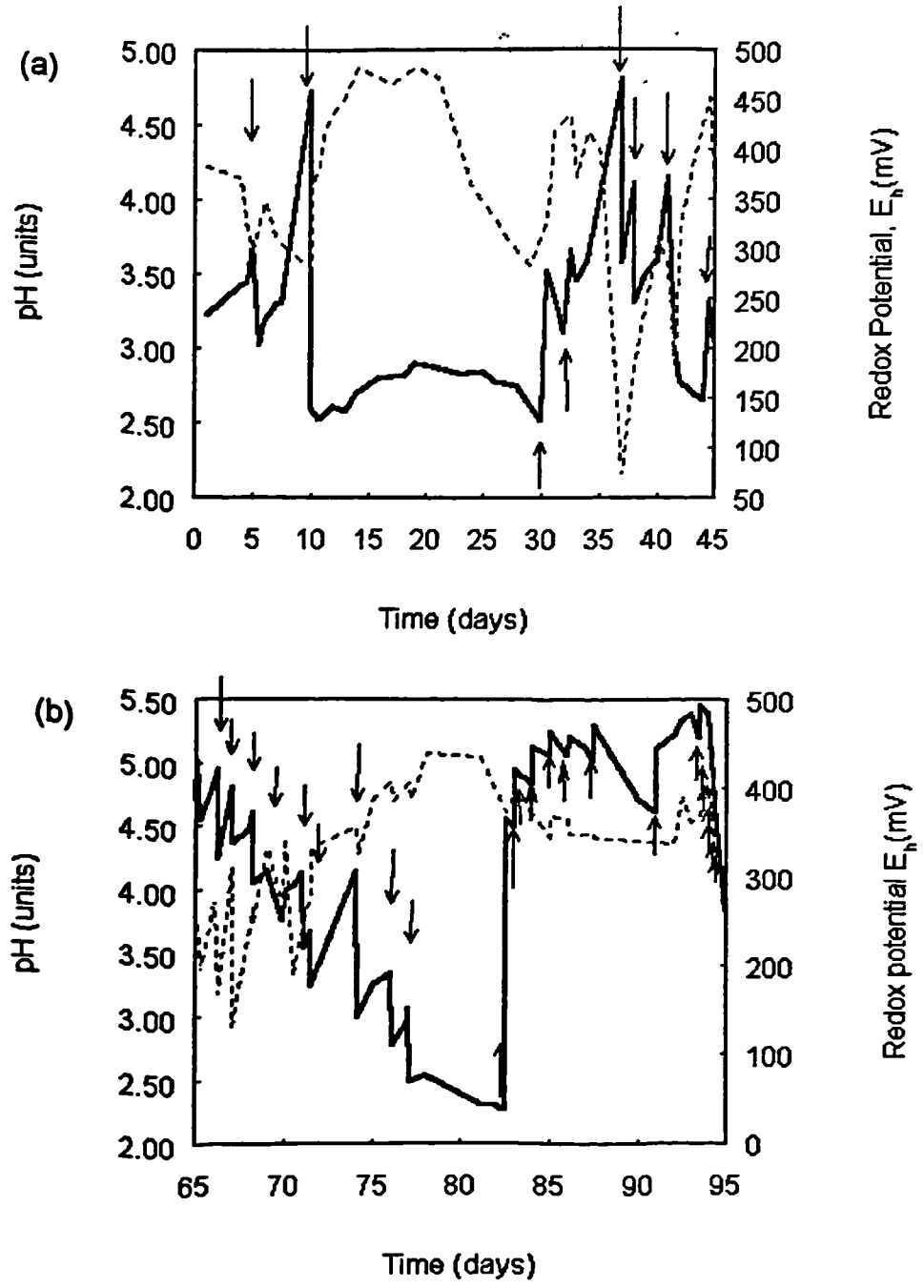


Figure 3.3. Alkali generation in Bioreactor 'M' (Montserrat enrichment) in a bench-scale bioreactor run in batch mode, following acidification with sulphuric acid. Changes in pH (—) and redox potential (——) are shown, with downward arrows indicating acidification events. The culture generated alkalinity following addition of acid (downward arrows) but following prolonged or sudden acid episodes (to ~ pH 2.5), net acidity was generated, despite periodic addition of sodium hydroxide (indicated by upward arrows in (a) and (b)).

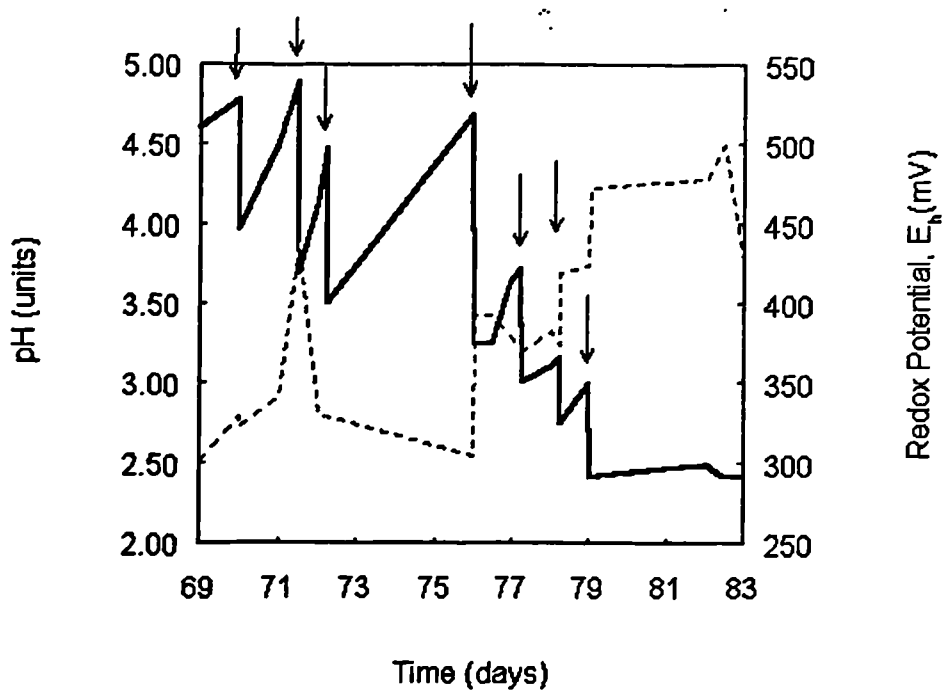


Figure 3.4. Alkali generation in Bioreactor 'C' (Cae Coch enrichment) in a bench-scale bioreactor run in batch mode, following acidification with sulphuric acid. Changes in pH (—) and redox potential (——) are shown, with downwardarrows indicating acidification events.

3.4 Sulphate reduction and glycerol utilisation by aSRB

3.4.1 Methodology

An experiment to examine sulphate reduction and glycerol utilisation, with no pH control, was set up using about 500 ml of a mixed culture obtained by enrichment of a subsurface streamer sample taken from the Cae Coch mine, using the medium described in sections 2.2 and 3.1. Samples were taken daily, centrifuged to remove iron precipitates and cells, and the supernatant analysed for sulphate and glycerol using methods described in 2.2.2 and 2.2.5 respectively (glycerol was measured using the colorimetric assay). Samples were analysed in duplicate and the results shown are calculated means.

3.4.2 Results

SRB activity in the bioreactor was evident after 5 days, indicated by an intense blackening of the vessel; at this point, observations began (Day 0; Figure 3.5). The results of the sulphate assays of samples showed that sulphate was depleted completely by Day 13, but disappeared most rapidly between days 7 and 10 (at pH ~5.0 - 5.4) and still further until day 13. Glycerol was undetected in the culture by day 11. It is noteworthy that pH increased during the phase of active sulphate reduction and catabolism of glycerol, and continued to rise slowly after the depletion of both.

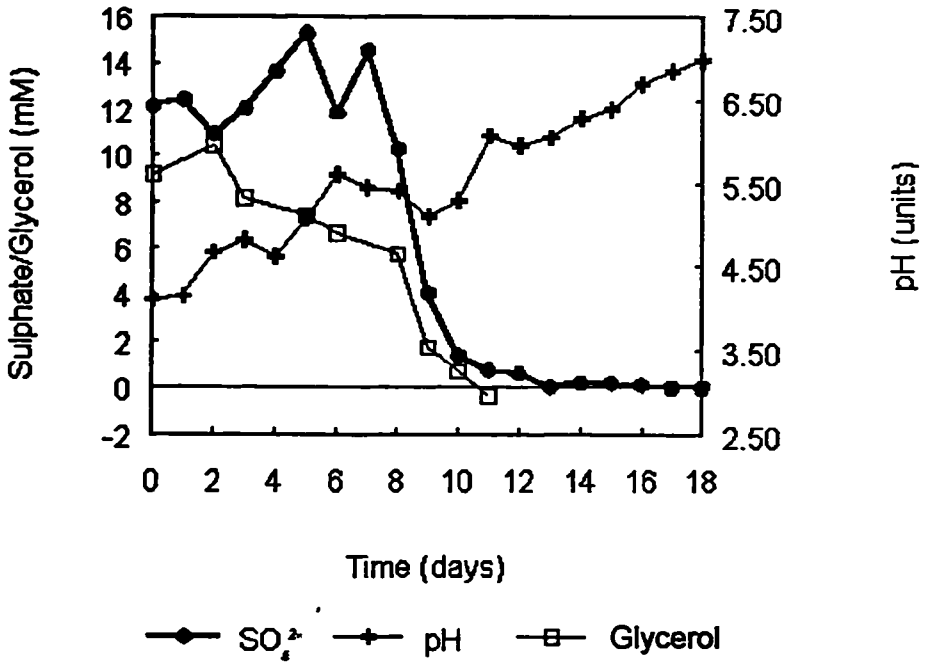


Figure 3.5. Sulphate reduction, glycerol utilisation and alkalinity generation by mixed aSRB culture (Cae Coch). Disappearance of sulphate and glycerol by day 11 and 13 respectively was observed.

3.5 Growth of aSRB and other acidophiles on solid media

3.5.1 Inoculation of glycerol/yeast extract overlay plates with bioreactor samples

3.5.1.1. Methodology

Samples taken from different enrichment cultures in bioreactors were used to loop-streak overlay medium containing glycerol (10 mM) and *Acidiphilium* SJH (section 2.1.1.4.1). The plates were incubated in anaerobic jars (Oxoid Anaerobic System; section 2.1.2) at 30°C until colony growth was visible.

3.5.1.2 Results

Growth of SRB on overlay plates was extremely variable; appearance of black colonies (tentatively identified as aSRB) took between 9 days and four months to appear. Plates streaked with samples from bioreactor 'A' (Parys enrichment) exhibited growth after three months, but were found to be contaminated with fungi. Fresh plates were inoculated and aSRB colonies were apparent after two months. SRB were separated from other microorganisms (non-SRB) by re-streaking colonies onto fresh plates; at this stage, the minimum time taken for appearance of black colonies was reduced to 9 days. Colonies of aSRB were obtained from samples from bioreactor 'B' (Cae Coch enrichment) despite the absence of obvious sulphate reduction in the bioreactor itself. Despite several attempts, no aSRB from samples taken from bioreactor 'C' (Cae Coch enrichment) grew on solid media. In general, aSRB from bioreactor 'M' (Montserrat enrichment) grew comparatively well on plates, but separating the black (SRB) colonies from other (white) colonies proved to be very difficult, and was only possible after 5 or 6 subcultures. Initially it was thought that two different types of aSRB from both bioreactors 'B' and 'M' had been found, due to slight differences in colony and cell morphologies, but these

differences were not consistent and seemed to vary according to plate/colony age.

Some of the plates containing mixed colonies were retained and stored anaerobically at 4°C, and others were used for sterile transfer of colonies to liquid medium. After between 5 and 10 subcultures, six bacterial isolates were obtained (three SRB and three non-SRB). These were labelled "P1", "CC1" and "M1" (SRB from Parys, Cae Coch and Montserrat enrichments, respectively), and ""PW1", WHG1" and "MA1" (non-SRB from the Parys, Cae Coch and Montserrat enrichment cultures).

3.5.2 Efficiency of different solid media

3.5.2.1 Methodology

A screening experiment was set up to investigate which of several solid media and anaerobic atmosphere conditions was most suitable for growth of the three mixed SRB cultures. These were: (i) an overlay plate containing ferrous iron (10 mM) and 0.02% yeast extract (pH 3.5) and either 10 mM, 1 mM or zero (to look for any stimulatory or inhibitory effects of glycerol); (ii) 'aged' or 'fresh' overlay plates: solid overlay media were made and either loop-streaked immediately ("fresh" plates), or incubated at 30°C for 2-3 days to encourage growth and activity of the *Acidiphilium* SJH in the underlayer before inoculation ("aged" plates); this was to assess whether overlay plates became more effective with maturation and (iii) comparison of two anaerobic jar systems, one of which (the `Anaerobic system') generates hydrogen (which can be used as an electron donor by some SRB) and the other (the *AnaeroGen* system) does not (section 2.1.2). Simple observations of whether any growth had occurred on the different media types/conditions were made; i.e. enumeration was not carried out at this stage. The medium giving the most prolific growth of aSRB was then used to grow aSRB from bioreactor samples.

3.5.2.2 Results

The results of the experiment (Table 3.2) suggested that "ageing" of the plates, i.e. allowing more time for SJH activity, did not enhance growth of aSRB colonies. Indeed, where growth did occur for a comparison to be possible, plate ageing appeared to have a negative effect. In general, the most successful growth of black aSRB colonies was observed on 'fresh' YE + 10mM glycerol medium, in both anaerobic systems. However, an exception to this was on plates inoculated with the Montserrat culture, where similar growth was observed on aged and fresh plates. These aSRB also grew well on the yeast extract overlay plate in the presence of hydrogen, but not in its absence. Growth of black (SRB) and white (non SRB) colonies was also obtained in the YE + 1 mM glycerol medium (hydrogen-free atmosphere) with the Cae Coch and Montserrat cultures, but black colonies did not appear in medium inoculated with the Parys culture. Indeed, in general, proportionately more white colonies (and less black colonies) were obtained from the Parys culture than others. Overall, neither of the two anaerobic systems was superior for the growth of the aSRB.

3.5.3 Growth of "non-SRB" on solid media

3.5.3.1 Methodology

White colonies that grew on solid media in the first screening experiment were streaked onto a variety of solid media. These were: (i) non-overlay FeTSB medium (section 2.1.1.4) (ii) overlay yeast extract medium, with or without 10 mM glycerol; (iii) TSB (0.025%, w/v) overlay medium (section 2.1.1.4), adjusted (before mixing with the agarose solution) to either pH 3.0 or pH 3.5. Inoculated plates were incubated (at 30°C) aerobically, microaerobically or anaerobically.

	TSB only						YE only						YE + 1mM Gly						YE + 10mM Gly					
	Fresh			Aged			Fresh			Aged			Fresh			Aged			Fresh			Aged		
	P	C	M	P	C	M	P	C	M	P	C	M	P	C	M	P	C	M	P	C	M	P	C	M
A																								
1	-	-	w	-	x	x	w	-	b b w	x	x	x	x	x	x	x	x	x	b b w	b w	b b w	w	x	x
2	-	-	-	w	x	x	-	x	x	x	x	x	x	x	x	x	x	x	b w	w	b b w	-	x	x
3	-	x	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	x	b w	x	x	w	x	x
B:																								
1	w w	w	b b w	-	-	-	w	w	w	x	x	x	w w	b b w	b b w	x	x	x	w b b	w w w	b b b w	w w w	x	b b w
2	w	x	x	-	x	x	-	x	x	x	x	x	w	b b w	b b w	x	x	x	w	x	b b b w	w w w	x	b b w

Table 3.2 Comparison of growth of aSRB enrichment cultures on different solid media, incubated (A) using the Oxoid `Anaerobic System' (which generates hydrogen), or (B) the Oxoid *AnaeroGen* system, which does not generate hydrogen. Key: b, growth of black (aSRB) colonies; w, growth of white (non-SRB) colonies; -, no growth; x, not tested. 1, 2 & 3 are duplicates/triplicates.

3.5.3.2. Results

The results of the screening exercise are summarised in Table 3.3. PW1 rapidly oxidised ferrous iron under microaerobic conditions, forming rusty-brown coloured colonies after about one week. In contrast, isolate WHG1 formed small white colonies under the same conditions. Neither of these two isolates grew under anaerobic conditions within two weeks. Following prolonged incubation of up to three months, tiny white colonies were obtained. These and other 'non SRB' isolates did not appear to be able to grow in the presence of oxygen, as no colonies were observed on plates incubated aerobically.

Strain	Atmosphere	Plate type	Growth
WHG1	Aerobic	FeTSB (pH 3.5)	-
		YEo	-
		Gly/YEo	-
PW1		Gly/YEo	-
		Gly/YEo (dup.)	+ (at origin)
MA1		Gly/YEo	-
		YEo	-
WHG1	Microaerobic	TSBo (pH 3.5)	++ (white colonies)
		Gly/YEo	+ "
		YEo	+ "
PW1		TSBo (pH 3.5)	++++ (rusty-brown colonies)
		Gly/YEo	++++ "
		Gly/YEo (dup)	++++ "
WHG1	Anaerobic	TSBo	-
		Gly/YEo	-
		YEo	-
PW1		Gly/YEo	-
		YEo	-

Table 3.3. Growth of non-SRB isolated from mixed cultures. TSBo = tryptone soya broth overlay plates; Gly/YEo = glycerol/yeast extract overlay plates; YEo = yeast extract overlay plates; "Dup" = duplicate.

3.5.4 Colony and cell morphologies

3.5.4.1 Methodology

Colony morphologies were observed using stereomicroscopy and cell morphologies were observed by making aqueous suspensions of bacterial colonies (wet mounts) and using phase contrast microscopy (section 2.3).

3.5.4.2 Results

(a) SRB (black colonies)

All of the aSRB that were observed at this stage formed large black colonies on solid media. P1, M1 and CC1 formed large (1-5 mm), black, domed, entire, colonies (as also described in section 6.3.1, Table 6.1). As the colonies grew older, their surfaces became shiny. Figure 3.6 shows some typical aSRB colonies (M1). The following cell types, obtained from the Parys, Cae Coch and Montserrat cultures respectively (as also described in section 6.3.1, Table 6.1), were observed using phase contrast microscopy (section 2.3.2) using wet mounts made from the colonies:

'P1': Large straight rods, often in pairs, approximately 3–6 x 0.5 μm, possible spore former, non-motile.

'CC1': Large rods, straight to curved, approximately 3–5 x 0.5 μm, spore former, sometimes motile. Sometimes oval/pear shaped.

'M1': Rods, straight to curved, approximately 2 – 5 x 0.4 μm, evidence for spore formation, occasionally motile. Occasionally oval/pear shaped.

(b) Non-SRB (white colonies)

On the original solid medium in which these organisms were grown, isolates 'PW1' and 'WHG1' were seen as small (approximately 1-3 mm) smooth, white, semi-opaque, domed, entire, regular colonies. Colonies of 'MA1' were small (approximately 1-2 mm), smooth, white, opaque, domed, entire, regular colonies. Figure 3.7 shows the contrasting colonies of 'M1'

(black) and 'MA1' (white) growing on glycerol/yeast extract overlay medium. Cells were observed using phase contrast microscopy:

'***PW1***': Long, (up to around 20 μm) thin, filamentous cells with a 'grainy' appearance, possibly due to gas vesicles or inclusions, possible spore-former. Occasionally seen as smaller rods. Non-motile.
'***WHG1***': Very similar to 'PW1' but finer filaments. Occasionally small, 'separate' rods seen (~2-4 μm). Spore-former. Non-motile.
'***MA1***': Bacilli/very short rods, ~ 0.5-1x 0.5μm. Non-motile.

3.6 Growth of aSRB in liquid media

3.6.1 Pure cultures of aSRB and associated organisms

3.6.1.1 Methodology

The liquid medium used routinely for isolation and subsequent culture of SRB was OFN-gassed glycerol/yeast extract/ferrous sulphate medium (section 2.1.1.2). A pH of between 2.5 and 4.5 was used, 3.5 being the usual initial pH of batch or bioreactor cultures. Single colonies grown on solid media were transferred into universal bottles (approximately 30 ml) and 1.5 ml eppendorf tubes, which were completely filled with medium to exclude oxygen, and incubated at 30°C. The same medium and method was used to attempt to isolate the acidophilic "non-SRB" (white colonies on plates) from the three cultures. If growth was successful, these cultures were used as inocula for subcultures, to maintain active cultures, and to "scale up" cultures, i.e. to grow larger volumes of active cultures for later experiments. This was achieved by pipetting a volume of culture into a larger volume of fresh medium so that the inoculum constituted approximately 10% of the culture volume. In the case of eppendorf cultures, the entire volume was transferred to universal bottles containing the same growth medium, which were incubated at 30°C. Where universal bottles provided the inoculum, culture was transferred to 300 ml glass bottles containing medium.

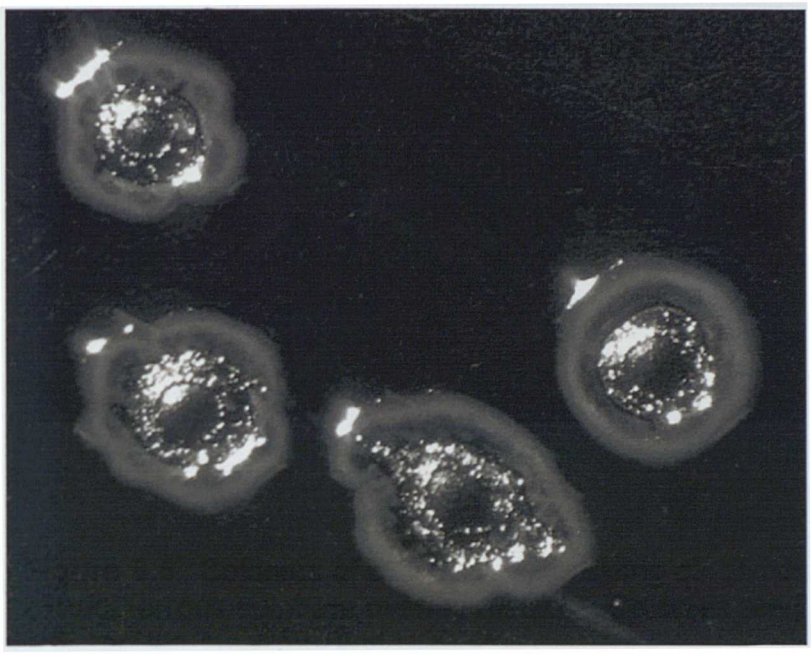


Figure 3.6 Colonies of aSRB ("M1") growing on glycerol/yeast extract/ferrous sulphate overlay medium. Colonies are 20 days old, and the magnification is X 32.

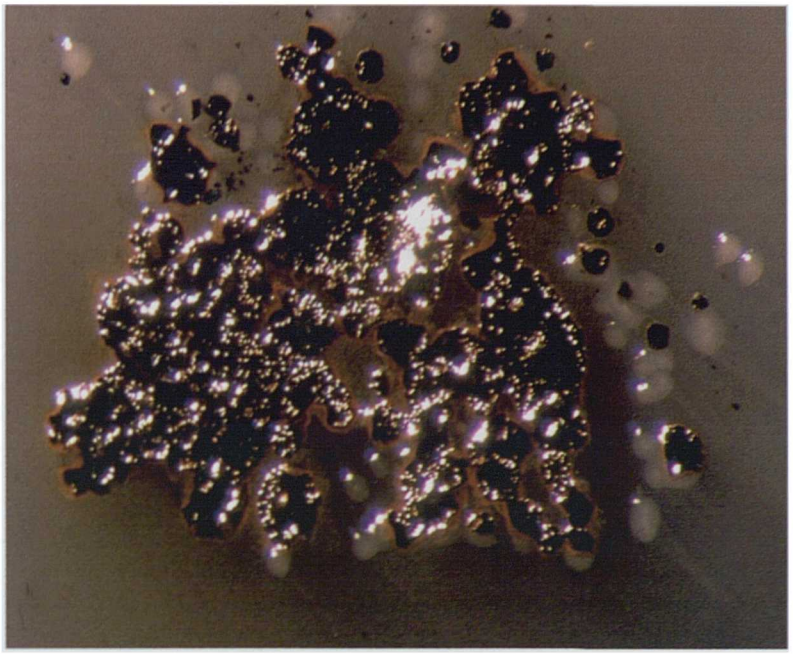


Figure 3.7 Black aSRB colonies ("M1") growing on glycerol/ yeast extract/ferrous sulphate overlay medium. Colonies are 20 days old, and the magnification is X 16.

3.6.1.2 Results

None of the universal bottles that were inoculated with aSRB colonies blackened after 3 months of incubation. However, some of the eppendorf tubes blackened after this time, and pure cultures of the Cae Coch and Montserrat aSRB (but not Parys aSRB) were obtained. These were successfully subcultured into larger volumes (after 1 – 3 months incubation). Only one of each of the universal bottles inoculated with the three different non-SRB resulted in growth of these bacteria, and scale-up of these cultures, attempted on several occasions, was not successful.

3.6.2 Effect of provision of solid particles and the use of a chemical reducing agent in liquid cultures

There were problems in getting consistent growth of the aSRB isolates in liquid media, though these bacteria could be readily subcultured on solid yeast extract overlay medium. In order to ascertain whether the 'liquid culture problem' was due to the lack of surfaces on which the bacteria could attach (known to be highly important in some SRB; section 1.7.5.3), liquid media containing a mixture of solid and porous glass beads were prepared. In addition, the possibility of enhanced growth in liquid media in which the initial redox potential was lowered by the presence of a chemical reducing agent was also tested.

3.6.2.1 Methodology

Two sets of universal bottles were set up, one set inoculated with 2.5 ml of the mixed Cae Coch aSRB culture ('CCB1') and the other with the mixed aSRB culture from Montserrat ('MB1'). These contained one of the following media:

1) Glycerol/yeast extract/ferrous sulphate medium, pH 3.5, with about 1 cm depth of an equal mixture of 1.5-2 mm diameter solid glass beads (source) and 0.2 mm porous glass beads (source), and 1 mM Na_2S;
2) Glycerol/yeast extract/ferrous sulphate medium, pH 3.5, with solid and porous glass beads, as above;
3) Glycerol/yeast extract/ferrous sulphate medium, pH 3.5, with addition of 1 mM Na_2S;
4) Glycerol/yeast extract/ferrous sulphate medium, pH 3.5.

The beads were heat sterilised at 160°C overnight. The universal bottles containing the cultures were completely filled and incubated at 30°C and observed daily.

3.6.2.2 Results

Within two days, the CCB1 culture had begun to blacken in medium (1), mainly around the beads at the bottom of the bottle, with the medium above the beads remaining clear. After three days, CCB1 in medium (3) blackened in the same way. Both of these continued to blacken, with the whole vessel becoming coated with ferrous sulphide. On day 7, CCB1 in medium (4) blackened and also, by day 9, CCB1 in medium (2). After two months, none of the MB1 cultures had grown.

3.6.3 Tolerance of aSRB cultures to chloride

In preliminary experiments, sulphuric acid was used routinely to control and modify pH in batch culture and bioreactor cultures. However, this obviously interfered with measurement of bacterial sulphate reduction in pH –statted cultures, where disappearance of sulphate (rather than the production of volatile sulphide) was required to be accurately measured. It was decided, therefore, to use hydrochloric acid to control pH in these cultures. However, it is known that some organic and inorganic anions

(including chloride) are toxic to many acidophilic bacteria (Norris and Ingledew, 1992) and therefore it was necessary to assess the effect of chloride ions on the growth of aSRB isolates.

3.6.3.1 Methodology

The ability of the aSRB to grow in the presence of chloride (Cl^-) was investigated by growing cultures of aSRB in medium containing different concentrations of potassium chloride. Universal bottles containing 10 mM glycerol/0.02% yeast extract/10 mM ferrous sulphate medium, pH 3.0, with additions of KCl (to final concentrations of 1, 2.5, 5, 10, 50, 100, 250, and 500 mM, and 1 M), were inoculated with 2.5 ml of a culture originating from the Cae Coch bioreactor. In parallel, universal bottles were also set up as described but including ~ 1 cm depth of glass beads. Culture bottles were completely filled and incubated at 25°C.

3.6.3.2 Results

After six days, complete blackening of the cultures containing glass beads, with 1-100 mM KCl, was observed. In the culture containing 250 mM KCl there was blackening only in the vicinity of the glass beads, and there was no blackening at all in cultures containing 500 mM or 1M KCl. No blackening occurred in cultures that did not contain glass beads (although after ten weeks some blackening was seen in the culture containing 1 mM KCl).

3.7 Discussion and Conclusions

3.7.1 Bioreactor cultures

Samples taken from acidic environments were used to inoculate bioreactors with medium containing an electron donor (glycerol) that had previously been used with success to enrich for sulphate reducing bacteria

at low pH (Johnson *et al.*, 1993). The majority of earlier studies had used organic acids (e.g. lactic acid) to enrich for aSRB, and had not been successful. However, organic acids were not used in the present study due to the potential toxic effects of undissociated organic acid molecules at low pH (Ingledew, 1982; Norris and Ingledew, 1992). Enrichments for SRB were obtained at pH 4, and subsequently these cultures were found to generate alkalinity following episodes of culture acidification. It was concluded that enrichments for acidophilic sulphate reducers had been successful, and that aSRB cultures had been obtained. The culture that was able to withstand the lowest pH and still generate alkalinity was in Bioreactor A (Parys mine enrichment), the low pH threshold being 1.73. The other cultures, in Bioreactor C and M, also demonstrated the ability to generate alkalinity, although pH thresholds were higher, at pH 2.4 and around pH 2.5 respectively. It was therefore concluded that the Parys aSRB from Bioreactor A were likely to be the most acidophilic or acid tolerant of the three cultures. All three mixed enrichment cultures, however, contained SRB that were much more acid tolerant than any that had previously been described.

However, not all of the enrichments were successful. It was not determined why aSRB did not apparently establish in Bioreactor B, which was inoculated with a mixture of surface and subsurface streamers from the Cae Coch mine. It may have been that the surface streamers contained facultative or aerotolerant anaerobes, which outcompeted the SRB for the growth substrate, and possibly utilised it in a fermentative metabolism. It is also possible that the SRB were inhibited by by-products of fermentation reactions, such as acetate. Another possible reason was that redox potentials were too high for sulphate reduction to be favoured. Indeed, Bioreactor 'C', the second Cae Coch culture, took a long time to establish, possibly due to the presence of considerable amounts of ferric iron in the streamer sample, which could result in unfavourable redox potentials of up to + 700 mV (section 1.7.4.1).

The redox values measured in these cultures were considerably more positive than those typically found in cultures of neutrophilic SRB. However, pH has a direct effect on measured redox potential, as shown by the Nernst equation for a sulphidogenic system (Pourbaix, 1966):

$$E = 0.252 + 0.0075 \log \{SO_4^{2-} / HS^-\} - 0.0675 (- \log \{H^+\}) \quad (3.1)$$

for the half-cell reaction (at 30°C):

$$HS^- + 4H_2O = SO_4^{2-} + 9H^+ + 8e^- \quad (3.2)$$

Therefore, redox potential is sensitive to pH variation, and measured redox potential is more affected by pH than by SO_4^{2-}/ HS^- couples by around 1 order of magnitude. This explains higher redox in acidophilic systems, which, in theory, will increase by approximately 50 mV for every unit decrease in pH. Thus, at pH 3, measured redox potentials will be about 200 mV higher than in a system at pH 7. However, the SO_4^{2-}/ HS^- couple is in itself greatly affected by pH: given that the pK_a of hydrogen sulphide is 6.88, the major product of sulphate reduction in the aSRB cultures is gaseous H_2S rather than soluble HS^-, which is more prevalent in neutrophilic SRB cultures. This would account for the increases in redox potential that were observed when the hydrogen sulphide gas was removed by sparging the culture with OFN. It was therefore concluded that redox potentials would be higher than expected in acidophilic cultures, but that this may not in fact be a limiting factor to the growth of aSRB.

The Montserrat culture (Bioreactor M) was different to the other cultures as it was able to generate alkalinity only following relatively small decreases in pH, but when subjected to a pronounced decrease in pH (pH 4.75 to 2.5; Day 10, Figure 3.4a), it took some time for sulphate reduction to become evident again (Days 10 – 30; Figure 3.4a), Indeed, following acidification of this enrichment culture by about 1.5 pH units or more, the

net trend exhibited was acid production (Figure 3.2b). The presence of a putative 'acidogenic' anaerobe in the mixed culture was considered to be the reason for this. This could be an acidophilic organism that was able, at low pH (3 or below), to outcompete the aSRB, utilising glycerol and producing an acid, e.g. as a fermentation product, causing pH to fall. It was concluded that the Montserrat aSRB was less acid tolerant (in the mixed culture) than the Parys or Cae Coch cultures, possibly due to this phenomenon.

In section 3.4, the relationship between sulphate reduction, glycerol utilisation and alkali production by the Cae Coch mixed aSRB culture is described (and shown in Figure 3.5). The data confirmed that reduction of sulphate and catabolism of glycerol occurred in the culture at low pH, and therefore, by inference, in the other bioreactor cultures. The culture pH was initially 4, and glycerol began to be depleted after 2 days, when the culture pH had risen to about 5. However, sulphate disappearance was not observed until days 7-8, by which time culture pH was 5.4. It is possible that other organisms in the mixed culture utilised some of the glycerol in the first few days, and then sulphate reduction became dominant in the system. It is not known why culture pH continued to rise slowly after both sulphate and glycerol was depleted. It may have been at least partially due to ammonification (i.e. the breakdown of organic nitrogen compounds, found in yeast extract, to form the ammonium ion, NH_4^+, at circumneutral pH), which also generates net alkalinity. However, it is probable that this phenomenon may have been occurring as a result of the formation of pyrite from ferrous sulphide, which is an acid-consuming reaction (section 1.5.2; equation 1.14).

3.7.2 Growth of aSRB on solid media

3.7.2.1 Growth of aSRB and screening of solid media

Acidophilic SRB from bioreactor cultures A, B, and M were grown (and eventually isolated from other organisms in the mixed cultures) on solid media, made using the overlay method (Johnson 1995a; Section 2.1.1.4.1). This method was used as it has been shown to promote colony growth of a variety of acidophilic organisms (Johnson, 1995a). In this technique, *Acidiphilium* SJH is incorporated into the underlayer of plates which are usually left to "condition" at room temperature for 2-3 days, to allow *Acidiphilium* SJH to consume agarose hydrolysis products which are considered to inhibit many acidophiles (Johnson, 1995a), i.e. a detoxifying effect. However, the results of this experiment suggested that "ageing" of the media, i.e. allowing more time for SJH activity, did not enhance growth of aSRB; indeed, it appeared to do the opposite. It is noteworthy that an exception to this was the Montserrat aSRB, which grew almost as well on the aged as on the fresh solid medium. These bacteria also grew well on the YE overlay medium in the presence of hydrogen, but not in its absence, which suggested it may be able to use hydrogen as electron donor. It may be that "ageing" of plates had some other effects which prevented growth, e.g. drying of the medium, or that growth of *Acidiphilium* SJH during the incubation period depleted the medium of organic substrates (this acidophile, as aSRB, is heterotrophic). It was concluded that, although lower (1 mM) glycerol concentrations would support growth, the use of higher concentrations would result in improved growth of aSRB, and that the choice of the anaerobic jar system used was of less consequence than the composition of the solid medium. It was recognised that further modifications and improvements were required to optimise growth of aSRB on solid media; these are detailed in Chapter 4.

3.7.2 Growth of non-SRB on solid media

The Parys acidophile 'PW1' rapidly oxidised ferrous iron (as indicated by 'rusty' orange-brown colonies) when grown on overlay media under microaerobic conditions. It was concluded that this isolate is a heterotrophic or mixotrophic iron-oxidiser, possibly related to another similar isolate ('CCH7'); Johnson *et al.*, 1992) found in a sample from Cae Coch during an earlier study. In contrast, it was concluded that isolate 'WHG1', which formed small white colonies under the same conditions, was an acidophilic heterotrophic bacterium. Isolate 'MA1' (the putative 'acidogen') did not grow in aerobic conditions. It is noteworthy that none of the isolates grew well on plates incubated anaerobically, even though they had been isolated from anaerobic plates in the first instance. This suggests that these bacteria are able to exist and grow under anoxic conditions but require some oxygen for optimal growth, or that there is some association or syntrophy with aSRB and/or other organisms in the various mixed cultures.

3.7.2.1 Colony and cell morphology

Morphological characteristics of microorganisms are generally too simple to serve as a basis for reliable identification or classification, although some differentiation may be possible, and it was useful in the early stages of the research to begin to recognise morphologies of the isolates and to notice any variations in cell size, shape or form. These were examined in more detail for some of the isolates in later work (section 6.2) through the use of SEM. Acidophilic SRB grew initially as white, then black (from the production of ferrous sulphide), smooth, domed, regular colonies which were usually > 1 mm in diameter. It was noticed that, for the isolates CC1 and M1, cell shape appeared to vary with the age of the colony or culture. In relatively young colonies or in cultures that had recently blackened, the cells appeared as large rods, straight to curved, which were occasionally motile in a twisting/rotating motion. In older, larger and more blackened

colonies, or in the case of older, more blackened liquid cultures, the cell shape was sometimes seen as oval or pear shaped, often with endospores, and were non-motile. The spores may have been produced in response to a number of factors; for example, an absence of nutritional requirements, which is possible in an older culture, or as a response to pH approaching neutrality. The Parys aSRB cells (P1) were straight rods that did not appear in a curved form. They were occasionally motile, in a twisting/rotating motion, and in older colonies or cultures, terminal endospores were present.

The 'non-SRB' isolates PW1 and WHG1 appeared to be quite similar morphologically, their filamentous growths superficially resembling those of actinomycetes, and also that of isolate CCH7 (Johnson *et al.*, 1992). Isolate CCH7 seemed to resemble most closely the (neutrophilic) iron bacterium members of the *Sphaerotilus-Leptothrix* group. These bacteria are filamentous, form streamer-like growths and consist of rods that vacate the filaments when conditions become suboptimal (Johnson *et al.*, 1992). This could explain the observations of both filamentous and rod-shaped cellular morphologies in PW1 and WHG1. MA1 did not exhibit any visible variations in morphology (pleomorphism) and, as it was a very small microorganism, it was not possible to make detailed observations using phase contrast microscopy alone. Difficulties in growing these organisms in isolation meant that further investigations were not possible.

PW1 oxidised iron rapidly in microaerobic conditions, and although it was not observed, it may be that WHG1 could also oxidise iron, but this was not observed in the duration of these experiments. It is likely that both of these organisms exist as part of mixed consortia in the AMD environments from where they originated, possibly in the microaerobic zone, and as such, may have some association with aSRB.

3.7.3 Growth of aSRB in liquid media

The standard procedure for isolating SRB has been to incorporate electron donor compounds into media containing sulphate, and to provide anaerobic conditions. Growth of aSRB did not appear to be this straightforward, as the majority of cultures failed to grow after prolonged (≥3 months) incubation. The reason for this was not known at this stage. Sub-optimal (relatively high) redox potentials were thought to be a possible reason for the failure of the pure cultures of aSRB to transfer consistently into fresh liquid media. It was also possible that the cultures were not completely deoxygenated; although culture vessels were filled completely to exclude oxygen, and deoxygenated medium was used, some oxygen may have been introduced during transfer of colonies. The transfer of colonies into a small (1.5 ml) volume of medium seemed to be more successful in growing pure cultures than using larger (30 ml) volumes, and this method was therefore employed subsequently on a routine basis.

There may be density-dependent factors that determine whether growth of aSRB cultures can occur at all. In quorum sensing, which is a mechanism used by Gram-positive and Gram-negative bacteria to regulate their gene expression, and resulting phenotype, cell culture density is "sensed" through production of cell signalling molecules (e.g. acylated-homoserine lactones, AHLs), and when a threshold concentration is reached, a signal transduction cascade is initiated, resulting in the expression of a number of target genes (Zavilgelsky and Manukhov, 2001; Nogrady et al., 2000; Greenberg, 2000; Stoodley et al, 2000; Bassler, 1999). It has also been suggested that quorum sensing may play a role in the development of biofilms, which also exhibit high cell densities (Williams and Stewart, 1994). Cell-to-cell communication and community behaviour appears to be the rule rather than the exception (Greenberg, 2000). It may be that aSRB cultures cannot grow, in pure culture at least, until a certain cell density is reached, at which point genes relating to, for example, sulphate activation

or oxygen reduction, are expressed, allowing growth via sulphate reduction to occur.

The use of a reductant such as sodium sulphide, sodium thioglycolate, titanium (III) citrate in the culture of SRB is a standard practice (section 1.7.3.1). However, it was not known whether it should be adopted in the culture of novel acidophilic strains. Both thioglycolic and citrate acids could conceivably inhibit SRB at low pH (the 'organic acid' effect; section 4.1 and Figure 4.1). In contrast, sulphide would be a less toxic reducing agent, in theory, at concentrations of <5 mM. However, sodium sulphide is strongly alkaline in solution, and therefore its use requires careful monitoring and measurement of culture medium pH. The incorporation of solid particles or a solid growth matrix for culturing SRB in laboratory and industrial-scale processes is well documented (section 1.7.5.3). The experiment described in section 3.7.2 was designed to test the effect of provision of surfaces for bacterial attachment (glass beads) in combination with sodium sulphide, as a chemical reducing agent. Although none of the MB1 cultures grew (for reasons unknown), the results obtained with the CC1 cultures suggested that use of a reductant such as sodium sulphide in liquid cultures of aSRB was advantageous, resulting in more rapid growth of aSRB. The data also showed that the inclusion of glass beads improved the growth of aSRB in liquid media. Further development of liquid media, and other experiments later in the research programme, made use of these initial findings.

An aSRB culture from Cae Coch (CC1) was screened for growth in medium containing varying concentrations of chloride (as potassium chloride), either with or without the inclusion of glass beads. The aSRB grew in cultures containing up to and including 250 mM potassium chloride when glass beads were included, but growth did not occur in any of the culture vessels without beads (with the exception of the culture containing 1 mM KCl, many weeks later). This clearly indicates that growth in high

concentrations of chloride ion is possible, as well as demonstrating the rapidity of growth when glass beads were incorporated into the cultures. It was concluded, therefore, that it would be possible to control pH with HCl in future experiments with bioreactor cultures; total concentrations much smaller than 250 mM would be required for control of pH. As there was no comparable growth in cultures without glass beads, It is possible that the establishment of a biofilm around such surfaces provides a "microniche" for the bacteria, conveying tolerance to toxins or pollutants such as chloride ion. Association with particles provides protection against the lethal effects of oxygen; Fukui and Takii (1990b) found that particle-associated *Desulfovibrio desulfuricans* were able to survive prolonged aeration, but free-living ones died rapidly. Immobilised aSRB, therefore, may prove to be more effective at bioremediation of AMD in continuous culture, and it was concluded that glass beads or other suitable matrices would be incorporated into further bioreactor experiments.

4. MEDIA FOR GROWTH AND ENUMERATION OF aSRB

4.1 Introduction

In Chapter 3, enrichment of aSRB from environmental samples and isolation of different organisms is described, together with experiments investigating the responses of mixed cultures of aSRB to low pH. The next stage of the research programme was to develop liquid and solid media for growing and maintaining pure cultures of aSRB. Methods and media adapted from those used to cultivate other acidophilic bacteria (Johnson *et al.*, 1987; Johnson *et al.*, 1993; Johnson, 1995a) were used initially. Subsequently, these were modified to be more appropriate for the growth of aSRB. Once established, media were tested for their effectiveness in isolating and enumerating aSRB and associated acidophiles directly from environmental samples (sediments from Parys Mountain; section 3.1).

Organic acids such as lactic acid typically present in laboratory media used to culture neutrophilic SRB (nSRB) (e.g. Postgate, 1984) cannot generally be used as substrates by acidophilic heterotrophs as they are usually toxic at comparatively low concentrations (around 1mM; Norris and Ingledew, 1992). Figure 4.1 illustrates the mechanism of toxicity. At low pH, weak organic acids are almost completely in the non-dissociated form; this depends upon on the pK_a of the particular acid (Table 4.1). The non-dissociated form is much more membrane-permeable than the dissociated form, and these therefore readily enter the cell. Inside the cell, at higher pH, the acids dissociate, resulting in a net influx of protons, and a gradual reduction in internal pH. In addition, the anions may interfere with cellular metabolism. Outside the cell, organic acids can also act as "uncouplers" (section 5.4.2), preventing ATP formation by inhibiting the proton gradient; protons that are pumped out can be taken up by the dissociated ion.

Organic acid	pK_a of acid
Acetic acid	4.75
Lactic acid	3.86
Formic acid	3.75
Propionic acid	4.87
Benzoic acid	4.19
Pyruvic acid	2.5

Table 4.1 Dissociation constants (pK_a) of some organic acids. At the pH shown for each acid, equilibrium between dissociated and non-dissociated forms is reached. Below this pH, the acids are mainly undissociated.

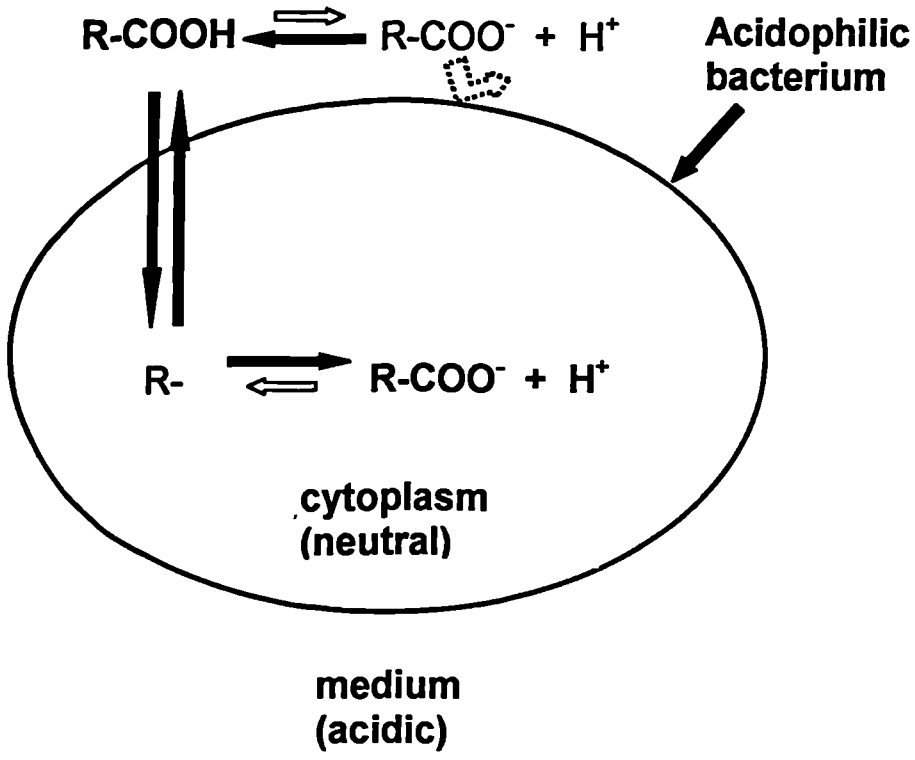


Figure 4.1 The proposed mechanism of toxicity of organic acids to acidophilic bacteria (Ingledew, 1982; adapted from Pronk, 1991).

It is noteworthy that incomplete oxidation of substrates used to grow aSRB, such as glycerol and ethanol, produce acetate (equations 5.8 and 5.6 respectively) which, in batch culture, can build up to concentrations exceeding 1 mM as the substrate is oxidised. Johnson *et al.* (1993) used glycerol/yeast extract medium for the isolation Table 4.1). The non-dissociated form is much more membrane-permeable than the dissociated form, and these therefore readily enter the cell. Inside the cell, at higher pH, the acids dissociate, resulting in a net influx of protons, and a gradual reduction in internal pH. In addition, the anions may interfere with cellular metabolism. Outside the cell, organic acids can also act as "uncouplers" (section 5.4.2), preventing ATP formation by inhibiting the proton gradient; protons that are pumped out can be taken up by the dissociated ion. It is noteworthy that incomplete oxidation of substrates used to grow aSRB, such as glycerol and ethanol, produces acetate (equations 5.8 and 5.6 respectively) which, in batch culture, can build up to concentrations exceeding 1 mM as the substrate is oxidised. Johnson *et al.* (1993) used glycerol/yeast extract medium for the isolation of SRB from acid streamers (section 1.8) under acidic conditions (as low as pH 2.9). No growth of these bacteria occurred when acidified Postgate's medium 'B' (Postgate, 1984), was used, which uses sodium lactate as the main carbon source. Gyure *et al.* (1990) in a study of acid strip-mine lakes, showed that concentrations of organic acid greater than 5 mM completely inhibited SRB activity in sediments at pH 3.8. However, Elliott *et al.* (1998), investigating the development of a bioremediation system for AMD, used Postgate's medium 'B' to enrich for SRB at pH 4.5, and used the culture in a reactor at low pH; SRB activity was obtained between pH 3.0 and 4.5 in medium containing 16.1 mM lactate. It was therefore suggested by these authors that organic acid toxicity is not the sole mechanism preventing growth of SRB in acidic conditions (Elliott *et al.*, 1998).

Initial investigations were carried out using glycerol as electron donor. Subsequently, substrates other than glycerol were investigated, in both

liquid and solid media, for their suitability for growth of aSRB. Routine, reproducible growth was required before further investigations, such as substrate utilisation (section 6.3) was possible. Other investigations, such as the requirement for yeast extract or vitamins were also carried out. In other experiments, ferrous sulphate was largely replaced with either zinc or aluminium sulphate in an attempt to limit the large increase in culture pH which was characteristic of positive aSRB cultures (section 4.2.1.3). In addition, a number of substrates were tested using ferrous sulphate-based medium to investigate whether they supported growth of aSRB isolates.

A variety of solid media were tested for growth of aSRB and associated acidophilic microorganisms. Initially, overlay solid medium that had been used to grow other acidophilic microorganisms (Johnson *et al.*, 1987) was modified for growth of aSRB. A new overlay medium, containing *Acidocella* WJB-3 in place of *Acidiphilium* SJH was eventually developed for use in growth and isolation of aSRB.

4.2 Liquid media for growth of aSRB

4.2.1 Growth of pure cultures of aSRB

4.2.1.1 Medium for routine growth of aSRB

4.2.1.1.1 Methodology

Initially, growth of batch cultures of pure aSRB cultures (section 3.6.1) was carried out using the liquid medium described in 2.1.1.1. An inoculum of pure aSRB culture (approximately 10% by volume) was pipetted into the medium, usually at poised at pH 3.5, and the culture was incubated in filled, screw-top glass bottles of up to 300 ml. Larger volume transfers, attempted in order to scale-up cultures for bioreactor experiments and molecular characterisation, were particularly difficult to achieve. A further development involved the addition of sodium sulphide to lower the initial E_h of the liquid media. Inocula from pure cultures (2 ml) was added to aliquots of medium (18 ml) in sterile universal bottles (28 ml volume), which were

loosely capped or stopped with sterile cotton wool and placed in anaerobic jars (section 2.1.2). Poraver beads (2-4 mm diameter; ~ 5 ml) were added to some cultures. The anaerobic jars were sealed and incubated at 30°C until blackening was observed.

4.2.1.1.2 Results

The modified sodium sulphide containing liquid medium proved to be much more effective for subculturing pure cultures of aSRB than previous media variants. Positive cultures were obtained after around 1-2 weeks. Pure cultures of aSRB from Parys (isolate 'P1') were obtained from transfer of single colonies into this liquid medium. However, after several subcultures using this approach, lack of successful transfer again became apparent. At this point it was decided to focus on the development of suitable solid media for aSRB.

4.2.1.2 Requirement of growth factors by aSRB

For a cost-effective, simple treatment system for AMD, it is advantageous to use a culture that does not have a requirement for complex growth substrates, i.e., that is capable of prototrophic growth. Yeast extract was included in the initial enrichment medium for aSRB in bioreactors, and subsequently in both solid and liquid media formulations, usually at 0.02% (w/v). The effect of different concentrations of yeast extract (0-0.05%) on sulphate reduction, pH, cell numbers, ferrous iron concentrations, glycerol utilisation and acetate production, was investigated. It was speculated that yeast extract were providing B vitamins and/or other compounds for metabolic functions of the bacteria, and that yeast extract might therefore be replaced with vitamins with little or no negative effect on growth. The effect of replacing yeast extract with a vitamin solution was therefore investigated.

4.2.1.2.1 Methodology

A pure culture of isolate M1 was inoculated into liquid medium (section 2.1.1.1), amended with 5 mM each of glycerol, potassium sulphate, and ferrous iron. A 10% inoculum was used, with a final volume of 20 ml, and the starting pH was found to be 4.0 – 4.5. Yeast extract added at 0% 0.001%, 0.002%, 0.01%, 0.02% or 0.05% (w/v, final concentrations). The universal bottles were incubated as described in 4.2.1.1.1, and when the cultures had blackened (after around ten days), samples were withdrawn. Cell counts were carried out using the THOMA chamber (section 2.3.2.1), and before carrying out other analyses (section 2.2), samples were filtered (0.2 μm cellulose nitrate, Millipore).

Subsequently, cultures of M1 and CC1 were set up as above but, in addition, samples were taken both prior to and following incubation, for measurement of pH, sulphate and cell counts. Additional cultures were set up with yeast extract added at 0.005%.

To test whether yeast extract could be substituted by a defined mix of vitamins, medium (18 ml) was inoculated with 2 ml of recently grown cultures of isolates M1, CC1 and P1, and a mixed Parys culture ("P2"), in duplicate, and incubated, using the methods and materials described above. The vitamin solution contained the following: 1 mg biotin, 5 mg *p*-aminobenzoic acid, 10 mg nicotinic acid, 5 mg calcium pantothenate, 15 mg pyridoxine dihydrochloride, 10 mg thiamine, and 5 mg cyanocobalamine in 100 ml distilled water; the solution was filter-sterilised. This was added to the cultures to a final concentration of 1 ml L^{-1} (Widdel and Bak, 1992), along with glycerol (final concentration 5 mM). In parallel, cultures with 0.02% yeast extract and 5 mM glycerol were set up, as well as controls containing 5 mM glycerol, to which neither yeast extract nor vitamin solution was added. Sulphate concentrations and pH were measured at the beginning and end of the incubation period.

4.2.1.2.2 Results

Figure 4.2 shows the effect of various concentrations of yeast extract on sulphate reduction, ferrous iron precipitation, pH, glycerol utilisation, acetate production, and cell numbers (i.e. changes in biomass). At 0% yeast extract, there was virtually no change in final pH (4.78, compared with an initial pH of 4.5). However, in cultures containing only 0.001% yeast extract, the mean final pH was 6.02. When comparing concentrations of residual sulphate, ferrous iron and glycerol in these cultures, a small but noticeable depletion was observed in those containing 0.001% yeast extract was apparent. More significant were the greater concentrations in acetate and the five-fold larger cell numbers in the latter than in the yeast extract-free control. In cultures containing 0.002% yeast extract, final glycerol concentrations were almost zero (0.33 mM), and the mean acetate concentration was 3.83 mM. Final sulphate and ferrous iron concentrations were also lower and the final pH was 6.21. At higher yeast extract concentrations, differences between cultures were less pronounced (Figure 4.2).

Figures 4.3 and 4.4 show results for the similar experiment with isolates M1 and CC1, respectively. With isolate M1, a significant increase in pH (to circumneutral), and a noticeable decrease in sulphate and ferrous iron concentrations occurred at 0.005% yeast extract. Above this concentration, further increases in yeast extract concentration did not appear to effect any further sulphate reduction, whilst ferrous iron concentrations were reduced to zero at 0.01% and above. However, cell numbers did not show any real increase between start to finish in any of the cultures except at 0.05% yeast extract. Similar results were found with isolate CC1, though noticeable changes occurred in 0.001% yeast extract medium. Obvious reductions in sulphate and ferrous iron concentrations, and a noticeable increase in pH occurred at this concentration compared with 0%. Cell numbers, as with the M1 cultures, did not show any

significant increases other than in the culture containing 0.05% (although in contrast to the M1 cultures, there were actual increases in all cultures, however small). pH rose to just over 7 at 0.002% yeast extract, and rose to 7.6 at 0.005 and 0.01%. The culture that was set up with 0.02% yeast extract had a much lower starting pH (2.72) and a higher sulphate concentration, which was probably due to an erroneous addition of ferrous sulphate solution (the stock solution was pH 2). However, this did not appear to inhibit the aSRB completely, as the pH was still observed to rise to 4.29 in this culture, and sulphate concentrations decreased from 16.6 to 12.7 mM.

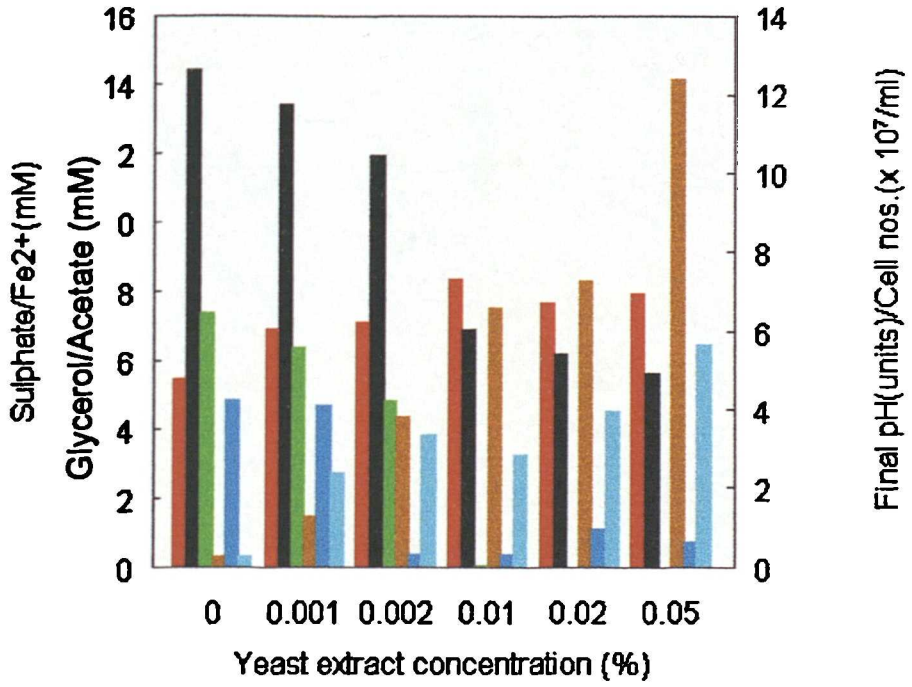
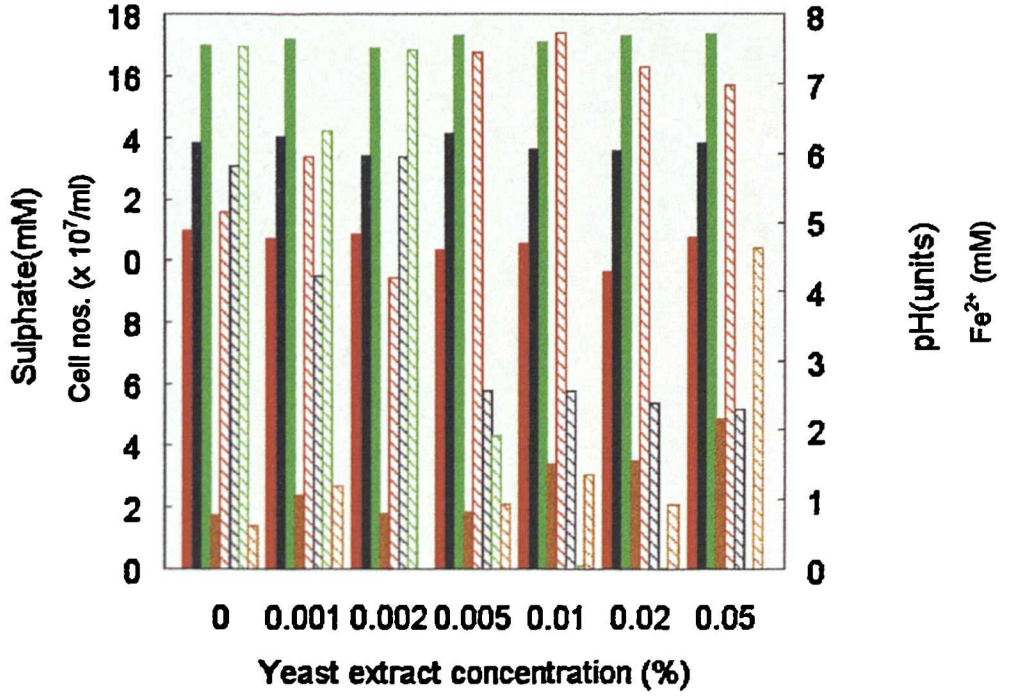
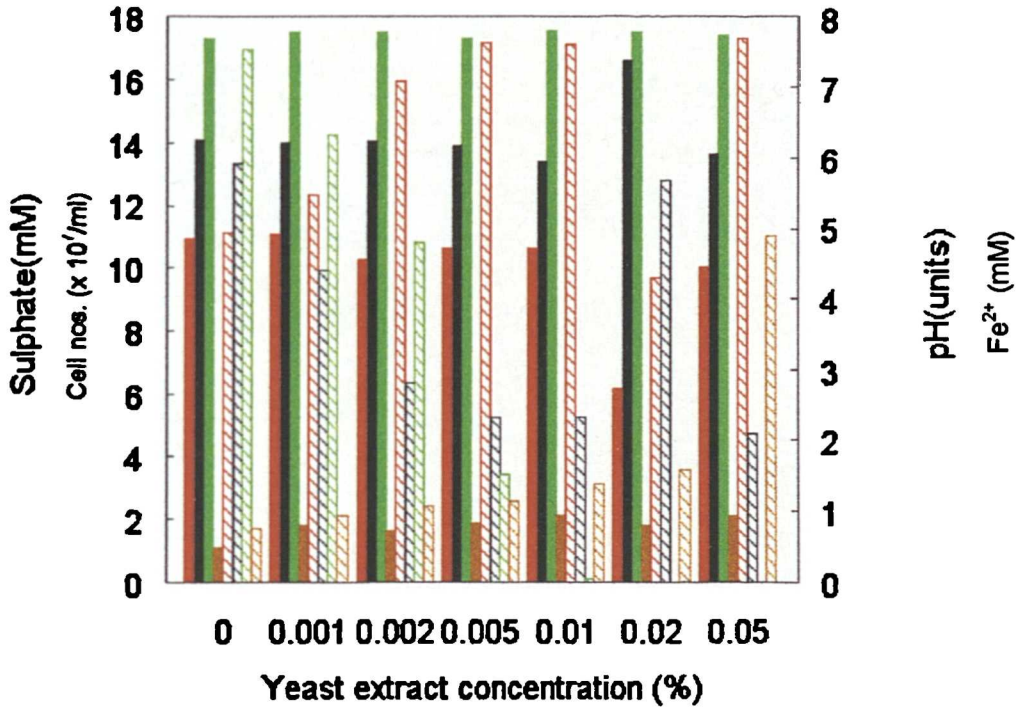


Figure 4.2 Effect of variations in yeast extract concentration on final sulphate (black), ferrous iron (green), glycerol (blue) and acetate (sky blue) concentrations, and final pH (red) and cell numbers (brown) in batch cultures of M1.





Figures 4.3 (top) and **4.4** (bottom). The effect of varying yeast extract concentration on final pH (red), sulphate (black) and ferrous iron (green) concentrations, and cell numbers (brown) in batch cultures of M1 (top) and CC1 (bottom). Solid lines indicate results of analyses at the start, and broken lines at the end of the incubation period, for each test concentration.

Sulphate reduction (measured by disappearance of sulphate) was comparable in cultures of all strains when compared with controls, when either vitamin solution or yeast extract was added (Table 4.2), although in cultures of CC1, M1 and P1, more sulphate was removed and a larger pH increase was obtained in vitamin- than yeast extract-containing medium. However, this was reversed with the mixed culture P2.

Culture	Test (yeast extract/vitamins/ control	Mean decrease in sulphate concentrations (mM)	Mean increase in pH (units)
CC1	+ Yeast extract	5.31	1.31
CC1	+ Vitamins	7.8	2.92
CC1	Control	0.62	0.81
M1	+ Yeast extract	6.61	0.81
M1	+ Vitamins	7.47	3.18
M1	Control	0.51	0.61
P1	+ Yeast extract	2.94	1.8
P1	+ Vitamins	4.96	2.29
P1	Control	1.69	1.70
P2	+ Yeast extract	3.96	2.73
P2	+ Vitamins	3.44	0.93
P2	Control	1.54	0.62

Table 4.2 Comparison of the effects of addition of yeast extract or a a defined vitamin mix to glycerol medium on the growth of pure cultures of aSRB (CC1, M1 and P1) and a mixed culture (P2).

4.2.1.3 Use of zinc- and aluminium sulphate in growth medium

It was found that, for cultures grown with ferrous sulphate–based medium (final concentration 5 mM), and 5 - 10 mM glycerol, the pH rose from initial

values of 3.5 – 4.5 to 6 or above at the end of the incubation period. An attempt was made to increase the buffering capacity of the growth medium, using two different approaches. In the first of these, zinc sulphate was added so that sulphidogenesis would result in the formation of solid-phase zinc sulphide (and concomitant proton production; equation 5.1) in order to counterbalance proton consumption resulting from sulphate reduction. The second approach was to include aluminium sulphate in the growth medium and to utilise the aluminium buffering system (equations 2.1 and 2.2).

4.2.1.3.1 Methodology

Zinc sulphate- and aluminium sulphate based media were prepared as described in sections 2.1.1.1.1 and 2.1.1.1.2, with the addition of glycerol (5 mM), ferrous sulphate (1 mM) and yeast extract (0.01%). Cultures of isolates CC1, M1, P1 and P2 were set up in universal bottles in duplicate and incubated at 30°C. The appearance of a pink-brown precipitate (of zinc sulphide) was taken as an indication that growth had occurred in zinc-containing medium. In these cultures, changes in sulphate concentration, pH, and cell counts were measured. Samples from cultures inoculated into aluminium sulphate-containing medium were analysed regularly for changes in cell numbers.

4.2.1.3.2 Results

In those cultures that grew in the zinc sulphate-containing medium (CC1 and P2), sulphate reduction and an increase in cell numbers was observed (Table 4. 3). Cells were motile and no spores were observed in these cultures. However, despite several attempts, successful subculturing in zinc-containing medium was not achievable. No growth of aSRB was observed in aluminium sulphate-containing medium.

Culture	Mean pH (start)	Mean pH (end)	Mean reduction in sulphate concentration (mM)	Mean increase in cell numbers (x 10^7/ml)
CC1	3.55	4.05	2.43	2.25
P2	3.52	3.54	2.45	1.64

Table 4.3 Summary of results from batch cultures grown in zinc sulphate-containing medium.

4.3 Solid media for growth and enumeration of aSRB

Initially, aSRB were grown on a solid medium adapted from a ferrous sulphate overlay formulation with yeast extract (0.02%) in place of tryptone soya broth and glycerol added at 10 mM. Whilst aSRB grew initially in large numbers using this medium, after a few subcultures, growth was poor or non-existent. In an attempt to improve reproducibility, 5 mM ferrous sulphate/5 mM potassium sulphate was used in place of 10 mM glycerol, and the pH of solution (i) (section 2.1.1.4.2) was adjusted to pH 5 before autoclaving, thereby producing a higher pH in the combined medium. Glycerol was used routinely as electron donor in this medium, though growth of aSRB was obtained in glycerol-free medium.

4.3.1 Comparison of overlay and non-overlay solid medium

4.3.1.1 Methodology

Freshly-grown colonies of isolates CC1 and M1 were loop-streaked onto yeast extract overlay medium (section 2.1.1.4.1 (i)) and yeast extract

medium (section 2.1.1.4.2), in duplicate, and incubated in anaerobic jars (Oxoid AnaeroGen™; section 2.1.2) at 30°C. Figure 4.5 shows both of the anaerobic systems for incubation of solid media.

4.3.1.2 Results

Colonies of aSRB were observed as blackened colonies after ~ 3 weeks, but only on the overlay plates. No growth occurred on non-overlay medium (Figures 4.6 (a) and (b)).

4.3.2 Comparison of sterilised and standard overlay media

An experiment was carried out to test whether viable *Acidiphilium* SJH cells were required to promote growth of aSRB on solid media.

4.3.2.1 Methodology

Yeast extract overlay medium and non-overlay medium was prepared as before. One batch of overlay plates was then sterilised by placing them on a short-wave UV transilluminator for 15 minutes. A second (non-sterilised) batch was set aside for incubation as usual. Non-overlay plates were included as a negative control. All plates were then loop-streaked with colonies of CC1 and M1, and incubated at 30°C in anaerobic jars for three weeks.

4.3.2.2 Results

No colony growth was visible on UV-irradiated overlay medium or non-overlay medium inoculated with isolate CC1, but small colonies (black and white) were present on the yeast extract overlay medium. With isolate M1, very small colonies were visible on the UV-irradiated overlay medium

Figure 4.5 Anaerobic systems for incubating solid media; the Oxoid AnaeroGen™ system (left) and Oxoid Anaerobic System (hydrogen-generating; right). Blackened plates indicate the formation of ferrous sulphide from sulphate reduction.

(a)

(b)

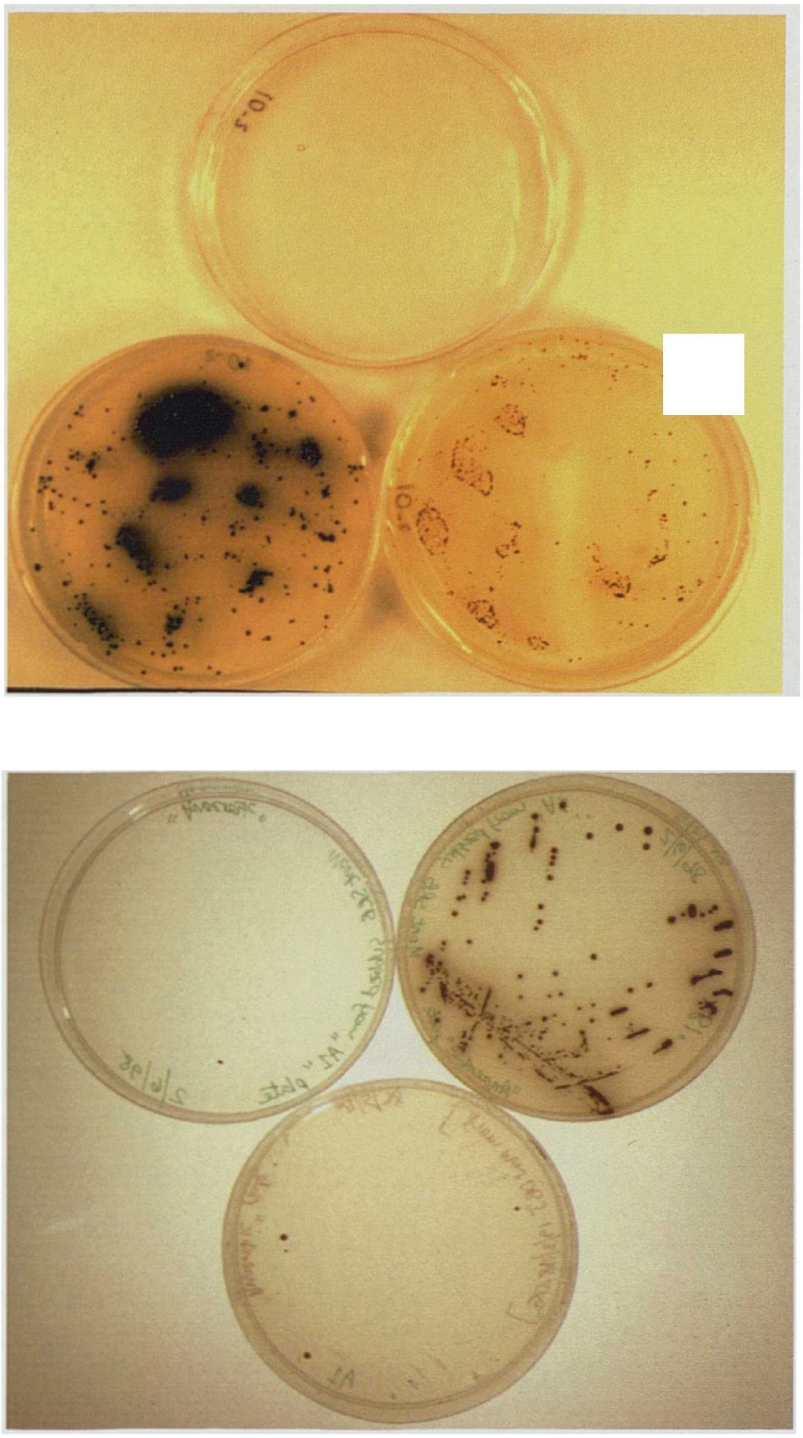


Figure 4.6 A comparison of aSRB growth with overlay and non-overlay solid media. **(a)** shows overlay (bottom left, and bottom right (sub-culture)) and non-overlay (top) media inoculated with CC1. **(b)** shows overlay (top right, and bottom (sub-culture)) and non-overlay (top left) media inoculated with M1.

though these were much small than those on non-irradiated plates. No growth was observed on the control non-overlay medium.

4.3.3 Effect of acid-washing of agarose

The precise role of *Acidiphilium* SJH in promoting growth of aSRB was unclear. As with the iron-oxidising acidophiles, its main role might be detoxification, i.e. removal of small-molecular weight, potentially toxic, organic compounds. If this were the case, then pre-washing the agarose could improve the plating efficiency of non-overlay plates, by removing loosely-bound low molecular weight molecules (Johnson, 1995).

4.3.3.1 Methodology

Agarose was acid-washed (WAYE medium; section 2.1.1.4.2), and used as a gelling agent in otherwise standard non-overlay plates. Variations included omitting yeast extract (AW medium) or adding glycerol (at 1 mM). Standard yeast extract overlay plates were prepared as a positive control. All plates were inoculated by loop-streaking from colonies of CC1, M1 and P1, and were incubated in anaerobic jars at 30°C.

4.3.3.2. Results

In all cases, there was a total absence of colony growth on AW medium, and only a very few (< 10) small colonies on WAYE and AW + 1 mM glycerol medium media. There was extensive growth of aSRB colonies on all standard yeast extract overlay plates.

4.3.5 Enumeration of aSRB and associated bacteria from AMD sediments using overlay media

A new overlay medium was developed using *Acidocella* WJB-3 as an alternative to *Acidiphilium* SJH in the gel underlayer. It was thought that this bacterial strain would remove pyruvic acid, a constituent of agar and derivative products, but at the same time not metabolise yeast extract or various other organic materials that might be used as substrates for aSRB (K.B. Hallberg, unpublished data). Together with *Acidiphilium* SJH overlay plates, this alternative approach was used to investigate the effectiveness of different solid media for supporting the growth of acidophilic microorganisms in subsurface sediments from the AMD site at Parys Mountain. It was also used to examine SRB populations in different zones of the AMD sediments.

4.3.8.1 Methodology

Experiment (i). The first experiment was set up to identify which type(s) of medium were the most effective for obtaining maximum colony counts of acidophilic bacteria in the AMD sediments. A sediment core was obtained from Parys Mountain as described in section 3.1 (i) (Figure 3.1). The pH profile of the core was measured using a flat-surfaced pH electrode (section 2.2.1.) Working inside an anaerobic chamber (section 2.1.2), a spatula of material taken directly from, and just around, a black zone mid-way down the core was weighed and placed into a test-tube containing 4.5 ml of sterile, anoxic basal salts. This was gently vortexed until the sediment was homogenised. A dilution series was carried out by transferring 0.5 ml of material from the initial suspension to another tube containing 4.5 ml of basal salts (10^{-1} dilution), vortexing gently to homogenise, and transferring again until dilutions up to 10^{-4} were obtained. Aliquots (100 µl) were spread onto the solid media. The following variants were tested:

(a) Yeast extract overlay (*Acidiphilium* SJH) medium (pH ~ 3.5), with the following added by spreading concentrated solutions onto the medium before inoculation:

(i) Glycerol (5 mM, final concentration)

(ii) Lactic acid (0.5 mM)

(iii) Galactose (2.5 mM)

(iv) zero addition

Different concentrations of substrates were used; in the case of glycerol and galactose, the concentrations used reflect the number of carbon atoms in a molecule of each substrate. "Carbon equivalents" were used. In the case of an organic acid such as lactic acid, much lower concentrations are used to prevent any possible toxic effects (sectionn 4.1).

(b) Yeast extract (non-overlay) media, as variants of the above.

(c) Yeast extract overlay (*Acidiphilium* SJH) medium (pH ~ 3.0)

(d) Yeast extract overlay (*Acidocella* WJB-3) medium, (pH ~ 3.5), with the following added to the molten medium:

(i) Glycerol (5 mM, final concentration)

(ii) Lactic acid (0.5 mM)

(iii) Galactose (2.5 mM)

(In addition, a set of yeast extract overlay plates (both *Acidiphilium SJH and Acidocella* WJB-3) was inoculated with diluted sediment samples under air, i.e. outside of the anaerobic chamber.

The pH of the different media was measured using a flat-surfaced pH electrode. All media were incubated in anaerobic jars at 20°C, and were removed after four weeks for colony counts. The dry weight of the sediment was obtained by weighing (to 2 d.p.), placing it into a crucible, drying it in an oven at 160°C overnight and weighing the cooled crucible. This was carried out in triplicate and the mean dry weight of a known wet weight of sediment was calculated.

Experiment (ii): In a second experiment, another sediment core was collected from the same location at Parys Mountain and a pH profile of the core recorded. (Table 3.1). With the core orientated vertically with the top of the core indicating the sediment surface, 'Zone 1' was the grey sediment zone immediately above a black zone ('Zone 2'), and 'Zone 3' was the grey zone immediately below Zone 2. Samples of sediment from all three zones used in a dilution series as described above. Dilutions 10^{-1} – 10^{-3} (sediment from Zones 1 and 3) and 10^{-2} – 10^{-4} (Zone 2) were used to inoculate solid media variants, using 100 µl of each dilution. All work was carried out in an anaerobic chamber (section 2.1.2). The following medium variants were tested in duplicate for each dilution:

(a) Zones 1-3:

(i) Yeast extract overlay (*Acidiphilium* SJH) plates, spread with glycerol (5 mM, final concentration)

(ii) Yeast extract overlay (*Acidocella* WJB-3) overlay plates, spread with lactic acid (0.5 mM);

(iii) Yeast extract overlay (*Acidocella* WJB-3) plates, including lactic acid (0.5 mM) added to the molten medium;

(iv) Non-overlay yeast extract medium, spread with galactose (2.5 mM, final concentration);

(b) Zone 2 only:

(i) Yeast extract overlay (*Acidiphilium* SJH) medium;

(ii) Yeast extract overlay (*Acidiphilium* SJH) medium, spread with galactose (2.5 mM, final concentration);

(iii) Yeast extract overlay (*Acidocella* WJB-3) medium, containing 5 mM glycerol;

(iv) Yeast extract overlay (*Acidocella* WJB-3) medium, containing 2.5 mM galactose.

All plates were incubated in anaerobic jars at 20°C, and were removed after three weeks for colony counts. They were then re-incubated for another three weeks to allow for possible further growth, and final counts were adjusted where necessary. Mean numbers of bacterial colony forming units (CFUs) per gram of dry weight of sediment were calculated.

4.3.8.2 Results

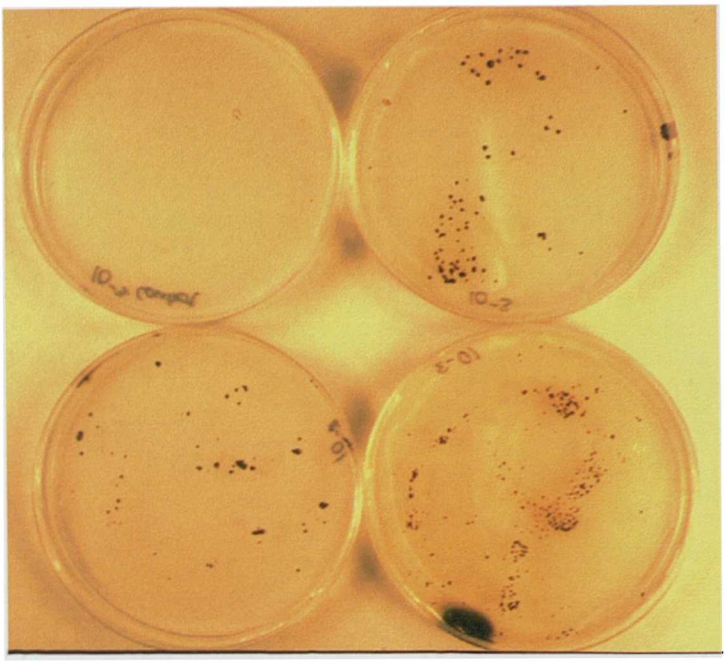
Experiment (i): The results from the first experiment are summarised in Table 4.4. The pH profile of the core was found to be very similar to that of the core described in section 3.1, with the highest pH found in the black sulphidic zone(s). The dilution series produced some unusual data in some cases, with some counts showing higher standard deviations than the means. This was probably because crowding on the 'lower dilution' plates prevented accurate enumeration. However, it was concluded that the largest total numbers of bacteria were obtained with the WJB-3 overlay medium + lactic acid (WJB-3 LAC), and the smallest lowest were obtained with the pH 3 SJH overlay medium. Much greater numbers of CFUs were achieved - by two to four orders of magnitude - with overlay medium that with non-overlay medium. Exposure of the inoculum to oxygen (i.e. inoculating outside of the anaerobic chamber) produced lower counts (1-3 orders of magnitude) than in corresponding plates inoculated inside the chamber, though the reverse was found using *Acidiphilium* SJH plates. Figures 4.6 - 4.8 show growth of aSRB on some of the solid media. On the majority of media, a mixture of P1- and PCP-like isolates were present, along with an unidentified bacillus ("UB"), with smaller numbers of PFB-like

isolates. It was noted that on the 10^{-1} dilution plate of the non-overlay medium YE + LAC, colonies of P1-, PCP-, PFB- and UB-like bacteria were present. On the WJB-3 GLY medium, only PFB- and PCP-types grew, with large spores observed in wet mounts of colonies of PFB. Only PFB-like bacteria grew on the YE, YE + GLY and the YE + GAL media, and in each case numerous large spores were seen.

Medium	CFUs/gram dry weight of sediment
WJB-3/ YE overlay	$7.7 \pm 2.3 \times 10^{8}$
WJB-3/ YE + GLY overlay	$1.4 \pm 1.3 \times 10^{8}$
WJB-3/ YE + GAL overlay	$1.3 \pm 0.04 \times 10^{9}$
WJB-3/YE+ LAC overlay	$3.9 \pm 4.9 \times 10^{10}$
WJB-3/ (aerobic inoculum)	$2.3 \pm 0.4 \times 10^{7}$
SJH/ YE overlay	$2.3 \pm 1.7 \times 10^{8}$
SJH/ YE + GLY overlay	$9.7 \pm 11.9 \times 10^{9}$
SJH/ YE +GAL overlay	$1.0 \pm 0.8 \times 10^{9}$
SJH/ YE + LAC overlay	$4.4 \pm 2.7 \times 10^{8}$
SJH/ aerobic inoculum	$1.5 \pm 2.2 \times 10^{9}$
YE (non-overlay)	$1.2 \pm 0.2 \times 10^{6}$
YE + GLY (non-overlay)	$1.8 \pm 1.2 \times 10^{6}$
YE + GAL (non-overlay)	$3.7 \pm 3.0 \times 10^{6}$
YE + LAC (non-overlay)	$9.9 \pm 5.1 \times 10^{6}$
SJH/ YE overlay pH 3	$< 10^{2}$

Table 4.4 A comparison of colony forming units (CFUs) obtained from inoculation of different solid media with material from AMD sediments. YE = yeast extract; GLY = glycerol; GAL = galactose; LAC = lactic acid.

4.7



4.8

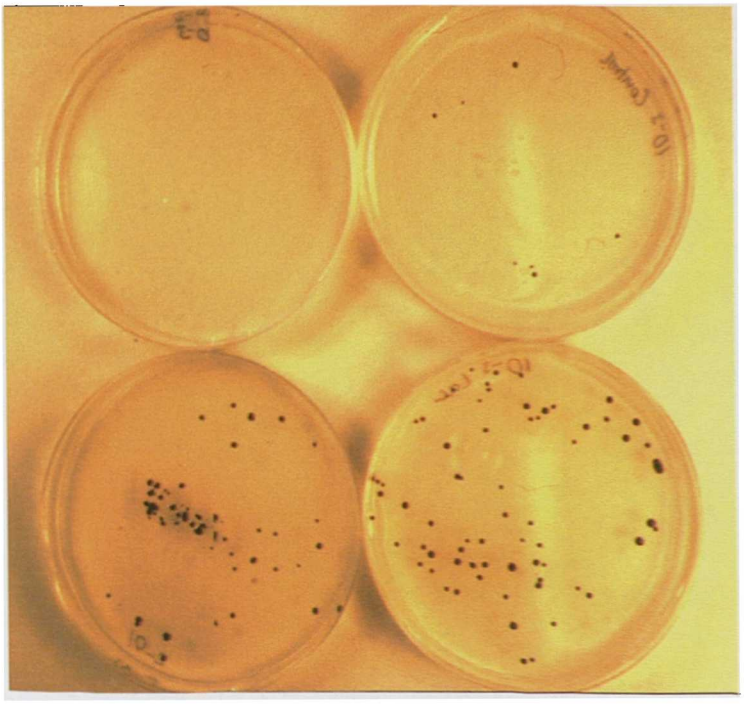


Figure 4.7 (top) and **4.8** (bottom). A comparison of aSRB growth on different overlay media. **4.7** shows petri plates containing (clockwise from top left): WJB-3 GLY; WJB-3 YE; WJB-3 LAC; and WJB-3 GAL media (all 10^{-3} dilutions). **4.8** shows petri plates containing (clockwise from top left): SJH YE ('acidic'); SJH YE ('aerobic' inoculation); SJH LAC; and SJH YE (standard) media (all 10-3 dilutions).

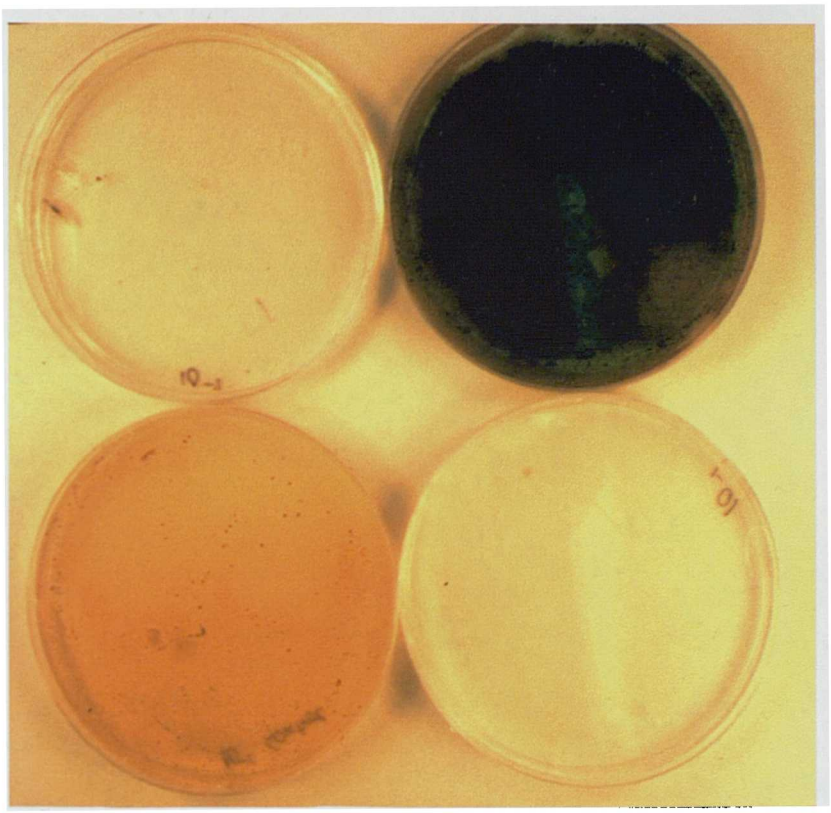


Figure 4.9 A comparison of aSRB growth on different solid media. Figure shows petri plates containing (clockwise from top left): YE (non-overlay); SJH YE (standard); SJH YE (pH ~ 3.0); and SJH YE (aerobic incubation), all 10^{-1} dilutions.

(ii) The results for the enumeration of bacteria from AMD sediment zones are summarised in Table 4.5. The largest numbers of bacteria grew on the WJB-3 medium amended with lactate (WJB-3 LAC), which was inoculated with material from Zone 2. The greatest diversity was found using WJB-3 LAC medium (Zone 1) and SJH medium amended with galactose (SJH GAL; Zone 2); five different strains were identified on each. In the former, the three characterised strains from the mixed bioreactor culture (Parys) P1, PCP and PFB were present, as well as an unidentified bacillus (UB) which grew as large (~8 μm in length) straight or curved cells, with terminal endospores. Colonies containing short (~3 μm in length) rods were also present (UR1). In the latter, UR1 and another, larger (~6 μm in length) rod-shaped organism (UR2) was found in addition to P1, PCP, and another sulphate reducer (USR). This formed much smaller, flatter colonies than the P1 type, with short (~4 μm in length), thick straight non-motile rods, some with terminal spores. The SJH medium amended with glycerol (SJH GLY; Zone 2) was the only other medium on which USR was found, and no SRB other than P1 and PFB were observed outside of Zone 2.

Overall, more diversity was found in the medium inoculated with Zone 1 sediments than with Zone 3. A third type of rod-shaped bacterium (UR3) grew on the SJH GLY medium inoculated with material from Zone 1. Cells were thick and oblong-shaped in appearance. No colony growth was obtained in the non-overlay medium (YE GAL) inoculated with material from Zones 2 and 3. A few yeast colonies (<5) grew on the WJB-3 LAC medium (Zone 1). Another observation was that the WJB-3 medium with lactate included in the medium produced growth of larger numbers of bacteria than when the substrate was spread onto the medium rather than being added to the molten medium.

Zone	Medium	Total nos. bacteria/g dry weight of AMD sediment	Types of bacteria present
1	WJB-3 YE+LAC	$4.1 \pm 1.52 \times 10^7$	P1; PFB ; PCP ; UB; UR1
	SJH YE+GLY	$1.1 \pm 0.18 \times 10^8$	P1; UR2; UR3
	YE+GAL (NO)	$3.7 \pm 0.89 \times 10^6$	PCP; UR1
2	WJB-3 YE+LAC	$4.6 \pm 2.21 \times 10^8$	P1; PCP; UB; UR1
	WJB-3 YE+LAC*	$3.5 \pm 1.3 \times 10^7$	P1; PCP; UB
	WJB-3 YE+GAL	$4.7 \pm 2.77 \times 10^7$	P1; PCP
	SJH YE+GAL	$7.2 \pm 3.4 \times 10^7$	P1; PCP; USR; UR1; UR2
	YE GAL (NO)	$<10^2$	N/A**
	WJB-3 YE+GLY	$2.7 \pm 1.1 \times 10^8$	P1; PCP; UR
	SJH (YE+GLY	$1.03 \pm 0.52 \times 10^8$	P1; PFB; PCP; USR
	SJH YE	$7.6 \pm 3.8 \times 10^7$	P1; PFB; UB; PCP
3	WJB-3 YE+LAC	$5.6 \pm 3.8 \times 10^7$	P1; PCP; UB
	SJH YE+ GLY	$2.6 \pm 1.6 \times 10^6$	P1; PCP
	YE GAL (NO)	$<10^2$	N/A**

Table 4.5 Summary of results from the investigation of bacteria in AMD sediment zones. YE = yeast extract; GLY = glycerol; GAL = galactose; LAC = lactic acid added to overlay (WJB-3 and SJH) media. P1, PCP and PFB are aSRB. UB = unidentified bacillus; UR1, UR2 and UR3 are unidentified rods; USR = unidentified sulphate reducer. *Lactic acid was spread over the medium, rather than added to molten medium. **No colonies grew on these media. NO = non-overlay medium.

4.4 Discussion and Conclusions

Growth of aSRB in liquid culture was, in general, very variable. Mixed cultures grew relatively easily in larger batch cultures (>100 ml), by simply subculturing with a heavy (~10%) inoculum into bottles completely filled with medium (to maintain enrichment cultures, data not shown), or in batch bioreactor systems (sections 3.2 – 3.4, and 5. 2). Pure cultures only grew in small batch cultures (~20 ml), and even this method was not always reliable, although there were improvements as growth media and handling techniques were refined. Attempts at scale-up of pure cultures of aSRB were unsuccessful, for unknown reasons. It is possible that aSRB grew more easily in mixed culture due to other bacteria utilising residual oxygen, or that fermentation reactions carried out by these or other organisms were providing by-products which were more easily utilised by the aSRB than the substrates provided in the growth medium (see section 1.7.5.2). As the individual members of the consortia were not identified, and were possibly novel organisms, the nature of any inter-relationships could not be determined. As described in section 3.7.3, growth of pure cultures in the laboratory may depend on density-dependent factors (e.g. cell signalling); further experiments would be required to investigate this.

Another possibility is that the use of sodium sulphide as a reductant was inhibitory. This appears paradoxical, as growth of aSRB in the liquid medium containing Na_2S was more easily achieved than when it was not used (section 3.6.2). However, commercial sodium sulphide may contain impurities which are inhibitory (Widdel, 1992a). A number of *Desulfotomaculum* spp. showed very poor growth in media reduced with commercial sodium sulphide compared to other reductants (Klemps *et al.*, 1985; Widdel, 1992); the inhibition was not thought to be due to sulphide itself as the added concentration (1.5 mM) was far below the concentration that is usually inhibitory to *Desulfotomaculum* spp. (7 to 10 mM; Cord-Ruwisch and Garcia, 1985), but was attributed to impurities in the

commercial-grade chemical. The liquid medium used routinely contained a lower concentration of Na_2S, i.e. 0.5 mM (section 2.1.1.1.1), but it is possible that inhibition occurred in some instances. Other reducing agents (e.g. sodium thioglycollate/sodium ascorbate) could not be used due to their toxic effects at low pH.

It was recognised that, if reproducible growth of pure cultures was not possible, physiological characterisation of aSRB, including substrate utilisation, would be problematic. However, as the method used to grow pure aSRB (strains CC1, M1 and P1) was initially very successful, an experiment to test utilisation of a number of possible substrates was attempted (data not shown). It was not possible to confirm positive or negative results from this experiment, as none of the cultures grew (including the putative positive controls), and it was concluded that another method would be required to test various physiological parameters (section 6.3).

Experiments with pure cultures demonstrated that the addition of yeast extract to liquid medium resulted in enhanced growth of aSRB compared with controls, as shown by sulphate reduction, increase in cell numbers, utilisation of glycerol, and generation of alkalinity. Concentrations of acetic acid were also noted to increase with increasing yeast extract concentrations, indicating that sulphate reduction was coupled to incomplete oxidation of glycerol in these strains. In cultures containing larger concentrations of yeast extract, acetic acid continued to be produced even after glycerol was depleted, suggesting additional incomplete oxidation of organic compounds contained in yeast extract. Following phylogenetic analysis of 16S rRNA sequences obtained from these bacteria, they were found to be most closely related to *Desulfosporosinus orientis* (section 6.4). This organism is a Group I SRB (in terms of its inability to oxidise organic substrates completely) (Table

1.2), and therefore the results of these experiments are consistent with this.

The addition of yeast extract to a concentration of only 0.001% resulted in improved growth compared with 0% controls. At 0.002%, almost all the glycerol added to the medium was depleted and pH increased to ~ pH 7. This concentration of yeast extract therefore appeared to be sufficient to promote growth of aSRB, and was therefore used in medium in later work, e.g. in bioreactor experiments (Chapter 5). With increasing concentrations of yeast extract (>0.002%), cell numbers increased beyond depletion of glycerol, suggesting that organic carbon from yeast extract was being utilised. However, this effect was not demonstrated in the repeat of this experiment, in which a noticeable increase in cell numbers was only found when yeast extract was added at 0.05%. It appeared that, in this case, sulphate reduction activity was occurring without a concomitant increase in bacterial growth. It is possible that acetate produced in these cultures (by inferral from the previous experiment) was acting as an uncoupler of respiration.

A yeast extract concentration of 0.005 - 0.01% was sufficient to cause enough sulphidogenesis to cause all ferrous iron added to be precipitated as ferrous sulphide. A concentration of 0.002% appeared to be necessary for growth of pure and mixed aSRB cultures. Although this is not a very high concentration, if the bacteria were auxotrophic for yeast extract, it could be disadvantageous to use these strains in a treatment system. However, the addition of a vitamin solution to glycerol medium resulted in more sulphate reduction and alkalinity generation in pure cultures (but not the mixed culture P2) compared with cultures with added yeast extract (section 4.2.1.2.2; Table 4.2). It is possible that trace amounts of yeast extract were carried over with the inoculum in these cultures, and the further addition of vitamins that may be essential or beneficial to metabolic functions may have resulted in the enhanced growth. Experiments with

washed cells would be necessary to establish whether it was the vitamins alone that promoted sulphidogenic activity by the aSRB. An estimation of the amount of sulphate reduction effected by utilisation of yeast extract by isolate M1 was made using the data from the initial experiment, assuming the initial sulphate concentration to be ~ 15 mM. Table 4.4 shows the decrease in concentration of sulphate for each yeast extract concentration tested, based on this assumption:

% yeast extract in culture	Change in sulphate concentration (mM)
0	0.5
0.001	1.5
0.002	3.0
0.01	8.1
0.02	8.7
0.05	9.4

Table 4.6 Change in concentration of sulphate in cultures of isolate M1 in which the concentration of yeast extract was varied.

Comparing cultures containing 0.01% and 0.02% yeast extract, there was a difference of 0.6 mM sulphate reduced, and the difference between 0.02 and 0.05% yeast extract was 0.7 mM sulphate reduced. Similarly, the additional sulphate reduced in cultures containing 0.01 and 0.05% yeast extract was1.3 mM. Using these data, the approximate mean value for the amount of sulphate reduction that occurred due to the addition of yeast extract was calculated as 0.385 mM/0.01% yeast extract. Using this value, the contribution of yeast extract to sulphate reduction in medium containing 0.002% (in bioreactor experiments, Chapter 5) or 0.02% (in other liquid and solid medium) is calculated to be low, but still significant.

In the aSRB cultures, yeast extract could be used as a carbon source (with glycerol acting as electron donor), or some components could be used as electron donors themselves. In addition, yeast extract could also be providing a compound required to for the growth and activity of aSRB (as with the vitamin solution). According to Widdel and Bak (1992), all mesophilic Gram-negative sulphate reducers known at that time grew in defined medium without the need for complex nutrients such as yeast extract or peptone, although such compounds were thought to be stimulate the growth of some species. However, Gram-positive *Desulfotomaculum* spp. which oxidised substrates incompletely were shown to not grow well in defined medium, and growth was promoted by yeast extract. As strains M1, CC1 and P1 are most closely related to the incompletely oxidising *Desulfosporosinus orientis*, the fact that they were stimulated by yeast extract is, therefore, not too surprising.

Zinc sulphate-containing medium, which was also used in a batch bioreactor experiment (section 5.2), was tested for growth of aSRB because the low solubility product of zinc sulphide means that, in theory, sulphate reduction in the cultures could take place without the resulting large increase in pH seen in ferrous sulphate-containing cultures. Two isolates (CC1 and P1) displayed sulphate reduction, alkalinity generation, and an increase in cell numbers in this medium, showing that these SRB could grow and reduce sulphate in a medium in which low pH was maintained. Growth was not observed in aluminium sulphate-containing medium, possibly due to the toxic effect of this metal, although soluble aluminium is likely to be present at elevated concentrations in environments where aSRB exist.

Solid medium used at the beginning of the research programme was modified to give a more favourable pH (~3.5 compared with 2.5 – 3) for the SRB isolates. This was found to give reliable growth of aSRB and was used routinely in subsequent work. The unamended yeast extract overlay

solid medium was found to support the growth of aSRB. A comparison of growth on overlay and non-overlay medium demonstrated unequivocally that overlay medium was required for growth, as no growth was obtained with non-overlay agarose medium with the same constituents.

The reason for the success of the overlay medium had not been established, and modified media were tested to investigate whether the role of *Acidiphilium* SJH was as a 'detoxifier' of the solid medium or as a 'substrate provider'.

The data from solid medium sterilisation (UV irradiation) and acid washing of agarose were inconclusive. The lack of colony growth on the UV-irradiated overlay medium suggested that either "detoxification" was being prevented, or that the aSRB were no longer able to utilise substrates produced by live *Acidiphilium* SJH in the gel underlayers. In theory, *Acidiphilium* SJH may, under anaerobic conditions, ferment galactose (the major monosaccharide component of agarose) or some component(s) in the yeast extract, forming small molecular weight acids or alcohols which the aSRB could then utilise. However, although *Acidiphilium* SJH is known to be a facultative anaerobe (Johnson, 1998) its only known strategy is anaerobic respiration using ferric iron as terminal electron acceptor. Attempts in the present study to grow this acidophile anaerobically in the absence of ferric iron were unsuccessful (data not shown). Acid-washing (AW) of the agarose was designed to remove the more acid-labile components of the agarose which might give rise to inhibition of aSRB, thereby mimicking any "detoxification" due to *Acidiphilium* SJH. However, there was no growth of aSRB on AW medium and minimal growth when either yeast extract or glycerol was added to it.

The *Acidocella* WJB-3 medium was developed as it was thought that pyruvic acid might be the source of toxicity in the non-overlay agarose-gelled media. *Acidocella* WJB-3 can utilise pyruvic acid, though it does not

utilise yeast extract or grow anaerobically. The initial medium used an *Acidocella* WJB-3 inoculum which had been grown in fructose medium, and the results obtained (in terms of colony growth of aSRB) was inferior to the conventional *Acidiphilium* SJH medium. In a modified approach, *Acidocella* WJB-3 were grown in 5 mM pyruvic acid medium, and the cells washed before incorporation into the gel underlayer. This modified *Acidocella* WJB-3 overlay medium proved to be effective for growing a large number and diversity of anaerobic acidophilic bacteria. In the two experiments which tested the effectiveness of different solid media promoting CFUs from AMD sediment, the largest counts were obtained on WJB-3 LAC medium. This was considered due to the efficiency of medium "detoxification", combined with the inability of *Acidocella* WJB-3 to utilise most of the organic substrates tested, or grow anaerobically. Lactic acid was possibly the most successful substrate for growing a wide range of organisms including aSRB (and indeed it has been the substrate of choice for growth of many nSRB; Postgate, 1984). A key factor here is likely to have been the relatively low concentration of lactic acid used (0.5 mM) since higher concentrations would, at least on theory, been toxic to aSRB as it is to most acidophilic bacteria (D.B. Johnson, unpublished data). In the natural environment, it is likely that aSRB are, like their neutrophilic counterparts, utilising small molecular weight organic acids as electron donors and carbon sources, but that the concentrations of these acids is always below the toxic threshold levels.

Some of the aSRB isolates were not particularly sensitive to oxygen, as plating them in an aerobic (laboratory) environment did not always cause CFUs to be less than when plating was carried out under strictly anoxic conditions (the anaerobic chamber). Some Gram-positive spore-formers such as *Desulfosporosinus orientis* can withstand oxic conditions to a certain extent (Widdel, 1992). However, lowering the pH of overlay medium to ~3.0 prevented growth of aSRB isolates almost completely. This is not altogether surprising as the pH of the sediment core, other than

in the first 5 cm or so, was substantially greater than 3.0 (see Table 3.1). As in previous experiments, overlay medium was found to be more efficient than non-overlay medium, but growth still occurred in the latter, indicating that many organisms in the AMD sediments (unlike the pure cultures of aSRB worked with for most of this project) are able to grow on the more simple solid medium.

In the investigation of bacteria in different sediment strata, greater numbers of CFUs were found in *Acidocella* WJB-3 medium with material from Zone 2 (the blackened stratum) than for Zone 1, although a greater diversity of colony types was observed in the latter. This demonstrated that aSRB exist in zones other than the high pH, sulphidic zones of subsurface sediments in AMD environments, and that they co-exist with a number of other organisms. Higher numbers, but fewer types of bacteria grew using this medium with material from Zone 3, suggesting that in deeper zones there are fewer acidophilic anaerobes, possibly as a result of lower availability of substrates. *Acidiphilium* SJH overlay medium (amended with either lactate or galactose) was the superior medium for isolating a diverse range of aSRB (Zone 2), which included an unidentified "sulphate reducer" (which formed black colonies) along with P1- and PFB-like isolates. This latter solid medium would be the most appropriate of those devised for isolating aSRB from mine water environments.

5: BENCH-SCALE SULPHIDOGENIC BIOFILM BIOREACTOR SYSTEMS USING ACIDOPHILIC SULPHATE REDUCING BACTERIA

5.1 Introduction

A number of industrial bioprocesses use SRB for treatment of wastewaters (section 1.5.2), including AMD, and the bioengineering aspects of the use and performance of nSRB in these systems are well understood. They have been shown to be an effective alternative for remediating such pollution, as demonstrated in bench-scale, pilot-plant and full-scale operations (e.g. Buisman *et al.*, 1996; Christensen *et al.*, 1996). SRB reactors can achieve very high sulphate conversion rates, in the order of 30 g sulphate per litre reactor volume per day (du Preez *et al.*, 1992; van Houten *et al.*, 1994). In these systems, synthesis gas (hydrogen, carbon monoxide and carbon dioxide) has been used, which is a relatively inexpensive source of an electron donor (hydrogen) and carbon source, as the gas is produced by various petrochemical processes. Such a system was used by van Houten and Lettinga (1995) for the treatment of AMD.

The potential for utilising acidophilic or acid-tolerant strains of SRB for the treatment of AMD has been recognised (Johnson, 1995b; Sen and Johnson, 1999), but as yet there has only been one study of their performance on a laboratory-scale (Kolmert and Johnson, 1999). In this, an average sulphate reduction rate of 0.25 – 0.30 g sulphate reduced L*day was achieved in fixed bed column bioreactors with immobilised bacteria. Biofilm reactors generally do not achieve the high sulphate conversion rates of some other reactor systems, e.g. UASB systems, but are more suitable in applications where a more robust system is required (Dvorak *et al.*, 1992; Hammack *et al.*, 1994a; Kolmert *et al.*, 1997; Kolmert, 1999). Evaluation of a novel bioreactor system at bench-scale level (typically 1 - 10 litre volumes) gives an indication as to whether a commercial-scale operation may be possible. Subsequent scale-up involves larger bioreactors; pilot plant reactors are usually 300-3000 litres

in size, while full-scale bioreactors are usually 10,000 – 500,000 litres (Brock, 1997). Scale-up is a complex procedure because microbial populations may behave very differently in large-scale bioreactors compared to bench-scale laboratory systems, as mixing, fluid dynamics and gas transfer can vary considerably with changes in the surface-to-volume ratio of the equipment. The process configuration is also based upon the performance of the process on a smaller scale.

In order to assess the potential of using aSRB in bioremediation systems, batch culture experiments were carried out in bioreactors using two mixed aSRB cultures. The objectives were: (i) to determine rates of sulphate reduction at different fixed pH, (ii) to find an optimum operating pH for the aSRB cultures, and (iii) to determine which culture would be most suitable for continuous mode experiments. Acid neutralisation potential was also investigated, i.e. the amount of acid required to control pH increase at different (controlled) pH. The effect of temperature and different growth medium on sulphate reduction rate was also measured in one of the batch systems. In addition, the cultures were monitored for changes in microbial populations at different operating pH values, to give an indication as to which organisms were the most acid-tolerant in the mixed cultures.

In subsequent experiments, a bioreactor was set up for continuous culture using varying flow rates and controlled (fixed) pH. Sulphide production was determined by trapping hydrogen sulphide gas sparged from the system. Substrate utilisation and production of by-products, (i.e. whether complete or incomplete oxidation was taking place) was also assessed.

5.2 Batch Culture Experiments

5.2.1 Methodology

5.2.1.1 Bioreactor experiments - batch

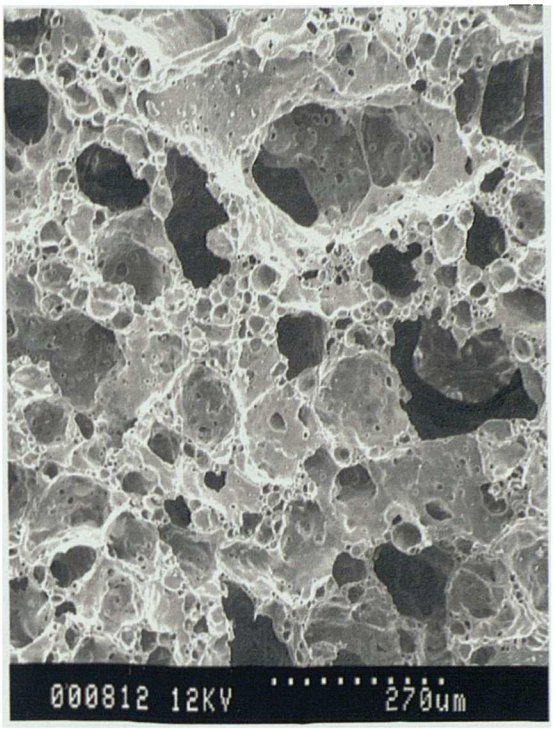
5.2.1.1.1 Bioreactor set-up

Mixed cultures of aSRB obtained from Parys Mountain and Montserrat samples were grown in batch cultures of ~ 500 ml total volume containing ~ 300 ml Poraver beads (2-4 mm diameter; Figures 5.1(a) and (b)). These were used as inocula for 2L bench-scale bioreactors. A schematic of the bioreactor set-up is shown in Figure 5.2. The LH series 502 system was used for the Montserrat culture (section 2.1.1.2) and the Electrolab system was used for the Parys culture (section 2.1.1.2; Figure 5.3). The bioreactor vessel was autoclaved with input tubing connectors, air filters (Whatman, 0.2 μm) and ORP and pH electrodes (section 2.2.1) before assembly. Acid and alkali (0.1 M HCl and NaOH or KOH) were heat-sterilised and the reservoirs were connected to the bioreactor vessel via silicone tubing and a pump on the control box. Mixing was achieved by an overhead stirrer agitating at approximately 40 rpm.

5.2.1.1.2 Culture media

In both the starter cultures and the bioreactors, medium contained basal salts solution (section 2.1.1.1) which was amended with trace elements (1 ml L^{-1}), yeast extract (0.002%), ethanol (10 mM), and $FeSO_4$ (10 mM). The latter was replaced in some cases with 8 mM $ZnSO_4$/1 mM $FeSO_4$/1 mM K_2SO_4 or 1 mM $FeSO_4$/9 mM K_2SO_4, the total added amounting to 10 mM sulphate added in each case.

(a)



(b)

Figure 5.1 Scanning electron micrographs (SEMs) of Poraver beads used for colonising aSRB in bioreactor systems (Kolmert, 1999). Scales are shown in µm. **(a)** A Poraver bead before colonisation; figure shows the porosity of the solid matrix. **(b)** The surface of a Poraver bead colonised with aSRB.

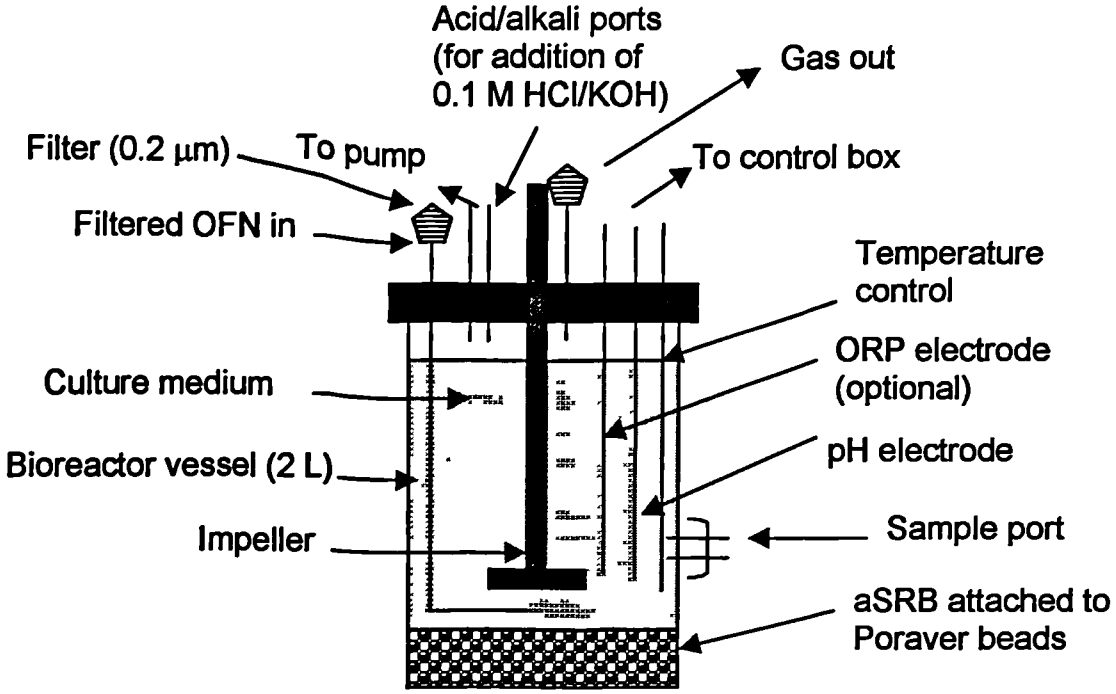


Figure 5.2 *Bioreactor set-up for batch culture experiments (not to scale)*

5.2.1.1.3 Experimental procedure

Initial experiments were carried out with a system inoculated with a mixed culture originating from a sample from Parys Mountain. The temperature was set at 30°C, the pH control was set at 4.0, and ethanol added through a septum in the top plate of the bioreactor. The culture was left for a week, and was then drained to just above the level of the beads, and fresh medium , sparged with OFN, and ethanol was added. A sample was taken immediately from the sample port into a sterilised universal (30 ml) bottle. This was assigned as Time (Day) 0 for a series of experimental 'runs', in which sulphate removal, acid (H^+) consumption and other parameters were measured, over a period of 1-2 weeks with samples taken each day. Each run was started after draining the bioreactor to just above the level of the beads, refilling the bioreactor, adjusting pH, gassing with OFN and addition of ethanol. The working volume, including the porous glass beads, was 1.8 L, equivalent to 1.56 L liquid volume (as the beads were

calculated to be approximately 88% pore space; Appendix 5.1). Replicate runs were carried out at pH 2.5, 3.0, 3.5, 4.0, 4.5 and 5.5 with both reactor systems. During the first run, the bioreactor cultures were not sparged with OFN, but in subsequent experiments, cultures were sparged with OFN twice daily for 5-10 minutes to remove H_2S. Other modifications included varying temperature and ferrous iron concentration, and addition of zinc (as sulphate) to remove hydrogen sulphide produced by the cultures as insoluble zinc sulphide.

5.2.1.1.4 Analytical Techniques

Samples were filtered through cellulose nitrate membranes, and sulphate, sulphide, ferrous iron, soluble zinc, and protein were analysed using the methods described in section 2.2. Bacteria were enumerated using a Thoma chamber (section 2.3.2), subdividing those appearing as characteristic *Desulfosporosinus*-like from others observed.

5.2.1.1.5 Monitoring Bacteria in Batch Culture

Bacteria present in the Montserrat mixed culture were enumerated using solid medium, in runs carried out at pH 3.0, 3.5 and 4.0. Samples taken at the beginning and end of an experimental "run" were diluted, plated and incubated (in replicate) as described in section 2.1. Counts of different organisms (M1, MCP and MR; sections 6.3 - 6.5) were based on variations in colony and cell morphologies.

5.2.1.2 Bioreactor experiments – continuous cultures

5.2.1.2.1 Bioreactor set-up

Figure 5.3a shows a schematic of the bioreactor set-up for the continuous culture experiments, and Figure 5.3b shows the actual bioreactor (Electrolab P350; section 2.1.1.2). The bioreactor vessel was autoclaved

as described in section 5.1.1.1 and inoculated with approximately 300 ml of Poraver beads (2-4 mm) which had been colonised with a mixed culture originating from Parys Mountain. It was then topped up with acid-washed, autoclaved Poraver beads to a level of approximately 1.2 L of the vessel, allowing the impeller to stir just above the level of the beads, and giving a working liquid volume of 1.65 L. The bioreactor was filled to a level of 1.8 L with medium, and was connected to a reservoir containing medium as well as a waste tank via a common multiple speed peristaltic pump. The waste tank (a 2L glass bottle) was set on ice during experimental runs to retard the growth of bacteria that had drained out. A gas vent was connected via a filter to a series of two collection bottles filled with 500 ml of 10 mM copper acetate) to trap hydrogen sulphide gas as copper sulphide.

5.2.1.2.2 Culture media

The standard medium in these experiments consisted of basal salts (1X); glycerol (5 mM); yeast extract (0.002%); glycerol (5 mM); potassium sulphate (9 mM); ferrous sulphate (1 mM), and trace elements (1 ml/L) (adjusted to pH 3.0 with sulphuric acid (25%)), and was autoclaved with all components other than the ferrous sulphate and glycerol, which were added after cooling. In experiments using acetic acid (added as sodium acetate) in place of glycerol as electron donor, the substrate was also added from a sterile stock solution following autoclaving, to a final concentration of 5 mM.

5.2.1.2.3 Experimental procedure

A series of experimental runs using medium supplemented with glycerol was set up to run at different fixed pH (2.5, 3.0, 3.5, 4.0) and different flow (dilution) rates (25 ml/h, 50 ml/h, 100 ml/h), at 30°C. As time did not allow for every combination of these parameters, all three flow rates were only tested at pH 4.0., and subsequent experiments were set at 25 ml/h.

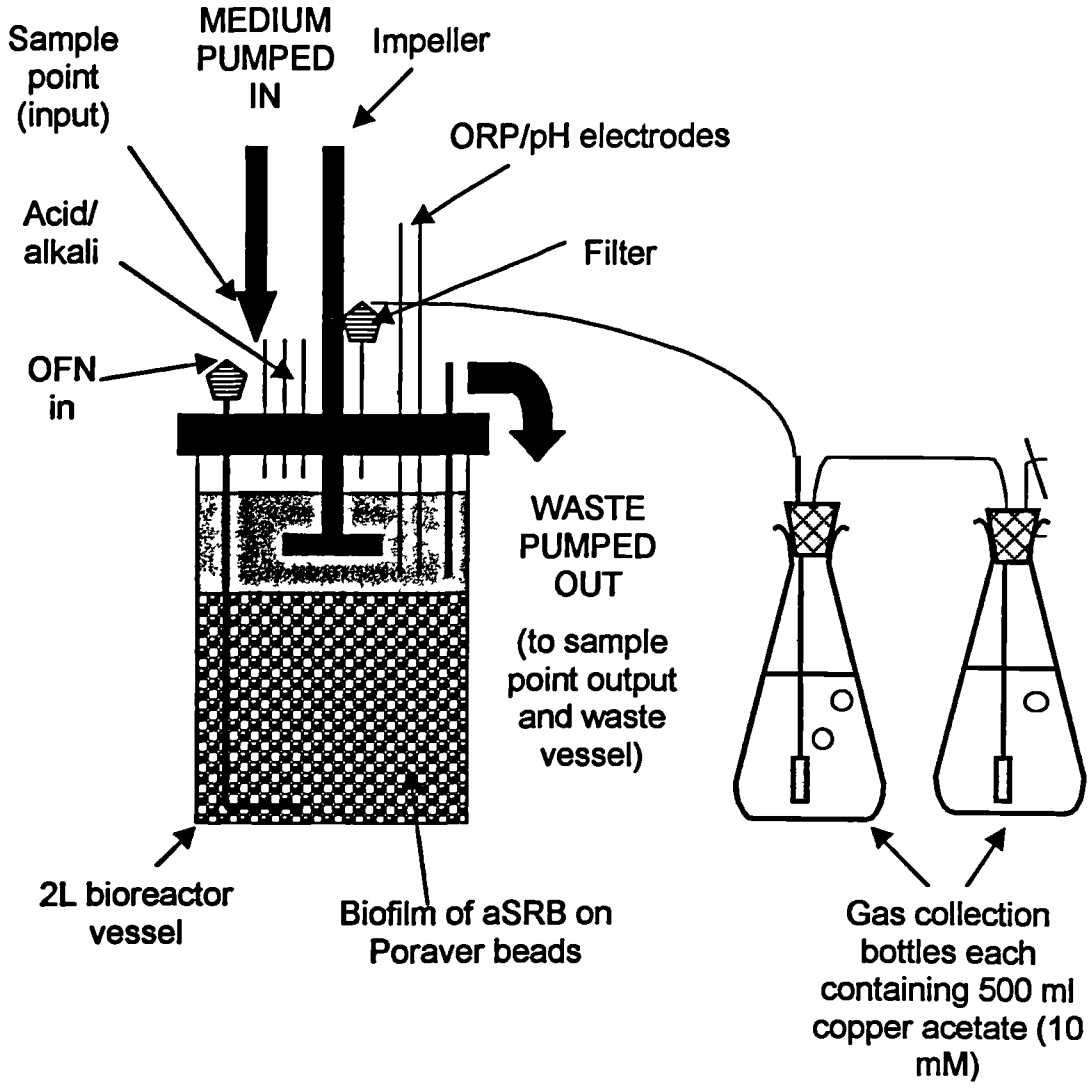


Figure 5.3a Bioreactor system set-up for continuous mode experiments.

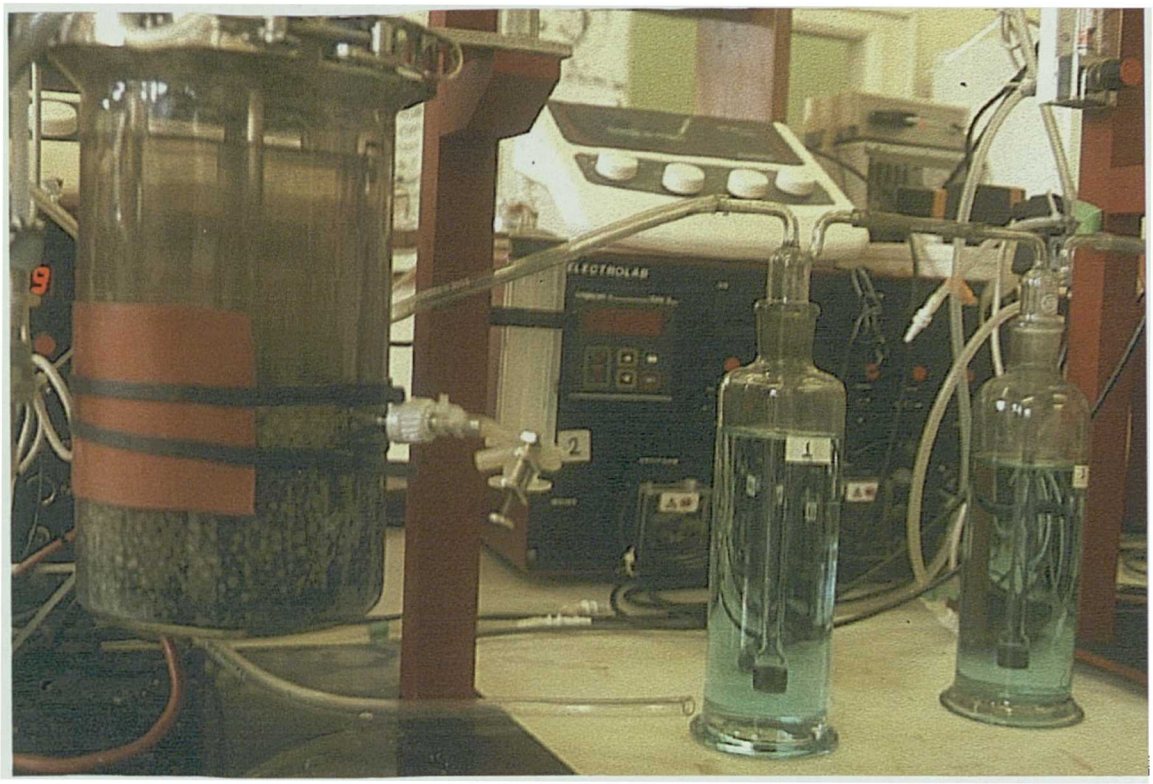


Figure 5.3 (b) Bioreactor system for continuous culture experiments with aSRB. The 2 L bioreactor vessel (foreground, left) was inoculated with a mixed culture, using Poraver glass beads for attachment of cells. The apparatus was connected to medium (input) and waste (output) vessels (not shown) as well as to two collection bottles containing a copper acetate solution (right).

Acetate-supplemented medium was also pumped into the bioreactor, to investigate whether the system could operate using the substrate. Experiments were carried out at either pH 4.0 (at flow rates 25 ml/h and 50 ml/h) or 4.8 (at 30°C and 22.5°C). For all experiments, data was collected at the beginning and end of each "day" (approximately 7 hours), and in most cases each set of operating conditions (i.e. different pH, flow rates, and temperature) were kept unchanged over a period of 7-10 days. Samples were taken from the inflow (medium) and the culture in the middle of each day, filtered, and analysed for sulphate, glycerol and acetate (acetic acid; the latter was determined only in the culture sample). Throughout each day, the bioreactor was sparged with OFN for 5 minutes at hourly intervals. The copper acetate solution in both collection bottles was sampled at the beginning (before the first sparge with OFN) and end of each day, and soluble copper measured using AAS (section 2.2.10). These data were used to determine sulphide generated by the system. At the end of each day, a sample was taken from the culture and the outflow liquor. Culture samples were filtered and analysed for sulphate, glycerol and acetate, and waste samples filtered and analysed for sulphate, sulphide, and pH. pH control was effected with 0.1 M HCl, connected to the bioreactor via pumps from the control unit. The volume of acid used for pH control during each day was noted and used to calculate H^+ consumed. When pH was adjusted at the start of a new experiment, the bioreactor was left to run until one reactor volume had been replaced at the new pH before beginning the sampling.

5.2.1.2.4 Analytical techniques

The analytical techniques used were as described in section 5.2.1.1.4. In addition, measurements were made of culture ATP levels (section 2.9).

5.2.1.2.5 Calculations

Sulphate reduction rates (SRR) were calculated in mmoles or μmoles SO_4^{2-} reduced L^{-1} h^{-1}. Sulphate reduction activity (SRA) was calculated using ATP as a measure of (active) biomass, in μmoles SO_4^{2-} reduced L^{-1} h^{-1} * μmole ATP. Hydraulic residence time (HRT), i.e. the mean amount of time that the culture was retained in the bioreactor, was calculated by dividing the working volume by the flow rate of the medium pumped in. (See Appendix 5.1 for calculation of estimated volume of the Poraver beads). The percentage of incomplete oxidation of glycerol was taken as the stoichiometric amount of acetate produced as a percentage of the amount of glycerol utilised by the culture. "Theoretical" sulphate reduced was calculated as the stoichiometric amount of sulphate reduced, in mM, from the incomplete oxidation of the utilised glycerol, added to the theoretical amount of sulphate reduced if the remaining glycerol was completely oxidised to CO_2.

5.2.1.2.6 Monitoring of Organisms in Continuous Cultures

Bacteria were enumerated using plate counts following serial dilution (in basal salts of the same pH of the experimental run) of bioreactor samples. Overlay plates (section 2.1.1.4 (a)) were spread with 100 μl of each dilution in an anaerobic chamber (section 2.1.2) and the plates incubated in anaerobic jars (Oxoid Anaero*Gen*™) at 30°C. For the samples taken from the acetate-fed runs, both SJH and WJB-3 overlay plates (section 2.1.1.4.(b)) were used for enumeration.

5.3 Results

5.3.1 Bioreactor experiments – batch cultures

5.3.1.1 Parys culture

Figures 5.4(a) and (b) shows results of analyses for a batch experimental run (i.e. a number of consecutive days of data) with the Parys culture at pH 4.0 over 22 days, and Table 5.1 (a) summarises data from all experiments with the culture (graphs of other experimental runs can be seen in Appendix 5.2). Sulphate reduction, measured by removal of sulphate, occurred in all experiments, but was noticeably less pronounced at pH 2.5, and was also lower at pH 4.5 than at either lower or higher pH (with the exception of pH 2.5). Ferrous iron concentrations fluctuated during all experiments but remained high (indicating that ferrous iron remained in solution rather than being precipitated as ferrous sulphide) except at pH 5.5, where the concentration fell to almost zero. In the first run at pH 3.5, where the culture was not gassed with OFN, ethanol concentrations fluctuated during the run but did not appear to decrease overall. In the next run, also at pH 3.5, where the culture was gassed with OFN, concentrations decreased by about 2 mM. At pH 4.0, concentrations decreased by about 3 mM (Figure 5.4(a)). Dissolved sulphide concentrations were only measured in initial experiments, where concentrations were found to be low (around 1 mM); in later experiments where gassing took place, dissolved concentrations were found to be even lower, i.e. approaching zero. Following this, analyses for dissolved sulphide were not performed in experiments with either batch culture, as concentrations of sulphide remaining in solution in samples were too low to be detected using the method available.

Acid 'consumption' occurred during all runs; the largest addition of acid required to maintain pH during a run (24.4 mmoles of H^+) was recorded at pH 3.5, and the least (0.3 mmoles) at pH 5.5).

Using pellets obtained from 1 ml of sample (i.e. planktonic bacteria) protein concentrations were found to fluctuate considerably from day to day during initial runs (see Figure 5.4 (b)), and no apparent trends or obvious differences were observed between runs. Cell counts gave equally ambiguous data, with numbers of all cells decreasing considerably during the first few days of the first run following system set-up, and then remaining relatively constant during all experiments. In the majority of samples, less "SRB" (*Desulfosporosinus*-like) cells were counted than other types, and in many counts, only a few distinct "SRB" cells were seen throughout the whole Thoma grid, making estimations of cell numbers using this method inaccurate.

Sulphate reduction rates in the Parys culture were lowest in the run at pH 2.5 and highest at pH 5.5, although Figure 5.6 shows that sulphate reduction in the Parys culture was more consistent over the pH range 2.5 – 5.5 than the Montserrat culture (section 5.3.1.2), with higher sulphate reduction rates demonstrated at pH 2.5, 3.0 and 5.5. The highest sulphate reduction rate of all the runs was observed in the final experiment at pH 5.5, in which medium containing 10 mM of ferrous sulphate was substituted for one containing 1 mM ferrous sulphate and 9 mM potassium sulphate.

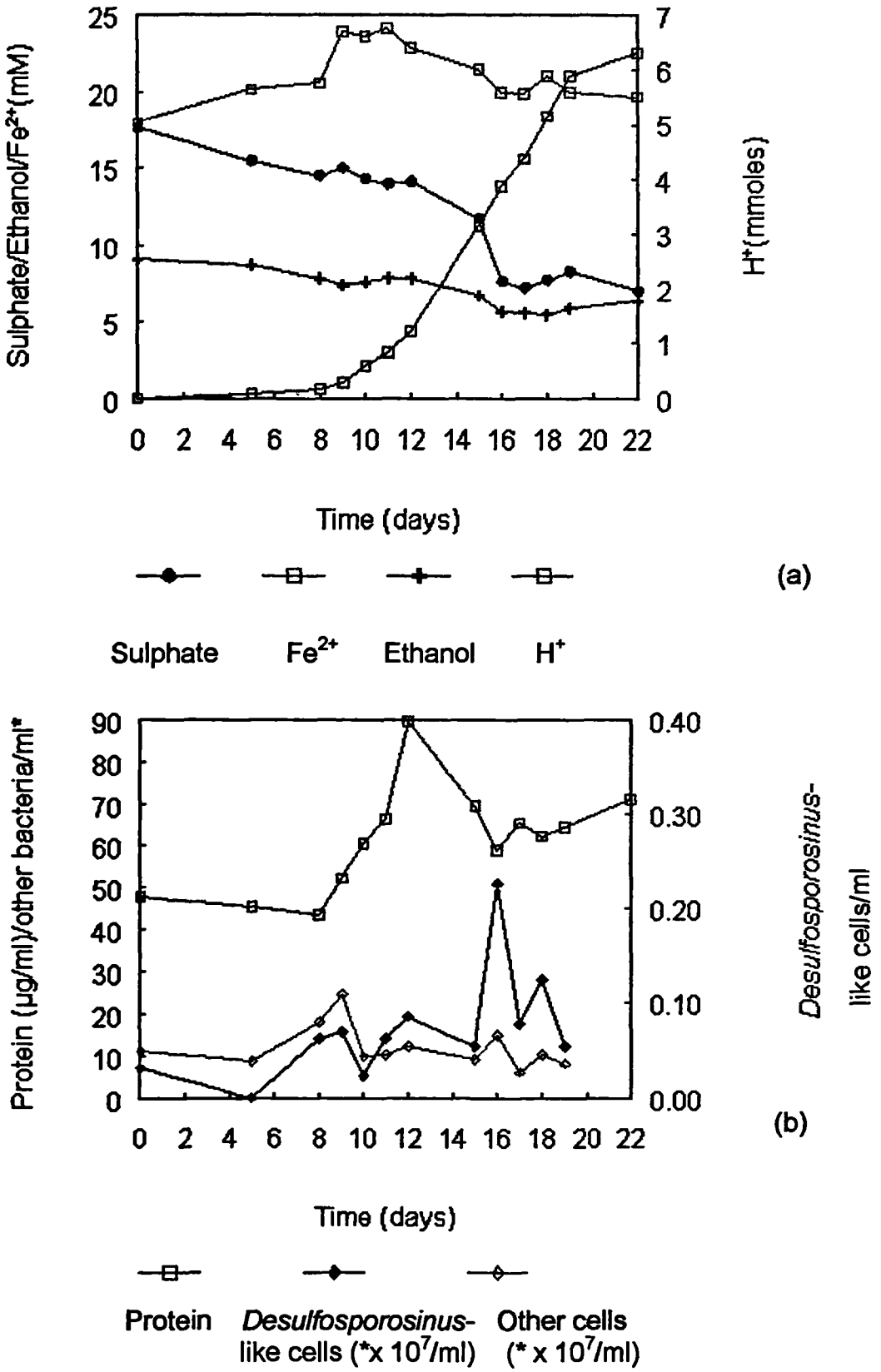


Figure 5.4 (a) (top) and **(b)** (bottom). Chemical and microbiological data for the mixed culture (Parys) system run in batch mode, pH 4.0. H^+ is expressed in mmoles required for pH maintenance (cumulative).

pH (units)	Duration of run (days)	Run modification (e.g. different medium)	Sulphate reduced (mmoles)	SRR* (μmoles $L^{-1} h^{-1}$)	H^+ input (mmoles)	Ethanol consumed† (mmoles)
2.5	9	-	4.6	21.1	10.3	nd
3.0	17	-	14,4	35.2	13.94	nd
3.5	10	No OFN sparging	8.3	34.5	9.5	nd
3.5	14	-	13.7	40.7	24.4	3.34
3.5	13	-	9.4	30.0	6.7	nd
4.0	22	-	18.0	34.1	6.3	4.81
4.5	11	-	7.3	27.8	4.21	nd
5.5	8	-	10.4	54.0	0.33	nd
5.5	5	1mM $FeSO_4$/ 9 mM K_2SO_4	7.0	58.6	1.52	nd

Table 5.1(a) Summary of data obtained from batch culture experiments with the Parys culture. * SRR= sulphate reduction rate; † nd = no data available.

5.3.1.2 Montserrat culture

Figure 5.5 shows data from the Montserrat culture run in batch mode at pH 4.0 over 15 days, and Table 5.1 (b) summarises data from all experiments with the culture (graphs of other experimental runs can be seen in Appendix 5.2). Sulphate reduction, measured by removal of sulphate, occurred in all runs, with the lowest rate at pH 2.5. Ferrous iron concentrations showed similar trends to those observed with the Parys culture. In the run with the zinc sulphate-containing medium, concentrations of zinc fell from around 9 mM to undetectable levels within six days, whereas ferrous iron remained at about 2mM throughout. In the first experiment at pH 4.0, ethanol concentrations decreased over the course of the experiment, indicating a consumption of around 8.5 mmoles (Figure 5.5; Table 5.1(b)). In the first experiment at pH 3.5, ethanol concentrations fell by just over 2 mM, i.e. around 3.1 mmoles were consumed by the system during the run (Table 5.1(b)). Acid 'consumption' occurred during all experimental runs; most acid (23.4 mmoles) was consumed at pH 3.5, and least (0.8 mmoles) at pH 4.0 and 20°C. In the Parys culture, there was a general pattern with acid consumption in that the higher the pH of the experiment, the lower the amount of acid consumed to control pH (section 5.3.1.1). No such general trend was observed with the Montserrat culture. Due to the problems encountered with analysis of sulphide, protein and cell counts in the initial experiments with the Parys culture, these parameters were not measured routinely in subsequent experiments with the Montserrat culture. Sulphate reduction rates in the Montserrat culture were lowest at pH 2.5 (7.27 µmoles L^{-1} h^{-1}). The highest rate was observed at pH 4.0, which appeared to be the "pH optimum" for this culture (Figure 5.6). However, Table 5.1(b) shows that, even at pH 4, a wide range of sulphate reduction rates was obtained in the batch culture experiments. In two experiments conducted at pH 4 with a time interval of 12 months, the sulphate reduction rate was 82.5 µmoles

L^{-1} h^{-1} in the first and 58.5 µmoles L^{-1} h^{-1} in the later run. Figure 5.6 shows a comparison of sulphate reduction rates in the Montserrat and Parys cultures. Whilst a higher rate was observed for the Montserrat culture at pH 4.0 and 4.5, rates were similar at pH 3.5, and, in all other cases, lower sulphate reduction rates were measured for the Montserrat culture.

5.3.1.2.1 Microbial populations in batch cultures

Table 5.2 shows bacterial counts for the Montserrat culture. In each case, there was an increase in numbers of all isolates (M1, MCP and MR; section 6.3) between the start and end of the run, the only exception being a decrease in numbers of the *Desulfosporosinus*-like M1 from 13 to 0 from the beginning to the end of the pH 4.0 run. The increase in total cell count was particularly evident in the longest run (22 days), at pH 3.0, where numbers increased approximately 5 times, from 1.1×10^5 to 6.4×10^5.

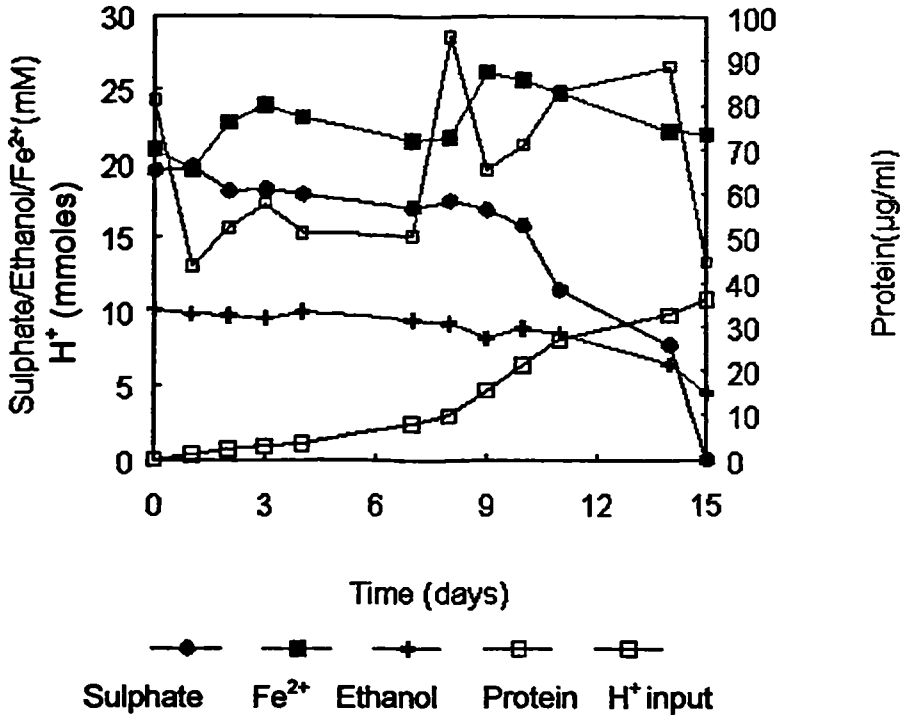


Figure 5.5 Chemical data for the mixed culture (Montserrat) system run in batch mode, pH 4.0. H^+ is expressed in mmoles required for pH maintenance (cumulative)

pH (units)	Duration of run (days)	Run modification (e.g. different medium)	SO_4^{2-} reduced (mmoles)	SRR* (μmoles $L^{-1} h^{-1}$)	Total H^+ input (mmoles)	Ethanol consumed (mmoles)
2.5	9	-	1.6	7.3	1.0	nd
3.0	22	-	8.6	16.3	11.9	nd
3.5	15	-	16.0	41.1	23.4	3.1
3.5	13	-	10.6	33.9	4.1	nd
4.0	15	-	29.6	82.2	15.2	7.5
4.0	7	-	9.8	58.5	10.1	nd
4.0	6	8mM $ZnSO_4$/1mM $FeSO_4$/1mM K_2SO_4	7.0	48.6	7.3	nd
4.0	10	1mM $FeSO_4$/9mM K_2SO_4	7.8	32.4	9.3	nd
4.0	10	1mM $FeSO_4$/9mM K_2SO_4; 20°C	4.1	17.0	0.8	nd
4.0	7	1mM $FeSO_4$/9mM K_2SO_4; 25°C	2.0	12.1	1.5	nd
4.5	10	-	10.1	42.0	6.8	nd
5.5	8	-	8.1	22.5	3.7	nd

Table 5.1 (b) Summary of data from batch culture experiments (Montserrat culture). See Table 5.1(a) for legend.

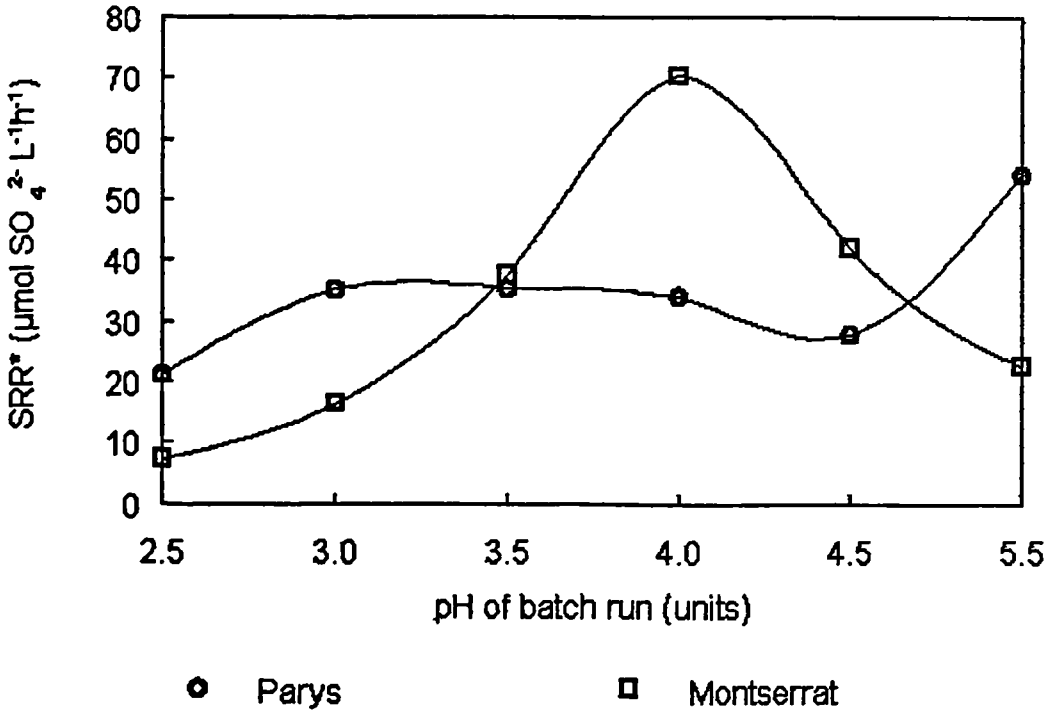


Figure 5.6 *Sulphate reduction rates (SRR) for batch experimental runs using mixed aSRB cultures from Parys and Montserrat. Results shown are calculated means using data from batch cultures operated with 'standard' medium and temperature.

Dur-ation of run (days)	pH	Start/ Finish	Nos. of isolate M1/ml	Nos. of isolate MCP/ml	Nos. of isolate MR/ml	CFUs/ml
22	3.0	Start	1.02×10^5	5.07×10^3	5.07×10^3	**1.1×10^5**
		Finish	5.31×10^5	8.2×10^4	2.6×10^4	**6.4×10^5**
7	3.5	Start	216	6.4×10^3	933	**7.6×10^3**
		Finish	2.1×10^3	1×10^4	1.5×10^3	**1.4×10^4**
7	4.0	Start	13	8.9×10^3	0	**8.9×10^3**
		Finish	0	1.04×10^4	2×10^3	**1.2×10^4**

Table 5.2 Cell counts calculated from plate counts of liquid samples taken from the Montserrat culture operated in batch mode. CFUs = colony forming units.

5.3.2 Bioreactor experiments – continuous cultures

Table 5.3 summarises the key data from results of experiments using the Parys culture run in continuous mode, and Figure 5.7 shows chemical and microbiological data from the run at pH 4.0 with the shortest HRT of 16.5 h (graphs for all other runs are in Appendix 5.3). In each case, sulphate concentrations in inflow medium were higher than in the bioreactor vessel and outflow liquor, indicating that sulphate reduction occurred during each experiment. In general, sulphate concentrations in the vessel and outflow liquor were similar, except for the pH 2.5 experiment where sulphate concentrations in the outflow were considerably smaller than in the bioreactor vessel.

Glycerol was utilised and acetate produced in all runs in which glycerol was supplied as substrate. In runs at pH 3.0 and pH 2.5, however, the amount of glycerol utilised gradually decreased over the period of the run, as indicated by its continuous accumulation in the bioreactor vessel. In the pH 3.0 run, there was a corresponding decrease in acetate concentrations, and in the pH 2.5 run the concentration of acetate remained considerably lower than in experiments carried out at higher pH.

Sulphide production and acid 'consumption' was evident in all runs; however, these were noticeably smaller (approaching zero) in experiments at pH 3.0, 2.5 (with glycerol as electron donor) and when acetate was supplied as potential electron donor.

There was an overall increase in total cell counts throughout the course of the glycerol-fed run at pH 4 (flow rate 50 ml/h), though numbers fluctuated considerably during the experiment. When the flow rate was increased to 100 ml/h, there was an initial rapid decrease in cell numbers (indicating washout) followed by a gradual increase to the end of the experiment (Figure 5.7). At pH 3.5, there was an overall decrease in cell numbers,

with considerable fluctuations during the run. Similar fluctuations, but an overall increase in numbers, was also found in the pH 3.0 run. At pH 2.5, there was a clear, gradual decrease in cell numbers during the course of the experiment. When acetate was used as sole carbon/energy source in the feed liquor, an initial (Days 1-3) increase in cell numbers was observed at a flow rate of 50 ml/h, followed by a marked decline and a slight recovery at the end of the run by which time the flow rate was lowered to 25 ml/h. In the acetate-fed run at pH 4.8, no apparent overall change in cell numbers was found at 30°C, and no data were available for the run at 22.5°C due to equipment failure.

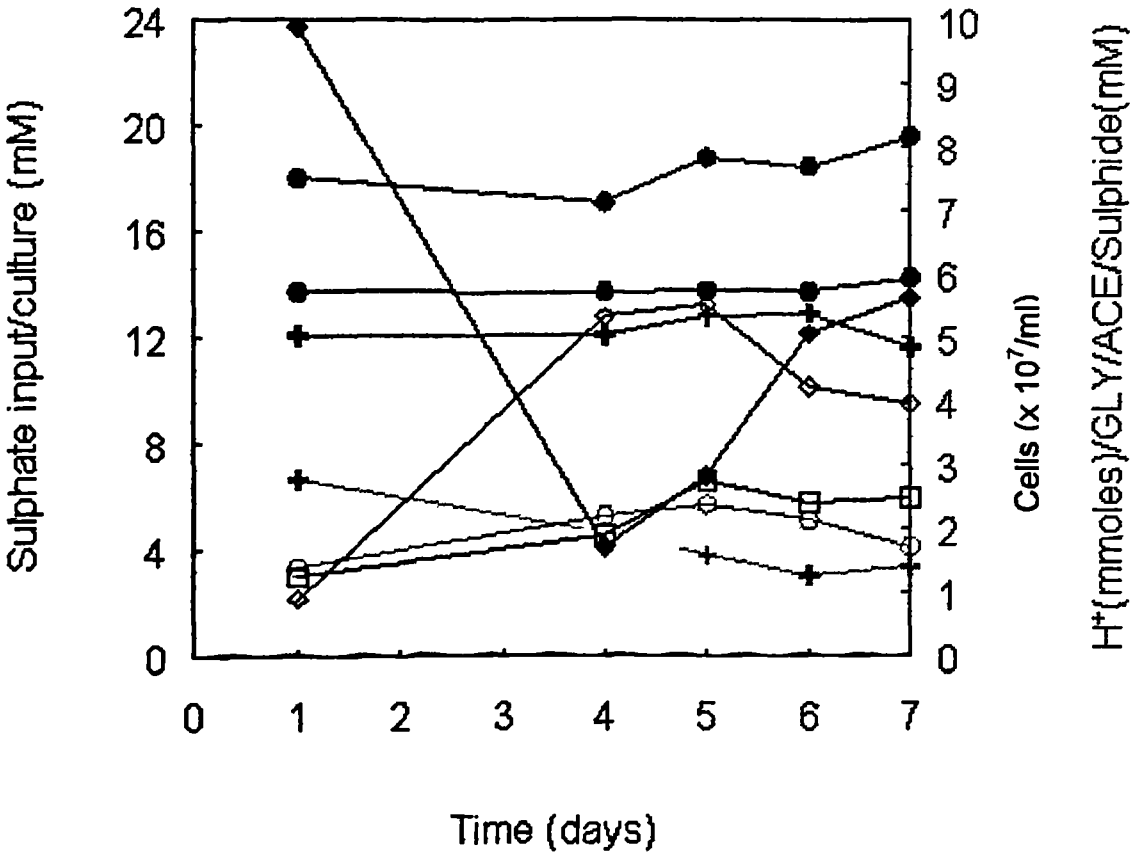
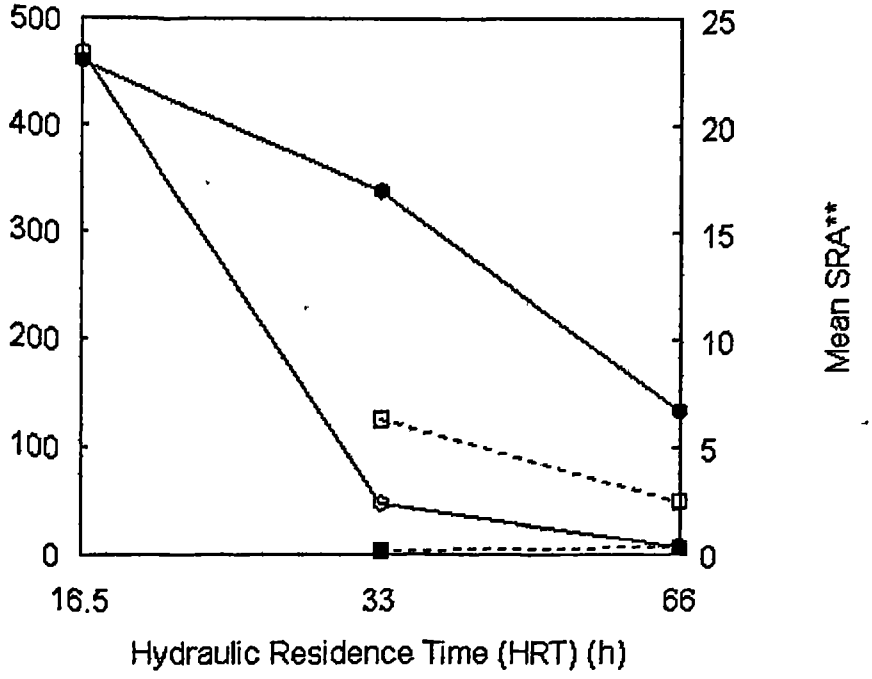


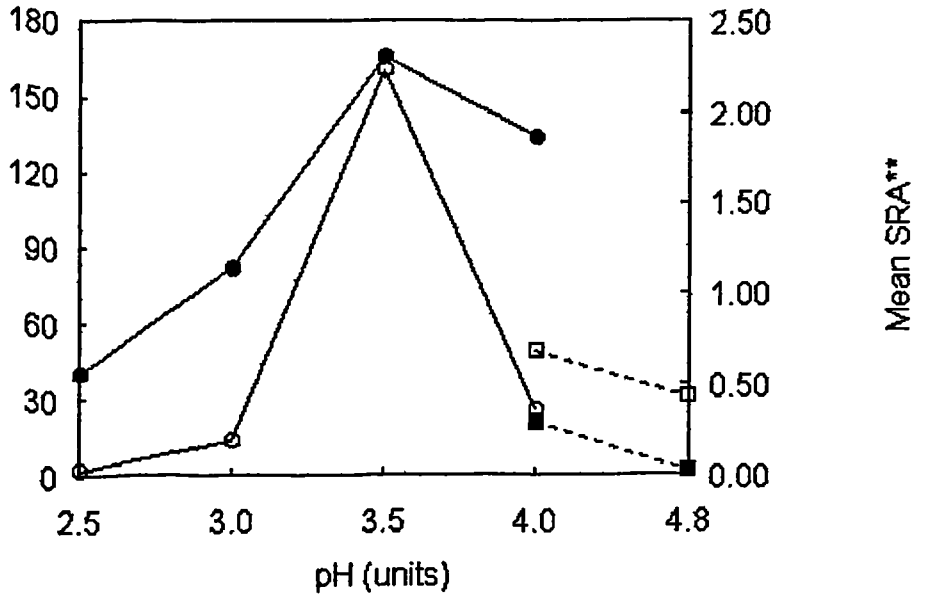
Figure 5.7 Chemical and microbiological data for mixed culture (Parys) run (continuous mode); pH = 4.0, HRT= 16.5 h. Key: ● [sulphate] (inflow); ● = [sulphate] (culture vessel); + = [glycerol] (inflow); + = [glycerol] (culture vessel); O = [acetate] (culture vessel); ◆ = cell nos. (x 10^7/ml); ◇ = [dissolved sulphide]; □ = H^+ addition (total in mmoles). GLY= glycerol; ACE = acetate.

Table 5.3 summarises the key data from the continuous culture experiments, showing calculated sulphate reduction rates (SRR), sulphate reduction activity (SRA) and sulphate conversion rates. These data are also shown in Figures 5.8 and 5.9. Figure 5.8 compares sulphate reduction rates (SRR) and sulphate reduction activity (SRA) for runs at pH 4, at the different flow rates and hence hydraulic residence times (HRT, in hours). For the glycerol-fed experiments, the highest SRR and SRA were found at the highest flow rate (100 ml/h, equivalent to an HRT of 16.5 h). The SRA fell noticeably at a HRT of 33h , although the relative decrease in SRR was small. At a HRT of 66h, both the SRR and SRA fell to much lower levels than at the higher flow rates tested. The SRA for the runs with glycerol and acetate were approximately equal at HRT = 66h, and were very low, although the SRR for the glycerol-fed run was noticeably greater at this HRT. The SRA for the acetate-fed run at HRT = 33h was almost zero. Figure 5.9 shows the mean SRRs and SRA at the different pH-statted runs, with HRT= 66h. The highest SRR and SRA were recorded at pH 3.5; SRA was considerably higher at this pH than at all others tested. The SRRs with acetate at pH 4 and 4.8 were similar to that found at pH 2.5 in the glycerol-fed culture, and the SRAs with acetate-fed (at pH 4.8) and glycerol-fed (at pH 2.5) cultures were almost zero. However, sulphate reduction was clearly still occurring in these runs, as the SRRs were well above zero. The data in Table 5.3 shows that the rates of sulphate removal (reduction) doubled between runs at pH 2.5 and 3.0, when glycerol was provided as electron donor. The rate increased more than two-fold when the pH of the experiment was increased from 3.0 to 3.5. At pH 4, rates of sulphate removal varied inversely with HRT, being being approximately twice as great at 33 h than at 66 h, and over 3 times faster at HRT = 16.5 h than at 66 h. SRA increased 10-fold when the HRT was lowered from 33 h to 16.5 h.

Run sequence order	pH (units)	Temp (°C)	Substrate	HRT† (h)	SRR*	SRA**	SCR*** (g SO_4^{2-} reduced L^{-1} day^{-1})
10	2.5	30	Glycerol	66	40.0	0.02	0.092
9	3.0	30	Glycerol	66	82	0.19	0.188
8	3.5	30	Glycerol	66	166	2.23	0.391
2	4.0	30	Glycerol	66	134	0.36	0.308
1	4.0	30	Glycerol	33	340	2.34	0.781
3	4.0	30	Glycerol	16.5	460	23.4	1.059
5	4.0	30	Acetate	66	50	0.29	0.113
4	4.0	30	Acetate	33	126	0.13	0.289
6	4.8	30	Acetate	66	31	0.02	0.071
7	4.8	22.5	Acetate	66	33	0.03	0.080

Table 5.3 Summary of data from continuous culture experiments with the mixed aSRB culture from Parys. Run sequence order refers to the chronological order in which runs were carried out. *Sulphate reduction rates (SRR) were measured in µmoles sulphate reduced L^{-1} h^{-1}. **Sulphate reduction activity (SRA) was measured in µmoles sulphate reduced L^{-1} h^{-1} * µmole ATP. ***Sulphate conversion rate (SCR) was calculated. † HRT = Hydraulic Residence Time (h).





re 5.8 (top) and **5.9** (bottom). Figure 5.8 shows changes in mean ıate reduction rate (*SRR), in µmoles SO_4^{2-} reduced L^{-1} h^{-1}, with ng HRT in continuous mode runs at pH 4.0 and 30°C. ● = mean (glycerol-fed culture); O = mean SRA (glycerol); ■ = mean (acetate); and □ = mean SRA (acetate). Figure 5.9 shows ı SRR and **SRA, in µmoles SO_4^{2-} reduced L^{-1} h^{-1} * µmoles ATP, ɔntinuous mode runs at pH 2.5, 3.0, 3.5, and 4.0 (glycerol), and nd 4.8 (acetate) (all at HRT=66 h and 30°C). Legend as for 5.8.

The acetate-fed run at pH 4.0 and HRT = 33 h exhibited sulphate removal rates similar to that of the glycerol-fed run at the same pH, though larger (66 h) HRT. The acetate runs at both temperatures displayed similar rates of sulphate removal over the course of the experiments. Figures 5.10 and 5.11 show the amounts of sulphate removed and hydrogen sulphide formed in each run. It can be seen again that the greatest amount of sulphate was removed in the glycerol-fed run at pH 4.0 and HRT = 16.5 h. Figure 5.10 shows that the largest amount of hydrogen sulphide formed (and trapped as copper sulphide) was in the glycerol-fed run at pH 4.0 and HRT = 33 h, followed by pH 4.0 and HRT = 16.5 h, although considerable daily fluctuations were found. In the pH 3.5 run, smaller fluctuations in sulphide production occurred throughout the experiment. The runs at pH 2.5 and those with acetate produced much smaller masses of sulphide, approaching or equalling zero, despite the fact that sulphate reduction did occur, albeit at low rates. Figures 5.12 to 5.18 show graphs of actual (i.e. difference in sulphate concentrations between the inflow and culture vessel) and theoretical (calculated from the amounts of glycerol that were oxidised, assuming incomplete oxidation to acetic acid; section 5.4.2, equation 5.8) concentrations of sulphate removed/reduced in these experiments. In the pH 4.0/50ml h^{-1}/glycerol run (Figure 5.12), actual sulphate removal was higher than the theoretical sulphate removed. In general, the actual amount of sulphate removed increased towards the end of the run. However, the percentage of incomplete oxidation gradually decreased from over 60% to around 40% from start to finish of the experiment. At the same pH with glycerol at 25 ml/h (Figure 5.13), however, actual sulphate reduced was around 1 mM less than the theoretical amount throughout, and incomplete oxidation stayed at around 40%. In the following run at pH 4.0 at 100 ml/h (Figure 5.14), actual sulphate reduced was only noticeably different to the theoretical amount on Day 1. The percentage of incomplete oxidation was higher in general than at lower flow rates (or higher HRT), staying above 60% until Day 6 of

7, the highest being 72%. However, it decreased almost linearly between 4 and 7.

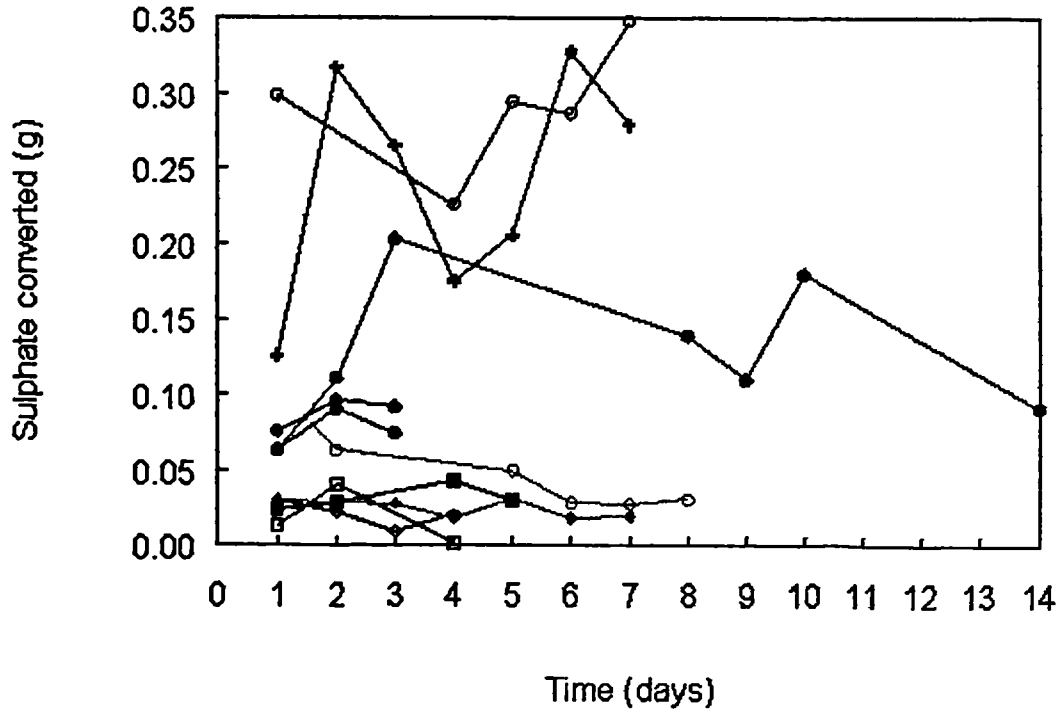


Figure 5.10 Mass of sulphate converted to hydrogen sulphide during each experimental run in continuous culture experiments. (+), (●), and (○) are for runs fed with 5 mM glycerol at pH 4.0 and 30°C, at HRT = 33, 66 and 16.5 h respectively; other glycerol-fed runs were (●) = pH 3.5, 30°C and HRT= 66 h; (○) = pH 3.0, 30°C and HRT= 66 h; (◆) = pH 2.5, 30°C and HRT= 66 h. (●), (■), (□) and (◇) are for runs fed with 5 mM acetic acid: (●) = pH 4.0, 30°C, and HRT = 33 h; (■) = pH 4.0, 30°C and HRT = 66 h; (□) = pH 4.0, 22.5°C and HRT= 66 h; and (◇) = pH 4.8, 30°C and HRT = 66 h.

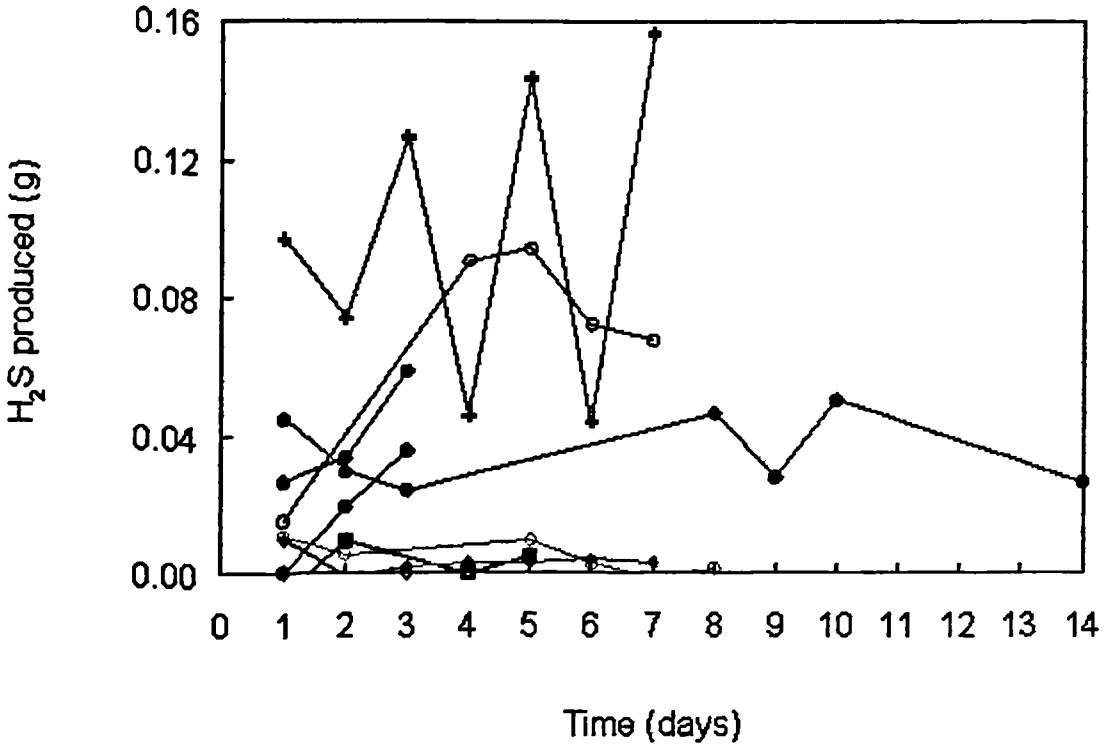


Figure 5.11 Mass of hydrogen sulphide precipitated as copper sulphide during each experimental run in continuous culture experiments. (+), (●), and (○) are for runs fed with 5 mM glycerol at pH 4.0 and 30°C, at HRT = 33, 66 and 16.5 h respectively; other glycerol-fed runs were (●) = pH 3.5, 30°C and HRT= 66 h; (○) = pH 3.0, 30°C and HRT= 66 h; (◆) = pH 2.5, 30°C and HRT= 66 h. (●), (■), (□) and (◇) are for runs fed with 5 mM acetic acid: (●) = pH 4.0, 30°C, and HRT = 33 h; (■) = pH 4.0, 30°C and HRT = 66 h; (□) = pH 4.0, 22.5°C and HRT= 66 h; and (◇) = pH 4.8, 30°C and HRT = 66 h.

Figure 5.15 shows the results for pH 3.5. On Day 2 there was a difference of just over 4 mM in actual and theoretical sulphate reduced. The percentage of incomplete oxidation did not fall below 50 or rise over 70%. At pH 3.0 (Figure 5.16), there was a difference of 3 – 4 mM between actual and theoretical sulphate reduced, and the actual amount of sulphate reduction stayed higher than the calculated amount until the last two days of the run. The actual sulphate reduced was only lower than the theoretical on Day 6. On Day 2, the percentage of incomplete oxidation was calculated as – 4.9, as the concentration of acetate in the culture was greater than the difference in sulphate concentration in the input and the culture. On Day 3, it rose to 46%, falling to 26% on Day 4, then rising to 34% and 54% on Days 7 and 8. At pH 2.5 (Figure 5.17), the actual change in sulphate concentration was lower than the theoretical amount on Days 1 – 4, becoming about 1 mM higher on Days 5 and 6, and was almost equal on Day 7 (around 1.5 mM). The percentage of incomplete oxidation was, in general, lower than in any of the other experiments with glycerol, ranging between just above zero and approximately 20 for all days other than Day 6, where it was 36. Figure 5.18 shows the actual and calculated data for the experiments with acetate. In all runs, with the exception of the 22.5°C experiment, the actual change in sulphate concentrations was higher than the theoretical. At pH 4.0, 30°C and 50 ml/h, the highest actual and theoretical changes were observed, with the two values being closest on Day 1 (2.07 and 1.85 mM respectively). With the same conditions but at 25 ml/h, the largest difference was on on Day 4 (2.41 and 0.42 respectively). At pH 4.8 and 25 ml/h at 30°C, the greatest difference in actual and theoretical changes in sulphate concentrations was most marked on Day 2; values were much closer on Days 1 and 3. At pH 4.8 and 22.5°C, the calculated amount of the change in sulphate concentrations was higher on all days other than Day 1: on Days 2 and 3 there were no changes in concentration between the input and the culture, suggesting that no sulphate reduction occurred.

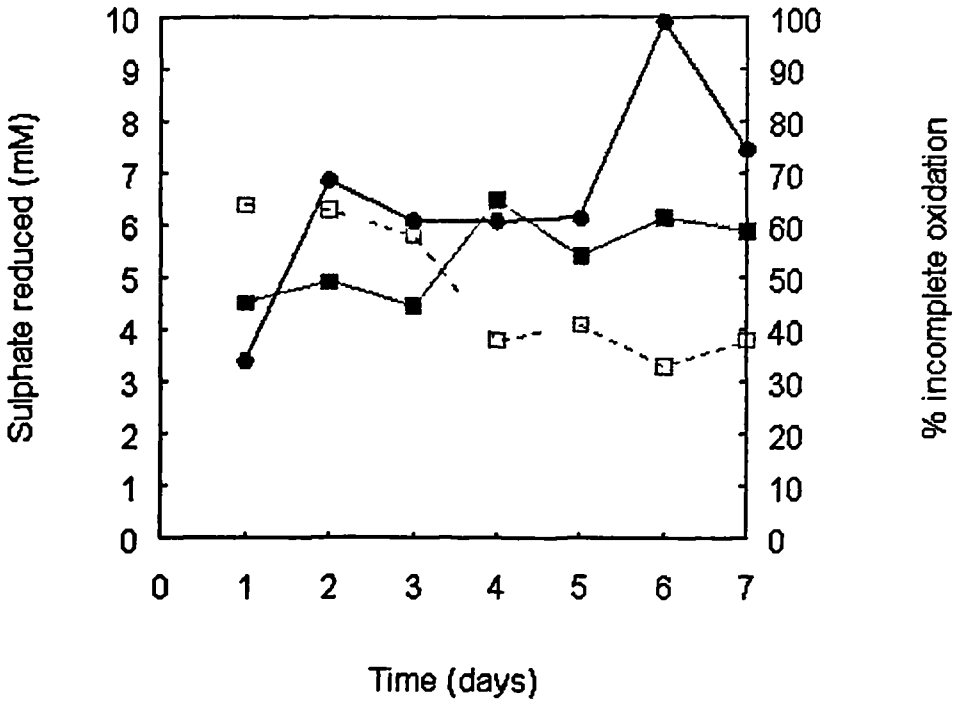


Figure 5.12 Actual (●) and theoretical (■, unbroken line) sulphate reduced, and % incomplete oxidation of glycerol (to acetate) (□, broken line) in continuous culture run at pH 4, 30°C and HRT = 33 h.

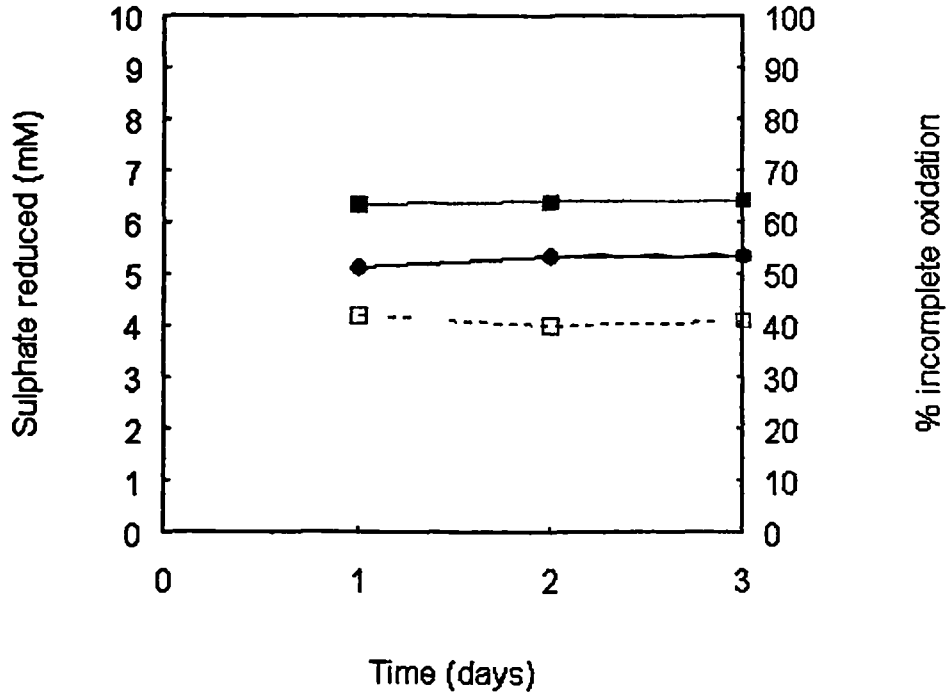


Figure 5.13 Actual (●) and theoretical (■, unbroken line) sulphate reduced, and % incomplete oxidation of glycerol (to acetate) (□, broken line) in continuous culture run at pH 4, 30°C and HRT = 66 h.

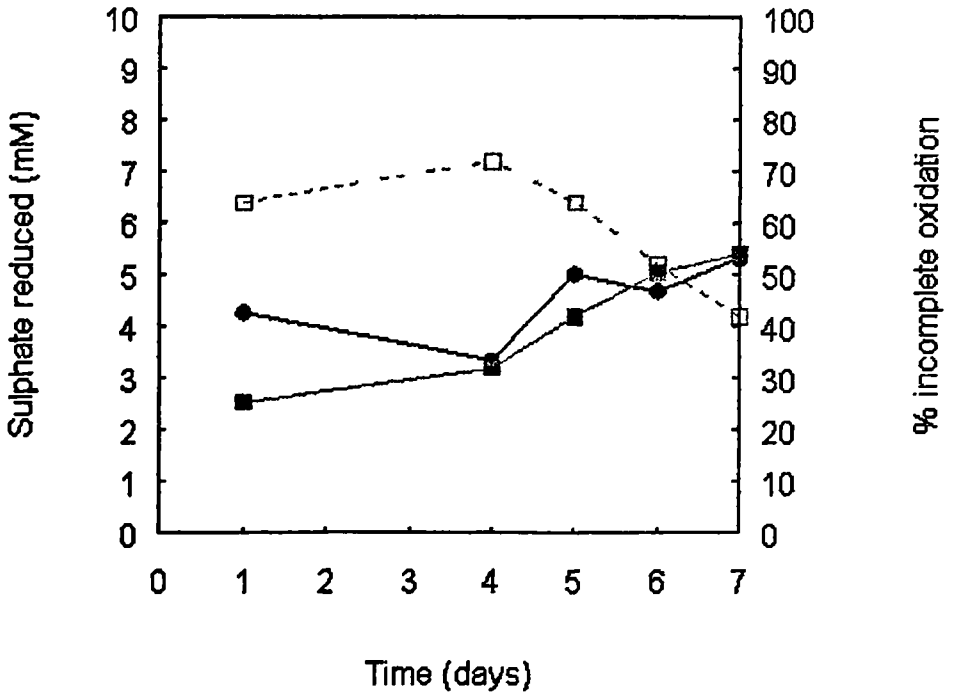


Figure 5.14 Actual (●) and theoretical (■, unbroken line) sulphate reduced, and % incomplete oxidation of glycerol (to acetate) (□, broken line) in continuous culture run at pH 4, 30°C and HRT = 16.5 h.

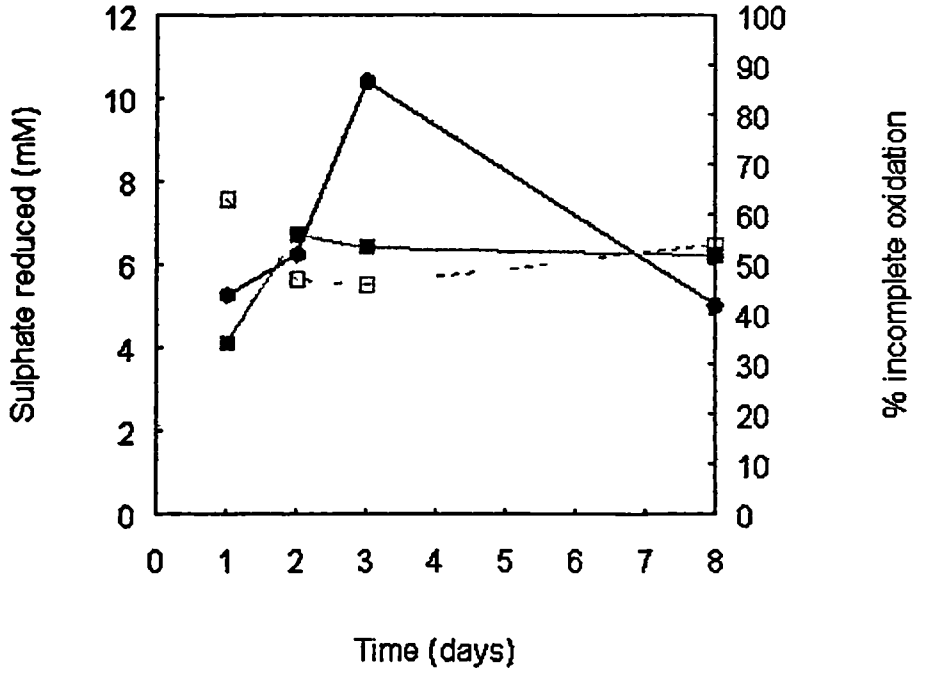


Figure 5.15 Actual (●) and theoretical (■, with unbroken line) sulphate reduced, and % incomplete oxidation of glycerol (to acetate) (, with broken line) in continuous culture run at pH 3.5, 30°C and HRT = 66 h)

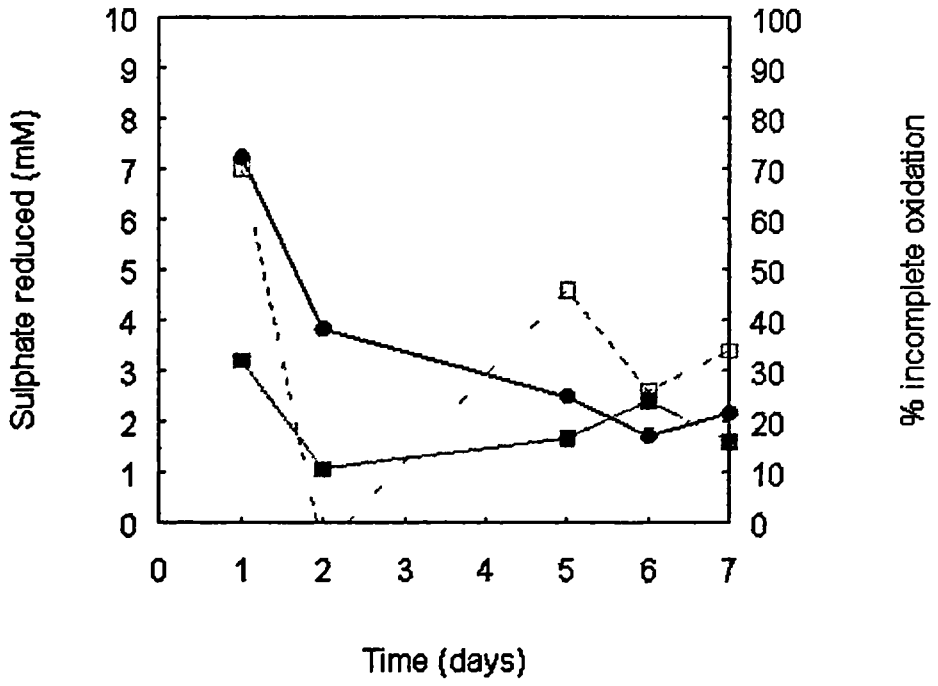


Figure 5.16 Actual (●) and theoretical (■, with unbroken line) sulphate reduced, and % incomplete oxidation of glycerol (to acetate) (□, with broken line) in continuous culture run at pH 3.0, 30°C and HRT = 66 h

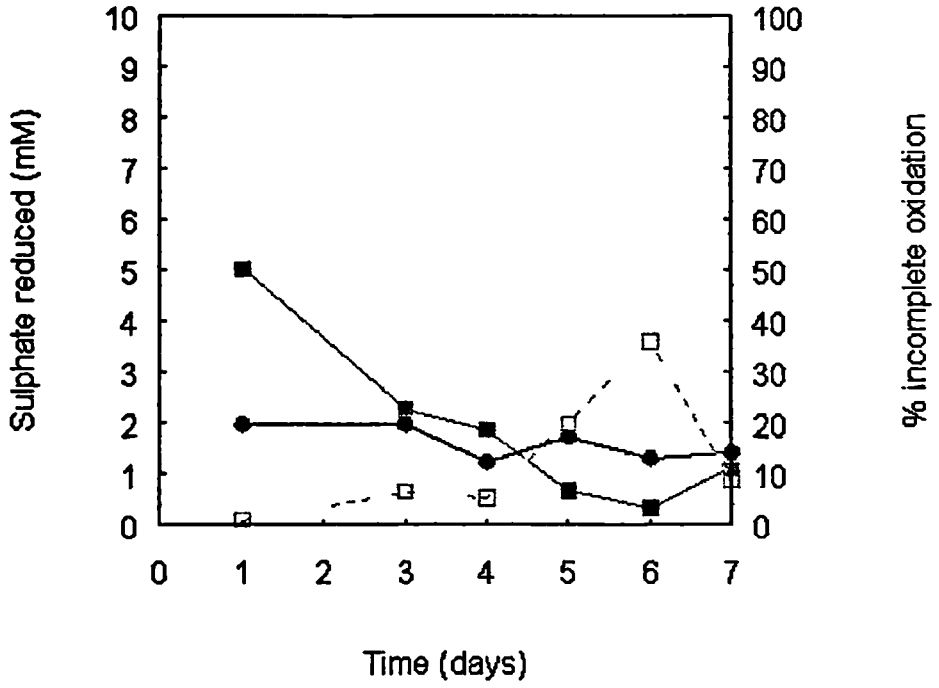


Figure 5.17 Actual (●) and theoretical (■, with unbroken line) sulphate reduced, and % incomplete oxidation of glycerol (to acetate) (□, with broken line) in continuous culture run at pH 2.5, 30°C and HRT = 66 h

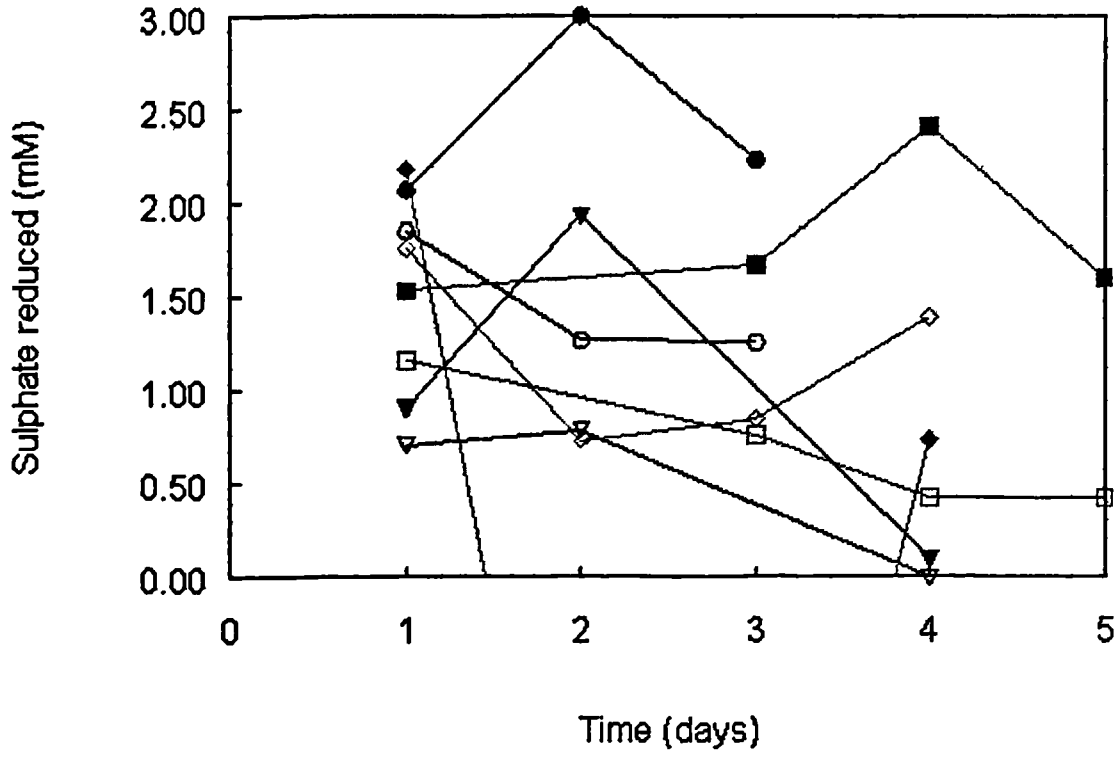


Figure 5.18 Actual (filled symbols) and theoretical (open symbols) sulphate reduced in continuous culture fed with 5 mM acetic acid. (●) and (○) = change in sulphate concentrations during run at pH 4.0, 30°C, and HRT = 33 h. (■) and (□) = from a run as above but with a flow rate of 25 ml/h (HRT= 66 h). (▼) and (▽) = from a run as above but at pH 4.8 and a flow rate of 25 ml/h. (◆) and (◇) = run at pH 4.8, 22.5°C and HRT = 66 h.

5.3.2.1 Populations in continuous culture

Table 5.4 shows results for enumeration of bacteria from serial dilutions of samples from the Parys bioreactor culture during continuous culture experiments (with glycerol). The highest numbers were counted from a sample taken shortly after set-up of the bioreactor, with a total count of 4 x 10^6/ml; however, no cells of the *Desulfosporosinus*-like P1 were counted at all. The next highest count was obtained for the beginning of the run at pH 3.5 (24/11/99): all the colonies counted appeared to be either P1 or PCP. This was followed by the count at the end of the pH 3.0 run (16/12/99), in which the highest counts of P1 over all runs were obtained. The counts at the end of the pH 4.0 (50 ml/h) run and the pH 2.5 run were 3.4 x 10^5/ml and 3 x 10^5/ml respectively, but a much higher proportion of the count was P1 in the pH 2.5 run (33/ml at pH 4.0 compared with 1.02 x 10^5/ml at pH 2.5). In general, higher counts of P1 were made at the pH of the run decreased; however, this also corresponded to the length of time the culture had been operative, as the higher pH experiments were carried out first. Higher total counts were obtained from the 100 ml/h run at pH 4.0 (25/10/99) than on the 25 ml/h run: this was evident on both types of solid medium. The highest numbers of PCP were counted at set-up, followed by pH 3.5. The highest numbers of 'unidentified' bacteria were counted in the sample from the 100 ml/h run, obtained using the YeO medium. No P1 were counted from the WJB-3 O plates.

Date	pH	Type of solid medium	Nos. of isolate P1/ml	Nos. of isolate PCP/ml	Nos. of uniden-tified*/ml	CFUs/ml
7/99	-	YEO	$<10^2$	3.9×10^6	5.3×10^4	**5.8×10^5**
9/99	4.0	YEO	$<10^2$	3.3×10^5	6.7×10^3	**3.4×10^5**
10/99	4.0	YEO	2.3×10^3	2×10^4	33	**2.3×10^4**
10/99	4.0	WJB-3O	$<10^2$	$<10^2$	1.1×10^4	**1.1×10^4**
10.5/99	4.0	YEO	4.5×10^3	1.2×10^5	7.6×10^4	**2.0×10^5**
10.5/99	4.0	WJB-3O	$<10^2$	6.9×10^4	$<10^2$	**6.9×10^4**
11-99	3.5	YEO	1.9×10^4	1.9×10^6	$<10^2$	**1.9×10^6**
12-99	3.5	YEO	1.4×10^5	2.1×10^3	4.5×10^2	**1.4×10^5**
12.5/99	3.0	YEO	6.2×10^4	1.2×10^5	$<10^2$	**1.8×10^5**
12.7/99	3.0	YEO	3.3×10^5	1.2×10^6	7.2×10^2	**1.4×10^6**
01/00	2.5	YEO	1.0×10^5	1.9×10^5	1.2×10^3	**3.0×10^5**

Table 5.4 Summary of results of the enumeration of bacteria from the mixed aSRB culture (Parys enrichment) during continuous culture experiments. CFUs = colony forming units. *"Unidentified" organisms refer to those whose identity was ambiguous, or were not recognised as any of the other known types. In two experiments, a second variant of solid overlay medium, WJB-3O, was tested during its development for its ability to support growth of aSRB culture organisms compared with YEO plates.

5.4 Discussion and Conclusions

5.4.1. Batch experiments

Sulphate reduction rates by the Montserrat culture were highest at pH 4.0. In contrast, there was not a clear pH optimum with the Parys culture. Although the highest rate was observed at 5.5, there was not such a clear pH optimum, and the system performed over a broader range of pH than the Montserrat culture. It appeared to be less sensitive to extreme acidity than the Montserrat culture, as sulphate reduction rates were noticeably higher at pH $\leq$3. At pH 2.5, the sulphate reduction rate was approximately three times greater with the Parys culture (21.1 µmoles L^{-1} h^{-1} compared with 7.3 µmoles L^{-1} h^{-1} for the Montserrat culture) and around twice the rate at pH 3.0 (35.2 µmoles L^{-1} h^{-} compared with 16.3). In addition, it was thought possible that the presence of a putative acidogen in the mixed culture from Montserrat (section 3.7.1) could render it less suitable for use in a continuous mode system. The Parys aSRB culture was therefore chosen for the continuous culture experiments.

In the batch experiment with the Parys culture that were carried out at higher pH (5.5), the highest sulphate reduction rate was obtained, and considerably less acid was required to maintain pH (Table 5.1b). Ferrous iron remained mostly in solution in experimental runs set at pH <5.5, but at pH 5.5 soluble ferrous iron concentrations dropped to almost zero. The formation of ferrous sulphide is acid-generating reaction (section 1.5.2; equation 5.1:

$$Fe^{2+} + H_2S \rightleftharpoons FeS + H^+ \qquad (5.1)$$

The solubility product K_{sp} is useful for calculating the aqueous solubility of sparingly soluble compounds under various conditions. The solubility product (log K_{sp}) of FeS is -18.8 (Diaz *et al*., 1977) which is greater than that of ZnS (log K_{sp} = -24.5) and CuS (log K_{sp} = -35.9). Sulphides with very low solubility products can be precipitated even from acidic solutions in

which the HS- ion concentration is suppressed by the presence of acid ions (Kelly, 1984; section 1.5.2). The hydrolysis reaction (equation 5.2) is highly dependent upon solution pH:

$$H_2S + H_2O \longleftrightarrow HS^- + H_3O^+ \quad (5.2)$$

The K_1 of hydrogen sulphide is 1.0×10^{-7} M and the K_2 ($HS^- \longleftrightarrow S^{2-} + H^+$) is 0.8×10^{-17} (Steudel, 2000). Therefore the concentrations of HS- present in solution will decrease significantly as pH declines, thereby dictating the pH at which metal sulphides will precipitate; those with very low solubility products (e.g. copper sulphide) will precipitate at much lower pH values than those with higher K_{sp}'s (e.g. ferrous sulphide).

$$M^{2+} + HS^- \longleftrightarrow MS + H^+ \quad (5.3)$$

The fact that an acid-generation reaction was occurring at pH 5.5 would account for the reduced amount of acid required to maintain culture pH. By lowering the concentration of ferrous iron, acid generation due to the formation of ferrous sulphide could be minimised. Experiments using 1 mM $FeSO_4$ + 9 mM K_2SO_4 in place of 10 mM $FeSO_4$ at pH 5.5, did result in additional input of acid (relative to the standard 10 mM $FeSO_4$ medium) to maintain culture pH. This was also the case with the pH 4 culture in which zinc sulphate (at 8 mM) substituted in part for ferrous sulphate. At pH 4, zinc sulphate precipitates but ferrous sulphide does not, due to the lower log K_{sp} of zinc sulphide.

The sulphate reduction rate in the presence of zinc sulphate was lower overall than with 10 mM $FeSO_4$, when measured over a period of several days (48.6 µmoles L^{-1} h^{-1} compared with a mean of 70.2 µmoles L^{-1} h^{-1} for the 10 mM FeSO4 cultures). However, the initial rate in zinc sulphate-containing medium was 61.8 µmoles L^{-1} h^{-1}, but this declined subsequently. The zinc sulphate precipitated out rapidly in these cultures,

at the time corresponding to the more rapid phase of sulphate reduction. The subsequent slower rate of sulphate reduction was possibly due to the fact that H_2S produced was not being removed as zinc sulphide but was remaining in solution and inhibiting bacterial activity. This being the case, the aSRB system might be more efficient in the present of metals such as zinc, which may be present in acidic wastewaters such as AMD.

Acid-generation, as opposed to 'consumption', could also come about at pH 5.5 due to the presence of dissociated acetic acid. Although acetate concentrations were not actually measured routinely in the batch experiments, occasional tests were carried out to check whether acetate was present in the cultures, and up to around 3 mM was generally detected (data not shown). This implies that ethanol was being incompletely oxidised to acetate (equation 5.6). Due to the pK_a of acetic acid (4.75), at lower pH, e.g. 3.5, acetic acid is 94% present as the undissociated acid, while at pH 5.5, 86% is present as dissociated acetate:(equation 5.4):

$$CH_3COOH \underset{pK_a = 4.75}{\rightleftharpoons} CH_3COO^- + H^+ \qquad (5.4)$$

In one of the earlier batch runs (pH 4.0), approximately 3 mM of ethanol was utilised, and therefore, up to 3 mM of acetate would be produced, which would correspond to 2.4 mM acetic acid, 0.6 mM acetate and 0.6 mM H^+.

It might, therefore, be advantageous to run a bioremediation system using aSRB at a pH <4 , below the point at which acid generation from the precipitation of ferrous sulphide and the dissociation of acetic acid becomes significant. This may make the system more robust to input of acidic liquors. Indeed, at neutral pH (7), i.e. when hydrogen sulphide and HS^- occur in equimolar concentrations, alkalinity is not generated by sulphate reduction (section 1.5.2, equation 1.11). In addition, if a system

was found that could perform effectively to treat acidic, metal-rich waste waters at pH 5 rather than 7, this would be sufficient to bring about significant reductions in running costs (Buisman, C., pers. comm.). Decisions regarding operating specifications will ultimately depend upon what the system is primarily designed to treat, e.g. increasing the pH of acidic waters or metal precipitation. (Acid-tolerant or aSRB could be very suitable for a role in the former, as part of a integrated multi-functional system designed to treat acidic waste waters).

An increase in biomass concomitant with sulphate reduction and alkalinity generation would be anticipated in bioreactor systems similar to those tested in the present work. However, monitoring an increase in biomass is complicated if it is mainly attached to a matrix in a biofilm within a closed system. Biofilms are dynamic structures, and planktonic cell numbers do not necessarily reflect changes in immobilised cell numbers; sampling the former may therefore provide data that is not representative of the biomass present. Processes of cell detachment from the biofilm can be linked to the environment within the biofilm or within the bulk liquid, and these influences can be physical (e.g. liquid velocity), chemical (e.g. nutrient availablity) or biological (e.g. production of enzymes that degrade EPS) and may induce physiological changes that lead to detachment (Moore *et al.*, 2000).

Increases in cell numbers were not found when counts were made with samples from batch bioreactor experiments; in general cell numbers stayed relatively constant with only minor fluctuations. Cell numbers of the *Desulfosporosinus*-like type (P1 and M1) were present in low numbers in the samples, although it is possible that these were almost exclusively attached cells, and if there were increases in attached cell numbers, they would not have been measured using the methods used. A different method for measuring the biomass would be required for future, similar experiments; for example, a small part of the matrix (e.g. one Poraver

bead) could be withdrawn and cells could be removed by sonication in buffer and counted. Measurement of cell protein provided limited useful information, probably for the same reasons, and the need to carry out numerous cell washes before carrying out the assay meant that the method became very inconvenient and lengthy. In addition, the presence of ferrous sulphide precipitates in the cell matrix (which did not respond to acid washing) appeared to be interfering with the assay. Some results were orders of magnitude in variation. It appeared that a different method was required to measure activity of the cultures, and therefore ATP in samples of planktonic cells was measured. However, enumeration of bacteria from samples taken at the beginning and end of runs in the Montserrat culture did show an increase in cell numbers at each pH tested. This could be due to an increase in biomass or an increase in detachment of bacteria from the biofilm, or a combination of the two. The greatest increase in numbers was found at pH 3.0; in this case a considerable increase in numbers of M1 was observed. It is not possible to conclude what proportion of this was attributable to biomass increase or detachment. However, as the runs began with higher "start" numbers in chronological order, it is possible that (attached) cell numbers were indeed increasing in the biofilm with time, and planktonic numbers were reflecting this. If biomass increase was indeed occurring with time, then sulphate reduction was coupled to carbon assimilation in the system, which would fulfil the requirements of an efficient treatment system using these bacteria. Low molecular weight compounds such as acetate can act as uncouplers preventing ATP synthesis by mopping up hydrogen ions (Alberts *et al.*, 1994), and sulphate reduction activity may occur without carbon assimilation, i.e. growth. If acetate were to accumulate in the system then it could slow down or prevent the coupled reaction. A method of removing acetate or incorporating an acetate-degrader into the system would then need to be considered.

Dissolved sulphide was not measured in any significant concentrations; in the run in which gassing was not carried out, concentrations were around 1 mM or less, and in later experiments they were found to be around the detection limit of the method used, i.e. virtually zero. This is not entirely unexpected, as at the low pH of the experiments, H_2S is volatilised very readily, much more so than in circumneutral systems (section 1.5.2, equation 1.9). Gassing or any leaks in the bioreactor system would allow for rapid removal of H_2S. Biogenic H_2S for "off-line" metal precipitation in a bioremediation system could therefore be easily obtained. Toxicity of H_2S may be a more important issue in systems that are circumneutral and therefore above the pK_a of H_2S (Hulshoff Pol *et al.*, 1998). This is because the inhibitory effect of sulphide is thought to be caused by unionised hydrogen sulphide, which easily permeates the cell membrane (Hulshoff Pol *et al.*, 1998), and may combine with cytochromes and other iron-containing compounds in the cell.

In those batch experiments with mixed aSRB cultures from Parys Mountain and Montserrat enrichments (section 3.2) in which ethanol concentrations were measured, more sulphate reduction occurred than could be accounted for. For complete oxidation of ethanol to CO_2 coupled to the reduction of sulphate to H_2S, the stoichiometry is 2 ethanol:3 sulphate (equation 5.5) and for incomplete oxidation to acetate it is 2:1 (equation 5.6):

$$2C_2H_5OH + 3SO_4^{2-} + 6H^+ \longrightarrow 4CO_2 + 3H_2S + 6H_2O \quad (5.5)$$

$$2C_2H_5OH + SO_4^{2-} + 2H^+ \longrightarrow 2C_2H_4O_2 + H_2S + 2H_2O \quad (5.6)$$

Due to the difficulties in monitoring changes in ethanol concentrations using the methods available, it is difficult to conclude how the resulting sulphate reduction was being driven. In addition, it is possible that some loss of ethanol was taking place due to the regular gassing of the system,

which was operating at a moderately high temperature (30°C). In the first batch run at pH 3.5 with the Parys culture, gassing of the culture with OFN was not carried out, in order to assess the effects on the system. A sulphate reduction rate similar to the other runs at pH 3.5, in which gassing was carried out, was obtained, but there was not a detectable decline in the concentration of ethanol. As this was the first run following the system set-up, the bacteria could conceivably have been utilising storage compounds from the enrichment culture inocula, but it seems unlikely that the observed rate of sulphate reduction could be sustained by this. In a subsequent run (with OFN gassing) ethanol concentrations did decline somewhat.

All runs were carried out at 30°C except for two, which were run at 20 and 25°C, at pH 4, with the Montserrat culture. The performance of the system was reduced at lower temperatures. The costs of running a full-scale treatment system at 30°C would be substantially higher than at 20 or even 25°C, but it may still be possible to run the system at 25°C by optimising all other factors. In fact, a reduction of just one or two degrees could decrease operating costs substantially (J. Boonstra, pers.comm).

The batch culture experiments were useful for assessing the potential of the cultures for bioremediation of acidic effluents, over what range of pH and temperature they were effective at sulphate reduction, and which pH was optimal for either culture. They were useful in identifying those parameters that were difficult to measure using the available methods. This was taken into account in the set-up of the continuous culture experiments as far as possible.

5.4.2 Continuous culture experiments

Continuous culture experiments were carried out in 2 L bench-scale bioreactor systems using glycerol as electron donor, at various flow rates of medium input (25, 50 and 100 ml/h) and pH (2.5, 3.0, 3.5, 4.0). The highest sulphate reduction rate (460 µmoles SO_4^{2-} L^{-1} h^{-1}), sulphate reduction activity (23.4 µmoles SO_4^{2-} L^{-1} h^{-1} * µmole ATP) and sulphate conversion rate (1.06 g SO_4^{2-} L^{-1} day^{-1}) was achieved at 100 ml/h (an HRT of 16.5 h) and pH 4. Activity of the system was therefore increased with a higher rate of substrate (medium) input. This conversion rate is not as high as in a commercial process such as Paques B.V.'s *Thiopaq®* (12 g L^{-1} day^{-1}), but this is to be expected at bench-scale, and scale-up and optimisation of the system might substantially increase this rate. However, the rate was greater than the average achieved by Kolmert and Johnson (1999) in a column biofilm bioreactor system using aSRB and nSRB (0.3 g SO_4^{2-} L^{-1} day^{-1}).; At pH 4, a lower reduction rate was obtained at a flow rate of 50 ml/h, or an HRT of 33 h (0.78 SO_4^{2-} L^{-1} day^{-1}), and lower still at 25 ml/h, or an HRT or 66 h (SO_4^{2-} L^{-1} day^{-1}), with sulphate reduction activity reflecting these data. The system reduced sulphate at all pH values tested, with the sulphate reduction rate at an HRT of 66 h decreasing by approximately half for each 0.5 pH unit decrease between 3.5 and 2.5 (166, 81.8 and 40.0 µmoles SO_4^{2-} L^{-1} h^{-1} respectively). The expected increase between pH 3.5 and 4.0 at this HRT was not evident, but when the HRT was decreased at an operating pH of 4, higher rates were achieved. It appears that pH 3.5 was an optimum in continuous culture at lower flow rates, but that higher flow rates improved performance when the system was operating at a higher pH (4.0).

The amount of hydrogen sulphide generated (and captured as CuS) by the Parys culture was less than expected in all experiments, given that for each molecule of sulphate reduced, 1 molecule of hydrogen sulphide is produced (equations 5.7, 5.8 and 5.9; Figures 5.10 and 5.11). As the

concentrations of dissolved sulphide in the outflow liquor were, in almost all cases, around zero, it was concluded that the apparatus was not 100% efficient in capturing biogenic hydrogen sulphide.

Enrichment, growth and isolation of aSRB had been achieved using glycerol as electron donor since the start of the current research programme. Johnson *et al.* (1993) had earlier used glycerol to isolate an SRB under acidic conditions (section 3.2). Glycerol is a major component of waste water from bioethanol production, i.e. distillery stillage (Qatibi *et al.*, 1991) and could be therefore obtained at relatively low costs. Anaerobic digestion is currently one of the main purification processes used on this type of waste water (Bories *et al.*, 1982). Due to these considerations, and as there had been difficulties in detecting ethanol using the methods used in the batch culture experiments, glycerol was used as carbon/energy source in continuous culture experiments.

For complete oxidation of glycerol to CO_2 coupled to the reduction of sulphate to H_2S, the stoichiometry is 4 glycerols :7 sulphates (equation 5.7) and for incomplete oxidation to acetate it is 4:3 (equation 5.8), with 1 molecule of acetate being produced for every molecule of glycerol oxidised:

$$4C_3H_8O_3 + 7SO_4^{2-} + 14H^+ \longrightarrow 12CO_2 + 7H_2S + 16H_2O \quad (5.7)$$

$$4C_3H_8O_3 + 3SO_4^{2-} + 6H^+ \longrightarrow 4C_2H_4O_2 + 4CO_2 + 3H_2S + 8H_2O \quad (5.8)$$

Oxidation of acetate coupled to sulphate reduction has a stoichiometry of 1:1 (equation 5.9), i.e. one molecule of sulphate is reduced for every molecule of acetate oxidised:

$$C_2H_4O_2 + SO_4^{2-} + 2H^+ \longrightarrow 2CO_2 + H_2S + 2H_2O \quad (5.9)$$

In all runs with glycerol, acetate was produced, with concentrations decreasing during the run at pH 3.0, and with very low concentrations measured in the pH 2.5 run. This indicated that incomplete oxidation of glycerol was being carried out by the aSRB and/or other species in the mixed culture, though acetic acid could also have been formed from reduction of CO_2 if any acetogenic bacteria were present. However, it was assumed that the concentration of acetate in the culture was produced from incomplete oxidation of glycerol (equation 5.8), and that, in the case of more glycerol being oxidised than acetic acid produced, this additional glycerol was being completely oxidised to CO_2 (equation 5.7).

Overall, incomplete oxidation of glycerol was found to be smallest (as a relative percentage) in the experimental run at pH 2.5 and an HRT of 66 h. and the highest at pH 4 and an HRT of 16.5 h. However, at lower pH, smaller amounts of glycerol were being oxidised overall. It appeared that a higher rate of dilution resulted in a slightly higher percentage of incomplete oxidation, at least at the beginning of the run, and that towards the end of the run there was a gradual increase in the amounts of glycerol being completely oxidised to CO_2. This was evident in runs with HRT of 16.5 h and 33 h. If there were, as it appeared, bacteria in the mixed culture that were capable of completely oxidising glycerol, it is possible that they were becoming more active during the course of these runs. Results were, however, somewhat ambiguous. Acetate concentrations gradually decreased during the course of the run at HRT of 33 h after an initial increase but in an experimental run with an HRT of 16.5 h, acetate concentrations increased with time.

Syntrophic relationships, where organisms in an ecosystem support each others existence, are particularly important in anaerobic systems. In aerobic systems, organisms can frequently degrade complex molecules completely to carbon dioxide and water without obligate relationships with other organisms (Brock, 1997). This is probably because the energetics of

complex materials with O_2 as electron acceptor is more favourable than fermentative catabolism of the same substrates. Fermentative catabolism predominates when conditions become anoxic and alternative electron acceptors scarce, but in many cases, syntrophic interactions are required (Brock, 1997). One of the most well studied syntrophic systems in fermentative metabolism is the fermentation of ethanol to acetate and methane by two organisms, an ethanol fermenter and a methanogen. The ethanol fermenter produces hydrogen and acetate in a reaction which has a positive free-energy balance; the hydrogen is then consumed by the methanogen in an energetically favourable reaction, and the overall energy yield is greater than -100 kJ/reaction (Brock, 1997). The methanogen is unable to utilise ethanol directly so both organisms benefit from the reaction. This syntrophy is based on a phenomenon known as interspecies hydrogen transfer. It is possible that one or more coupled fermentation reactions were occurring in the bioreactor systems, which could account for some of the glycerol utilised. Sulphate reducing bacteria may switch from deriving energy from sulphate reduction to fermentation in the environment, according to whether sulphate is limiting, or may utilise fermentation products from other organisms, such as hydrogen (which was utilised by the aSRB isolates P1 and M1; section 6.3.2). However, growth yields from fermentation are substantially less than those from sulphate reduction. It is possible that glycerol was being fermented by one or more organisms in the mixed culture. For example, glycerol can be fermented by *Acetobacterium* spp. (equation 5.10):

$$4C_3H_8O_3 + 2HCO_3^- \longrightarrow 7C_2H_4O_2 + 5H^+ + 4H_2O \qquad (5.10)$$

Qatibi *et al.* (1991) found that, in the absence of sulphate, glycerol utilisation was characterised by the transient formation of 1,3-propanediol before propionate and acetate, and that the presence of sulphate as a terminal electron acceptor apparently diminished the production of 1,3-propanediol by a mixed microbial culture (including SRB). They found that

species of *Desulfovibrio* were the main glycerol degraders; *Desulfovibrio* species can utilise glycerol and diols such as 1.2- and 1,3-propanediol by interspecies hydrogen transfer (Qatibi *et al.*, 1989). However, *D. carbinolicus* has been shown to oxidise glycerol anaerobically in the presence of sulphate (Nanninga and Gottschal, 1987). The investigation by Qatibi *et al.* (1991) agreed with the concept that anaerobic degradation of reduced products of classical fermentations such as alcohols or fatty acids usually requires the reduction of electron acceptors (e.g. sulphate; Widdel, 1988), or a syntrophic association of, for example, fermenting bacteria and methanogens, or SRB and methanogens.

In the experimental runs with acetic acid/acetate, as with glycerol, more sulphate was reduced than expected (Figure 5.18), except in the low temperature (22.5°C) run. The experiments were carried out to determine whether the mixed culture could oxidise acetate (the oxidation of acetate by SRB has been discussed in detail in section 1.7.4.3). The mean SRR with acetate at pH 4 was higher than with glycerol at pH 2.5 and 3.0 (Figure 5.9). In fact, the mean SRR at an HRT of 33 h with acetate was around three times the SRR at an HRT of 66 h with glycerol. It appeared that, at pH 4, the mean SRA was approximately the same as with glycerol at the highest residence time (66 h), even though the SRR was much lower, suggesting that the culture was able to generate ATP using acetate at this pH. However, in theory, acetic acid could be toxic to the cells at this pH (section 4.1; Ingledew, 1982; Pronk, 1991; Norris and Ingledew, 1992). At pH 4.8, which is the approximate pK_a of acetic acid there is less undissociated acetic acid. than at pH 4.0 (equation 5.4). Therefore, it was thought more likely that utilisation of acetate would be possible at pH 4.8 than at 4.0, or that a higher SRR and SRA would be obtained. However, this was not apparently the case. At pH 4.8, sulphate reduction rates were the lowest of all the acetate-fed runs. Sulphate conversion rates were markedly lower than at pH 4 (Table 5.3) and sulphide production was around zero (Figure 5.11). Slightly higher SRR, SRA and sulphate

conversion rate was found at 22.5°C than at 30°C; it is possible that different bacteria in the culture have different temperature optima, but a range of temperature would need to be tested to make firm conclusions. The mode of the inhibitory effects of uncouplers was outlined in 5.4.1. It is possible that the cells in continuous culture were able to tolerate higher concentrations of acetic acid/acetate, and to utilise it to some extent, because under these conditions they were more metabolically active than under nutrient-limited conditions or in batch culture. When cells are starved, resting or inactive, they are more vulnerable to the effects of uncouplers such as acetate (Alberts *et al.*, 1994). In addition, in acidic conditions, the presence of "extra" hydrogen ions outside of the cell may have meant that the proton gradient remained despite any dissipation from redissociation of the weak acid, allowing for sulphate reduction and ATP formation to continue.

For both batch and continuous experiments, the possibility that the yeast extract in the medium was providing the cultures with enough additional organic carbon to fuel the sulphate reduction that was not accounted for by oxidation of ethanol, glycerol or acetate was considered. However, it was calculated (using data in an experiment with batch cultures in section 4.2.1.2) that the inclusion of 0.01% yeast extract reduced the concentration of sulphate in a culture by an average of 0.385 mM. Given that the concentration of yeast extract in the medium was 0.002%, it is unlikely that the yeast extract made a significant contribution to the "excess" sulphate reduction exhibited by the systems.

Bacteria can be free-living (planktonic) but in most natural ecosystems they grow on interfaces as biofilms (Beveridge *et al.*, 1997). On these biofilms they may take advantage of the nutrient concentrative effects of the interface (Loeb and Niehof, 1977), but in addition they may be conferred protection from predators and toxic agents by the biofilm (Beveridge *et al.*, 1997). In the bioreactor systems, samples of planktonic

bacteria were used for dilution series to enumerate bacteria from the mixed cultures, to look for any patterns in population shifts between identified organisms. Populations in the mixed culture during continuous culture varied with the pH of the run, but total numbers were highest just after the system was set-up. Numbers of *Desulfosporosinus*-like cells, P1, were below detection: this could be because they were attached to the Poraver in the inoculum. In the first run (9/99) at pH 4, numbers of P1 increased slightly to 33/ml (Table 5.4). In the following run, numbers had increased again, to 2.3 x 10^3/ml (as quantified by the use of the yeast extract overlay medium). This could be explained by either a build-up of biomass of P1, or detachment of attached cells, or both. In the 10/99 run, numbers increased slightly to 4.5 x 10^3/ml, and in the pH 3.5 runs that followed, numbers increased again. When pH was lowered to 3.0 (12.5/99), there was an initial decrease in numbers from the previous run at pH 3.5, followed by an increase after the system had run for three days at pH 3.0. At pH 2.5 (01/00), numbers decreased slightly but were around the same as those found at pH 3.5. It is not known what effect pH has on attachment or detachment of these cells; adverse conditions, such as carbon limitation, may cause detachment, promoting migration to more favourable conditions (Delaquis *et al.*, 1989; Wolfaardt and Cloete, 1992) or may cause attachment to, or migration within, the biofilm by dormant or fragmented cells; Sanders *et al.*, 1994; Bass *et al.*, 1996). Marshall (1985) speculated that detachment of a biofilm may be a response to the need for dispersion. Active detachment and relocation in a flowing situation may be considered part of a survival strategy, offering a distinct survival advantage in several ecological niches (Bass *et al.*, 1998). Numbers of P1 may have increased due to biomass increase in the biofilm with time, and whilst these were highest at pH 3.0, mean SRA was highest at pH 3.5, suggesting that at lower pH some cells may have become inactive, possibly due to the effects of acetate. In addition, at higher pH, more FeS precipitates in the biofilm would have been present. In natural environments, bacteria are very often closely associated with the minerals

which they have helped to develop (Beveridge et al., 1997), which in this case is FeS (and also to some extent FeS_2). This might partially account for the lower numbers of planktonic cells of P1 at higher pH.

Isolate PCP was highest in numbers just after the system was set up, and was clearly lowest in numbers in the second run at pH 3.5 (12/99); numbers were almost half of those found in the previous run at 3.5 (11/99). Numbers of PCP rose again at pH 3.0 (12.75/99) and dropped off slightly at pH 2.5. This suggested that, at pH 3.5, PCP was either actively attaching to the biofilm, or being washed out. It may be that this bacterium was being outcompeted by the SRB at this pH if SRA was highest (it was concluded subsequently that PCP is not a dissimilatory sulphate reducer; Chapter 6). The number of "other" cells was highest at pH 4.0 (not at set-up as with the other organisms counted), and appeared to be lowest at pH 3.5. Numbers rose significantly between pH 3.0 and 2.5, whereas P1 and PCP decreased in numbers at this pH. It is possible that different members of the mixed microbial culture have different pH optima, which may at least partially account for this. As the identities of these bacteria are not known, however, very little can be concluded from the data.

The WJB-3 overlay plates were included to test their effectiveness for growth of organisms from the mixed culture, compared with the YEO plates; a full experiment was not carried out as the medium was still being developed at this stage. They were found to be not as effective as the yeast extract overlay plates for growth of all of the organisms at pH 4.0: no colonies of P1 or PCP were counted in the first run at pH 4, and in the second, only PCP were observed, whereas P1, PCP and "other" organisms all grew on the YEO medium.

Many, if not most treatment systems employ immobilised cultures for treatment of acidic and/or metalliferous waste waters (section 1.7.5.3). Elliot *et al.* (1998) operated a system with SRB under acidic conditions for

the treatment of AMD, using sand as a matrix for a biofilm (section 1.7.5.3). Porous glass beads such as Poraver (Figure 5.1(a)) provided a large surface area for colonisation by aSRB (Figure 5.1(b)) and other microorganisms in the mixed culture. Bacteria in biofilms can be more metabolically active than their counterparts in the planktonic phase; the close proximity of cells of different species within biofilms results in closer metabolic associations than between free-living cells, and as a result, improved degradative efficiency (Wolfaardt *et al.*, 2000). Therefore, the bioreactor systems evaluated in the present study are considered to have benefited from the inclusion of the Poraver beads.

6. PHYSIOLOGICAL AND PHYLOGENETIC CHARACTERISATION OF aSRB AND ASSOCIATED ACIDOPHILIC BACTERIAL ISOLATES

6.1 Introduction

Characterisation of bacteria has traditionally been concerned with the investigation and description of criteria based on morphology, biochemistry, and physiology. Phylogenetic relationships among SRB have been inferred by classical comparative techniques: by the analysis of similarities and differences in biochemical pathways, physiological requirements, pigments, cytochrome *c* characterisations, G + C contents, and rRNA:DNA homologies (Pfennig *et al.*, 1981; Widdel and Pfennig, 1984; Devereux and Stahl, 1993). However, the fact that SRB are a very diverse group of bacteria (Widdel, 1988) led to the recognition that the assessment of these criteria, whilst still valuable for comparative analysis, may not be sufficient for determinative purposes. The term "sulphate reducing bacteria" is an appropriate description of common physiology, but it obscures the evolutionary diversity of the organisms. Bacteria generally lack an abundance of physiological and morphological characteristics that are phylogenetically interpretable (Devereux and Stahl, 1993), and a molecular approach is often necessary to clarify their phylogeny. Classification of organisms based on evolutionary history may be inferred by comparing sequences of homologous biopolymers with a constant rate of fixed mutations, such as the 16S rRNA molecules. The phylogeny emerging from these comparisons is often, but not always, consistent with classification based on biochemical and physiological criteria.

Identification and enumeration of SRB is important in both ecological and economic contexts. Assessments of populations in the environment or in mixed communities in the laboratory assist in furthering an understanding of natural relationships. The SRB are of major importance in microbially influenced corrosion (MIC) (section 1.7.6). They are also used in a number

of important industrial bioprocesses (section 1.5.2). Identification of isolates and their genes using molecular techniques may provide some clues as to nutritional requirements, metabolic by-products, and enzyme kinetics, which are crucial factors in the design of such processes. In addition, bacterial activity can be monitored in polluted sites using molecular techniques. Once identified, the strategies that these organisms have evolved to exist (and proliferate) in these polluted environments can be exploited through their use in new bioprocesses. In many instances, these bacteria are found to be novel species or strains.

Isolation of pure cultures of SRB is complicated by requirements for anaerobic techniques and further constrained by the long generation times of many species (Devereux and Stahl, 1993). Consistent growth of aSRB in the current research programme was not always achieved. The novel aSRB isolates (and other bacteria that were possibly sulphate reducers, since they formed colonies that were either black/brown or had a black/brown centre, presumably from the formation of ferrous sulphide) isolated from mixed bioreactor cultures (Chapters 3 and 5) were grown using liquid or solid media, according to whichever was possible. Colony and cell morphologies were determined, isolates were Gram-stained and, following the development of solid overlay medium using *Acidocella* WJB-3 (section 2.1.1.4.1 (b), and Chapter 4), growth was tested using a variety of substrates. The 16S rRNA genes of the isolates were sequenced, and the sequences obtained were used to determine their phylogenetic position relative to other sequenced bacteria.

6.2 Colony and cell morphologies, and Gram staining

6.2.1. Methodology

Colony and cell morphologies were determined using microscopy as described in sections 2.3.1-2.3.3. Gram staining was carried out using the method described in section 2.1.3.

6.2.2 Results

Table 6.1 summarises the colony/cell morphologies and results of Gram staining for the seven isolates that were characterised. Figure 6.1 shows colonies of (a) P1 and PCP, and (b) MCP and MR on solid medium, and Figure 6.2 shows scanning electron micrographs of isolates P1 and PFB.

6.3 Substrate Utilisation

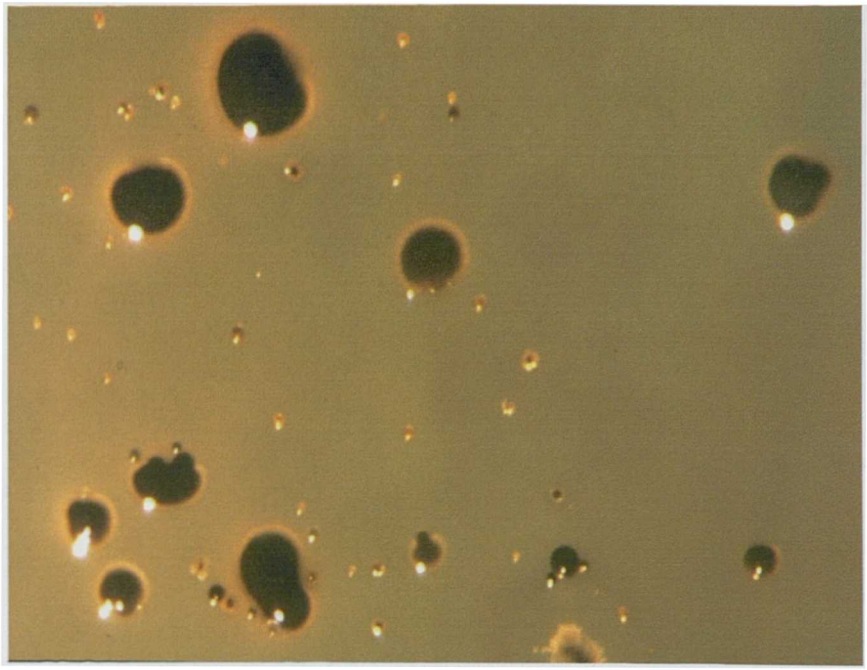
6.3.1 Methodology

Recently grown colonies of the six isolates (on 0.02% (w/v) yeast extract overlay medium) were loop-streaked onto *Acidocella* WJB-3 overlay solid medium (section 2.1.1.4.1), containing different potential organic substrates, and which had a final pH (measured with a flat-bed pH electrode; section 2.2.1) of 3.6 – 3.8. Petri plates containing 0.002% (w/v) yeast extract solid medium, were incubated at 30°C for approximately 1 hour to dry the surface) Appropriate volumes of sterile stock solutions of organic substrates were applied and spread over the medium (assuming the volume of the gelled yeast extract medium to be 30 ml in each plate). Plates were incubated upright at room temperature for 1-2 hours, allowing solutions to be absorbed into the gel. The following substrates were used (final concentrations in parentheses): lactic acid (0.5 mM); acetic acid (0.5 mM); pyruvic acid (0.5 mM); mannitol (5 mM); glycerol (10 mM); ethanol (15 mM); methanol (20 mM); glucose (5 mM); galactose (5 mM); ribose (5 mM), and glycine (10 mM) (as described in section 4.3.8.1, different concentrations of these substrates were used to obtain equal 'carbon equivalents', with the exception of the organic acids). Hydrogen was tested as an electron donor by incubating unamended (yeast extract) plates using the Oxoid Anaerobic System. All other plates were incubated in the Oxoid Anaero*Gen* system (section 2.1.2), and all plates were incubated at 30°C. Plates were inspected for growth after six weeks, re-incubated, and inspected again after a further two weeks. Growth was assessed visually,

Isolate	Characteristics
P1	Large (1-5 mm), black, domed, entire colonies. Large (~ 3 – 6 x 0.5 μm), thick straight rods, often in pairs, terminal endospores, sometimes motile. Gram-positive.
PFB	Large (1-5 mm), black or white, domed or umbonate, entire or crenated colonies, growing deep into agarose. Large (~ 8–20 x 0.5-1 μm), thick straight rods, spores and/or vesicles sometimes present, sometimes motile. Gram-positive.
PCP	Small (0.5-3 mm), white and rough or white and smooth with black centres, domed or umbonate colonies. Short (~ 2 μm) rods/cocci, rods sometimes clubbed, non-spore-former, non-motile. Gram-positive.
M1	Large (1-5 mm),black, domed, entire colonies. Large, thick rods, straight to curved, ~ 3–5 x 0.5 μm, terminal endospores, occasionally motile. Sometimes large oval/pear shaped cells with central or terminal endospores. Gram-positive.
MCP	Small (0.5-3 mm), white or white with black centres, domed or umbonate colonies. Clusters of cocci or club-shaped rods, non-spore-former, non-motile. Gram-positive.
MR	Small (0.5-3 mm), dark brown (occasionally white), rough, effuse/flat or slightly raised, irregular or crenated colonies. Large (~ 5-7) μm thin rods, terminal spores, non-motile. Gram-positive.
CC1	Large (1-5 mm), black, domed, entire colonies. Large, thick rods, straight to curved, ~ 3–5 x 0.5 μm, terminal endospores, occasionally motile. Sometimes large oval/pear shaped cells with central or terminal endospores. Gram-positive.

Table 6.1. A summary of colony/cell morphologies and Gram-stain results for aSRB and other isolates obtained from mixed bioreactor cultures.

(a)



(b)

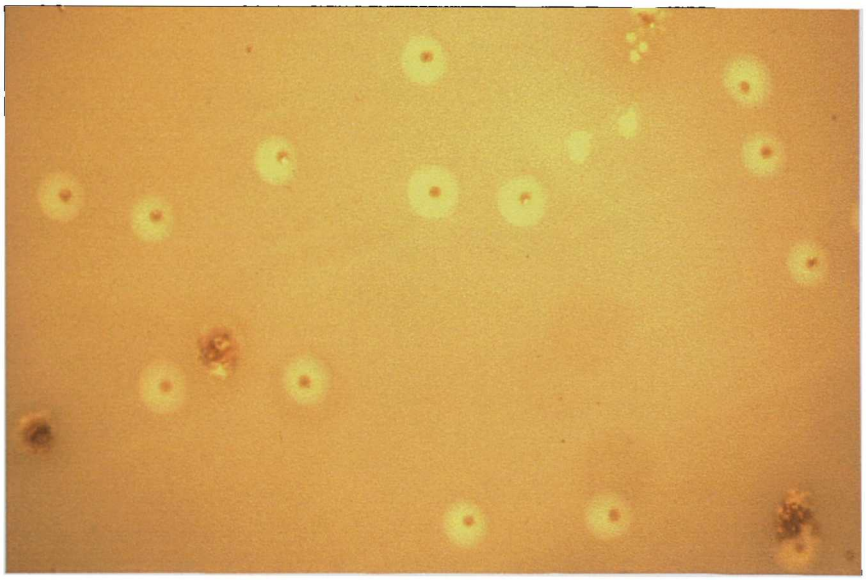
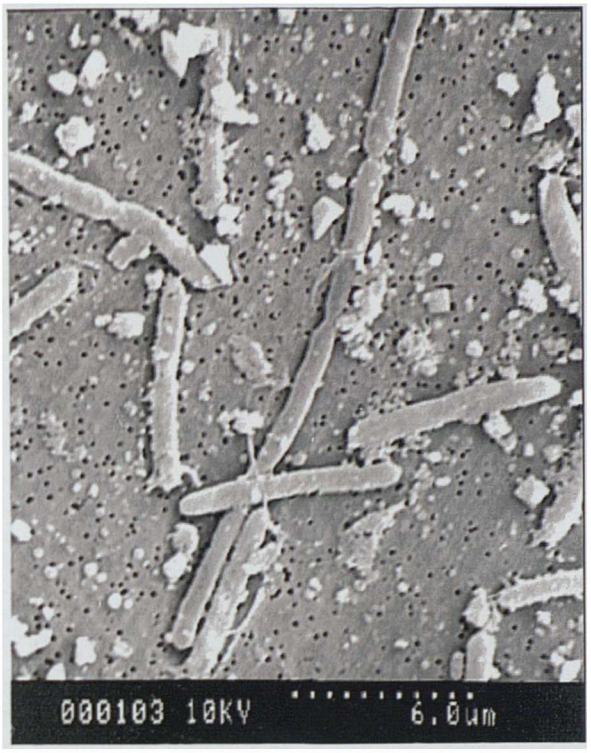


Figure 6.1 (a) Colonies of isolates P1 (large black colonies) and PCP (smaller colonies with dark centres); magnification X 16. **(b)** Colonies of isolates MCP (white with dark centre) and MR (brown/black); magnification x 20.

(a)



(b)

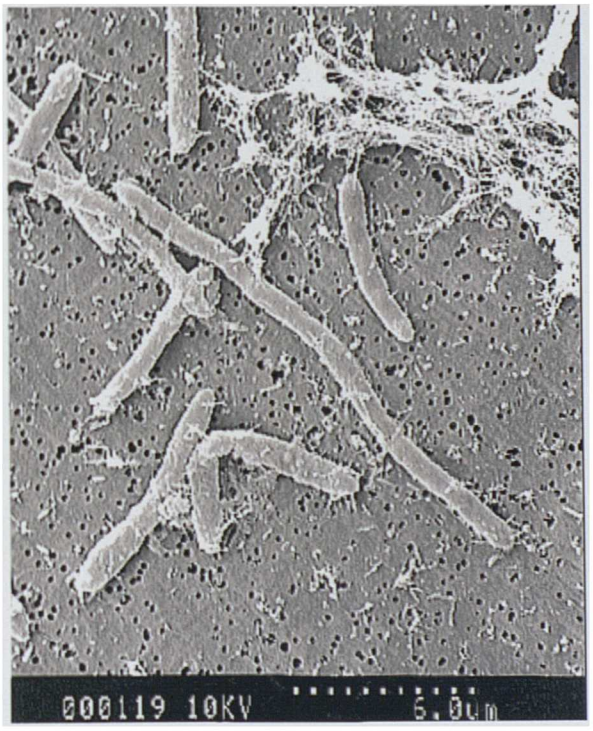


Figure 6.2 Scanning electron micrographs of the isolates (a) P1, and (b) PFB. The scale (in µm) is shown below each micrograph.

by comparing colonies on plates containing potential organic substrates with control plates containing 0.002% yeast extract. Each test was performed in duplicate.

6.3.2 Results

The results of the substrate utilisation experiment are summarised in Table 6.2. The aSRB isolate P1 appeared to grow best on the lower molecular weight alcohols and hydrogen, although some growth occurred with lactic and pyruvic acid, and glycine. Isolate PFB, although considered to be a sulphate reducer, grew as white colonies, though some blackening appeared around the edges of colonies grown on galactose amended medium after eight weeks of incubation. A powerful, slightly sweet odour, similar to that of low molecular weight volatile organic acids such as propionic and butyric, was characteristic of all plates on which PFB had grown well. This isolate exhibited most extensive growth on media amended with glucose, galactose and mannitol. In contrast, the other putative Parys aSRB (isolate PCP) did not show appear to grow on any of the substrates tested, except ribose. However, isolate PCP grew on white colonies on ribose medium rather than the characteristic "white with black centre" colonies seen on 0.02% yeast extract medium, though cell morphologies were identical in both cases. The Montserrat (aSRB) isolate M1 grew best on medium containing mannitol, though enhanced growth (relative to the control plates) was also found with ethanol and glucose. In contrast, isolate MCP did not grow particularly well on any of the substrates tested, though there was evidence of limited growth on lactic acid, glycerol, ethanol and glucose. Isolate MR grew on medium containing all of the substrates tested except acetate and methanol. Its characteristic colonies were white rather than brown when grown on medium containing mannitol, glucose and ribose; brown colonies were seen in other cases where growth occurred.

Substrate	Isolate					
	P1	PFB	PCP	M1	MCP	MR
LA	⊕Ø	Ø	–	⊕	⊕	+
AC	–	–	–	–	+	–
PY	⊕	Ø	⊕	+	+	⊕
GLY	⊕	Ø	+	+	⊕	+
ETH	⊕⊕	+	+	⊕⊕	⊕	⊕
MET	⊕⊕	–	+	–	–	–
MAN	⊕⊕⊕	ØØØ	⊕	⊕⊕⊕	–	ØØØ
GLU	–	ØØ	+	⊕⊕	⊕	ØØØ
GAL	–	ØØØ	+	⊕	+	⊕
RIB	–	Ø	ØØ	–	+	ØØØ
GLN	⊕	–	+	–	+	+
H_2	⊕⊕⊕	+	⊕	⊕⊕	+	⊕

Table 6.2 Substrate utilisation in isolates from mixed aSRB cultures (from Parys and Montserrat enrichment cultures) determined by colony growth on WJB-3 overlay solid medium. LA=lactate; AC=acetate; PY=pyruvate; GLY= glycerol; ETH=ethanol; MET=methanol; GLU=glucose; GAL= galactose; RIB=ribose, and GLN=glycine. Growth was compared with a control plate to which no substrate was added: ⊕ = black colonies; Ø = white colonies; + = growth comparable with control, and - = no growth or less than observed in controls. Each test was performed in duplicate.

Overall, there was a noticeable absence of growth of all isolates on medium containing acetic acid, and growth on glycerol was less pronounced than expected from earlier work where glycerol had been found to be utilised as electron donor in pure cultures of aSRB (section 4.2.1.2).

6.4 Biomolecular characterisation of isolates

6.4.1 Methodology

The 16S rRNA gene sequences of isolates P1, PFB, PCP, MCP, and MR were obtained using the methods described in sections 2.4.2 to 2.4.9, at University of Wales Bangor (UWB). For isolates M1 and CC1 only, chromosomal DNA extraction and purification was carried out using the method described in section 2.4.1, and DNA samples of M1 and CC1 were sent to the University of Birmingham for PCR amplification and cloning of their 16S rRNA genes, as well as plasmid DNA purification and sequencing. Partial sequences of the 16S rRNA genes were obtained (~ 700 b.p.), and the closest known relatives were determined using BLAST (section 2.4.9) at UWB. To investigate the relatedness of the four *Desulfosporosinus*-like organisms to each other, sequences were subjected to a 2-way BLAST alignment for each combination, and percentage sequence identities were generated.

6.4.2 Results

The results of the BLAST similarity searches are summarised in Table 6.3. Isolates P1, PFB, M1 and CC1 were found to be members of the low G+C branch of the Gram positive bacteria, and were phylogenetically all members of the family *Peptococcaceae*, moderately related to the strain "Blif" of the recently renamed genus *Desulfosporosinus* (Stackebrandt *et al*, 1997). Isolate PFB was most closely related to strain "S8", showing a higher (97%) sequence identity.

The phylogenetic trees obtained for the aSRB strains are shown in Figures 6.3 and 6.4, and in Figure 6.9 with the other isolates. The sequence information for M1 and CC1 was obtained around eighteen months prior to that of the other isolates, and therefore Figure 6.3 shows a more general tree showing relationships between these isolates and a wide range of sulphate reducers (Hallberg *et al.*, unpublished data). Figure 6.4 shows a phylogenetic tree constructed using sequences of P1 and PFB, with others known to be related to *Desulfosporosinus*, i.e. members of *Desulfotomaculum* and *Clostridium*. *Bacillus subtilis* was included for comparison. Results of the multi-sequence alignments for the partial sequences of isolates M1 and CC1 with those of isolates P1, PFB and the type strain of *Desulfosporosinus orientis* are shown in Figure 6.5. This displayed large areas of sequence similarity, demonstrating that these strains are almost identical to each other and, as isolates P1 and PFB, are most closely related to sequenced bacteria of the genus *Desulfosporosinus*. Table 6.4 shows the relatedness of the *Desulfosporosinus*-like organisms to each other. It shows that isolates P1, M1 and CC1 show high 16S rRNA sequence relatedness to each other (99%), while PFB shows low sequence relatedness to all of the other three isolates (92% with isolates M1 and CC1, and 94% with isolate P1).

Isolates MCP, PCP and MR, which were originally considered (from colony morphologies) to be possible sulphate reducing bacteria) were, in fact, found to be most closely related (on the basis of comparative 16S rDNA sequences) to *Luteococcus* sp. CCUG 38120T, *Cellulomonas gelida* and *Bacillus niacini*, respectively. Isolates MCP and PCP were identified as members of the high G+C Gram positive bacteria, but exhibited only 93% and 94% sequence identity with their respective closest relatives.
Figure 6.6 is a phylogenetic tree showing relationships between isolates MCP and related organisms, rooted with *Streptomyces scabies* as an outgroup. Multi-sequence alignments (with sequences producing significant alignments from the BLAST search), and subsequent

phylogenetic analysis and tree construction show that MCP branched alone but grouped with a cluster containing *Luteococcus* sp. CCUG38120 and *L. japonicus.* Figure 6.7 shows phylogenetic relationships between isolate PCP and related organisms. Whilst the BLAST search revealed the closest relative of PCP to be *Cellulomonas gelida* (Table 6.3), multi-sequence alignments and tree construction revealed that PCP branched alone but clustered with *C. flavigena* (the type strain for the genus) and *Cellulomonas* sp. 792, separately from *C. gelida* and *C. uda.* Figure 6.8 shows the phylogenetic relationship of the isolate MR with its closest related organisms. The BLAST search assigned *Bacillus niacini* as the closest known relative to MR, but multi-sequence alignments and phylogenetic analysis resulted in a tree in which MR branched alone but clustered with *B. niacini* and *B. pseudomegaterium.* Figure 6.9 is a tree showing relationships between all of the isolates.

Isolate	Source	Nearest neighbour*	Family	% Homology	Reference
P1	Parys Mt.	*Desulfosporosinus* sp. Blif 16S rRNA gene	Peptococcaceae	95	Stackebrandt *et al.*, 1997
PFB1	Parys Mt.	*Desulfosporosinus* sp. S8 16S rRNA gene, partial sequence	Peptococcaceae	97	Robertson *et al.*, 2000
M1	Montserrat, W.I.	*Desulfosporosinus* sp. Blif 16S rRNA gene, (through 700 b.p.)	Peptococcaceae	94	–
CC1	Cae Coch mine	*Desulfosporosinus* sp. Blif 16S rRNA gene (through 700 b.p.)	Peptococcaceae	95	–
PCP	Parys Mt.	*Cellulomonas gelida*	Cellulomonadaceae	94	Rainey *et al.*, 1995
MCP	Montserrat, W.I.	*Luteococcus* sp. CCUG38120^T	Propionibacteriaceae	93	Collins *et al.*, 2000
MR	Montserrat, W.I.	*Bacillus niacini*	Bacillaceae	96	Goto *et al.*, 2000

Table 6.3 A summary of the isolates obtained from mixed aSRB cultures. * The nearest neighbour was determined by conducting a BLAST search (Altschul *et al.*, 1997) on the National Centre for Biotechnology Information (NCBI) database.

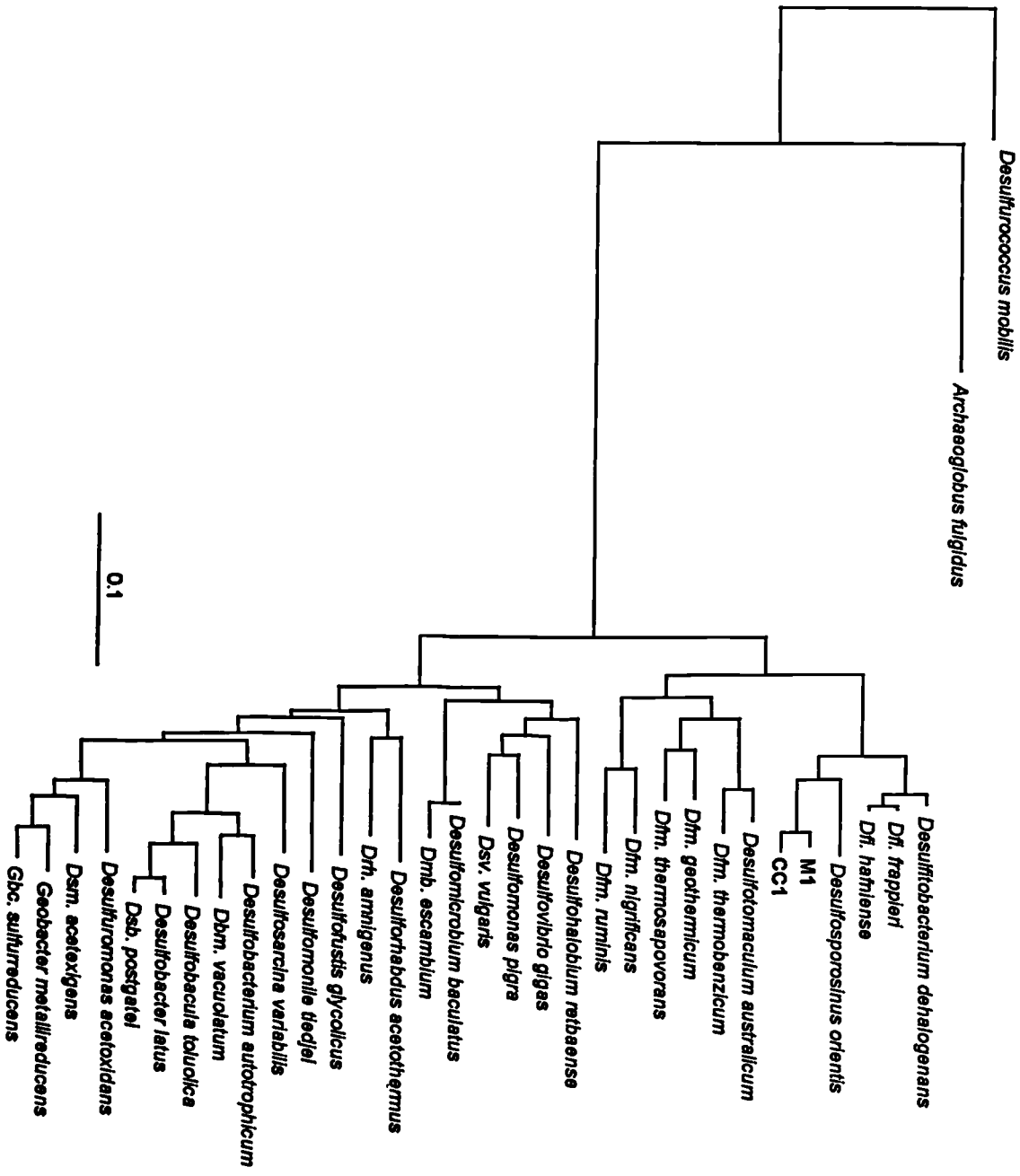


Figure 6.3 A phylogenetic tree of selected sulphate reducing microorganisms including aSRB isolates CC1 and M1. (Hallberg *et al.*, unpublished data). Branch lengths are proportional to the calculated evolutionary distances. The bar indicates 0.1 substitutions per site.

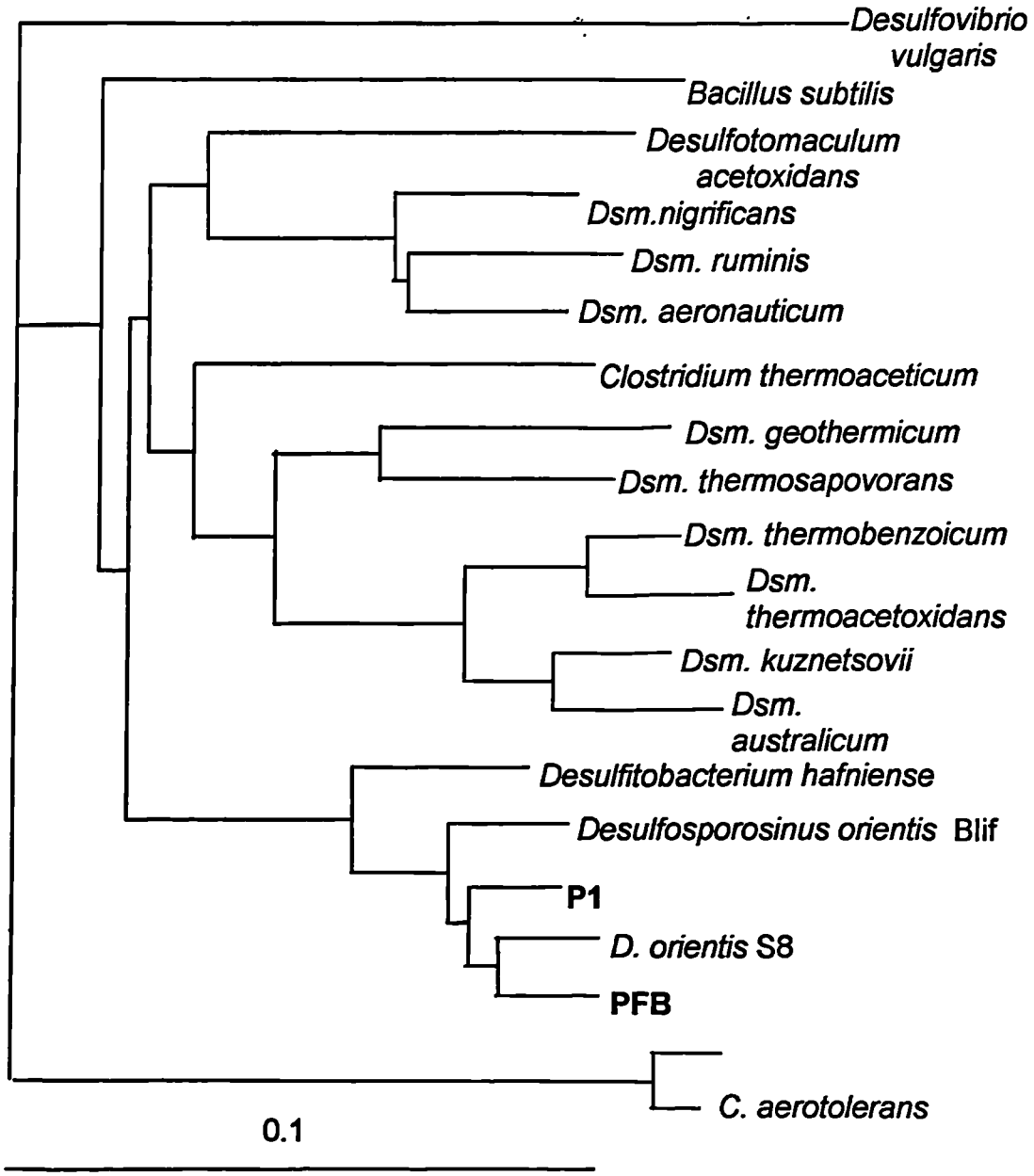


Figure 6.4 Phylogenetic relationships of isolates P1 and PFB and other selected SRB (*Desulfosporosinus* and *Desulfotomaculum*) and *Clostridium* spp. *Bacillus subtilis* is included for comparison, and the tree was rooted using the SRB *Desulfovibrio vulgaris*. Branch lengths are proportional to the calculated evolutionary distances. The bar indicates 0.1 substitutions per site.

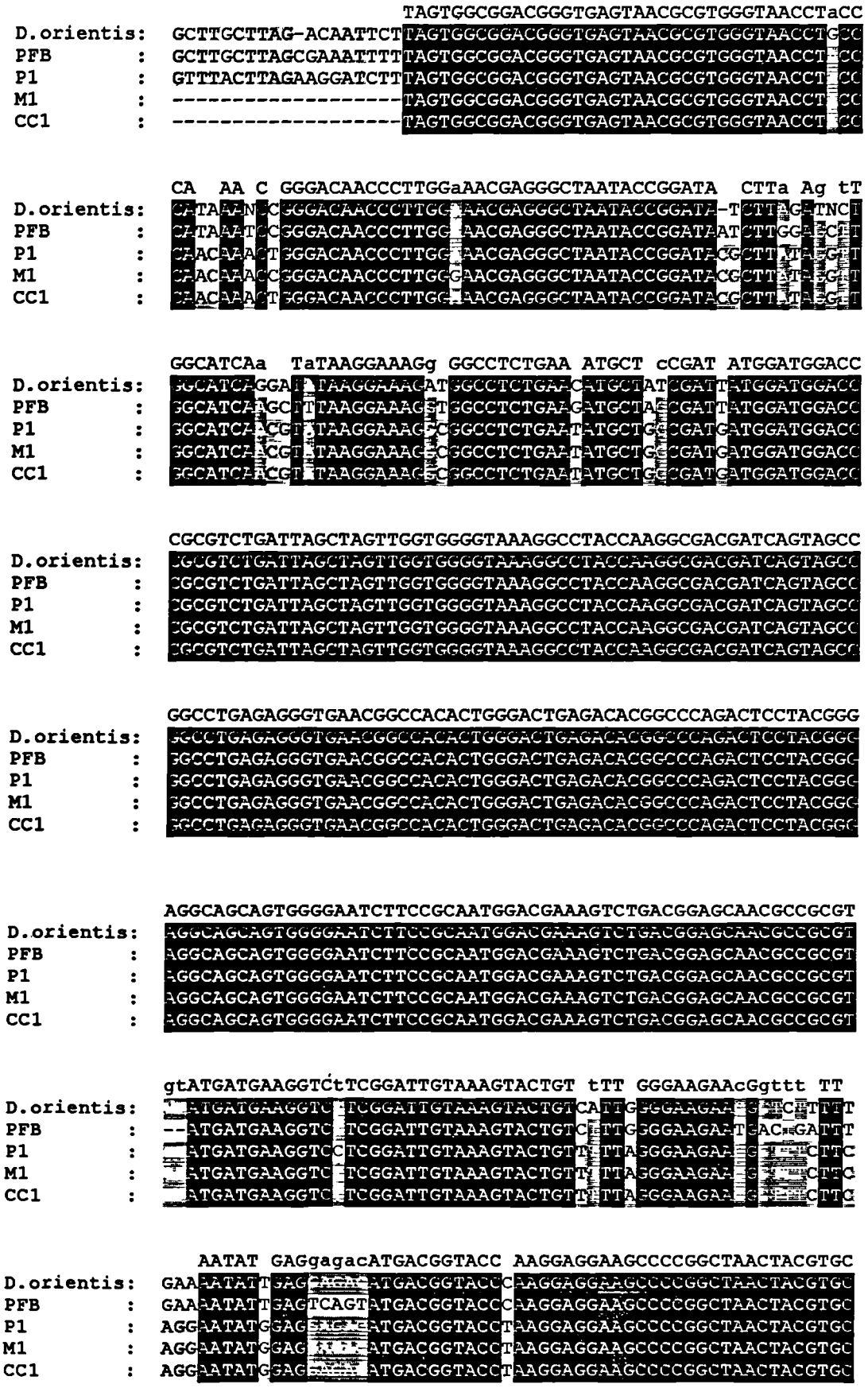


Figure 6.5: continued overleaf

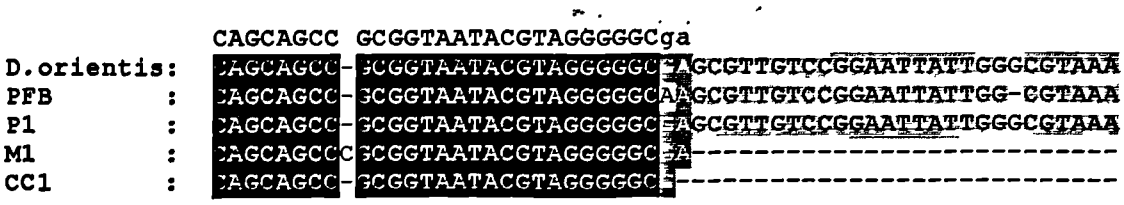


Figure 6.5. A comparison of partial 16S rRNA sequence data for aSRB isolates PFB, P1, M1 and CC1 with *Desulfosporosinus orientis* (type strain). All the isolates are closely related (areas shaded in black) but M1 and CC1 are almost identical over the region of ~ 500 b.p.

Isolate	M1	CC1	P1	PFB
M1	_	99	99	92
CC1	99	_	99	92
P1	99	99	_	94

Table 6.4 The results of alignments of the available 16S rRNA sequences from the *Desulfosporosinus*-like aSRB isolates using 2-way BLAST. Numbers shown are the resulting percentage sequence identities.

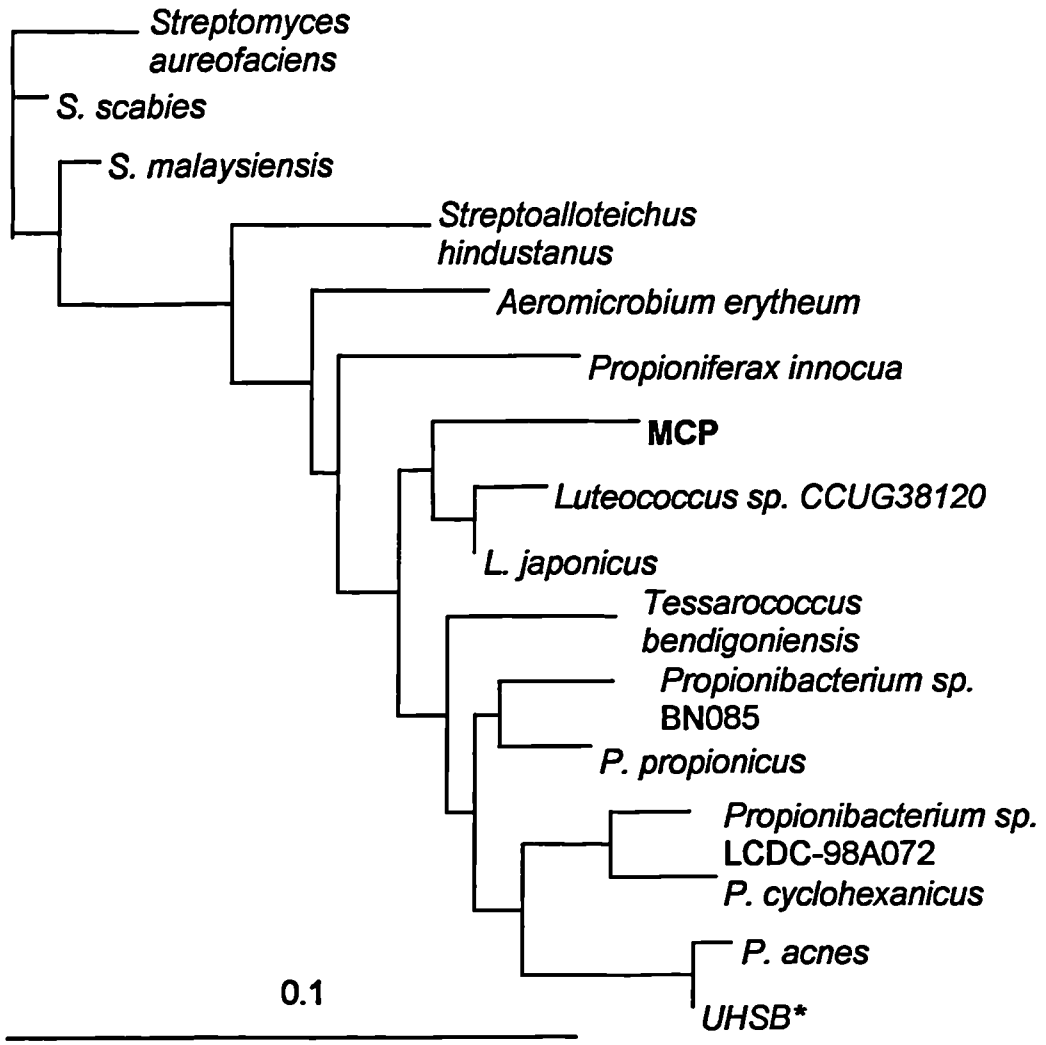


Figure 6.6 Phylogenetic tree showing relationships between isolate MCP and related organisms, rooted with *Streptomyces scabies* as an outgroup. Branch lengths are proportional to the calculated evolutionary distances. The bar indicates 0.1 nucleotide substitutions per site. (*UHSB = Uncultured hydrocarbon seep bacterium.)

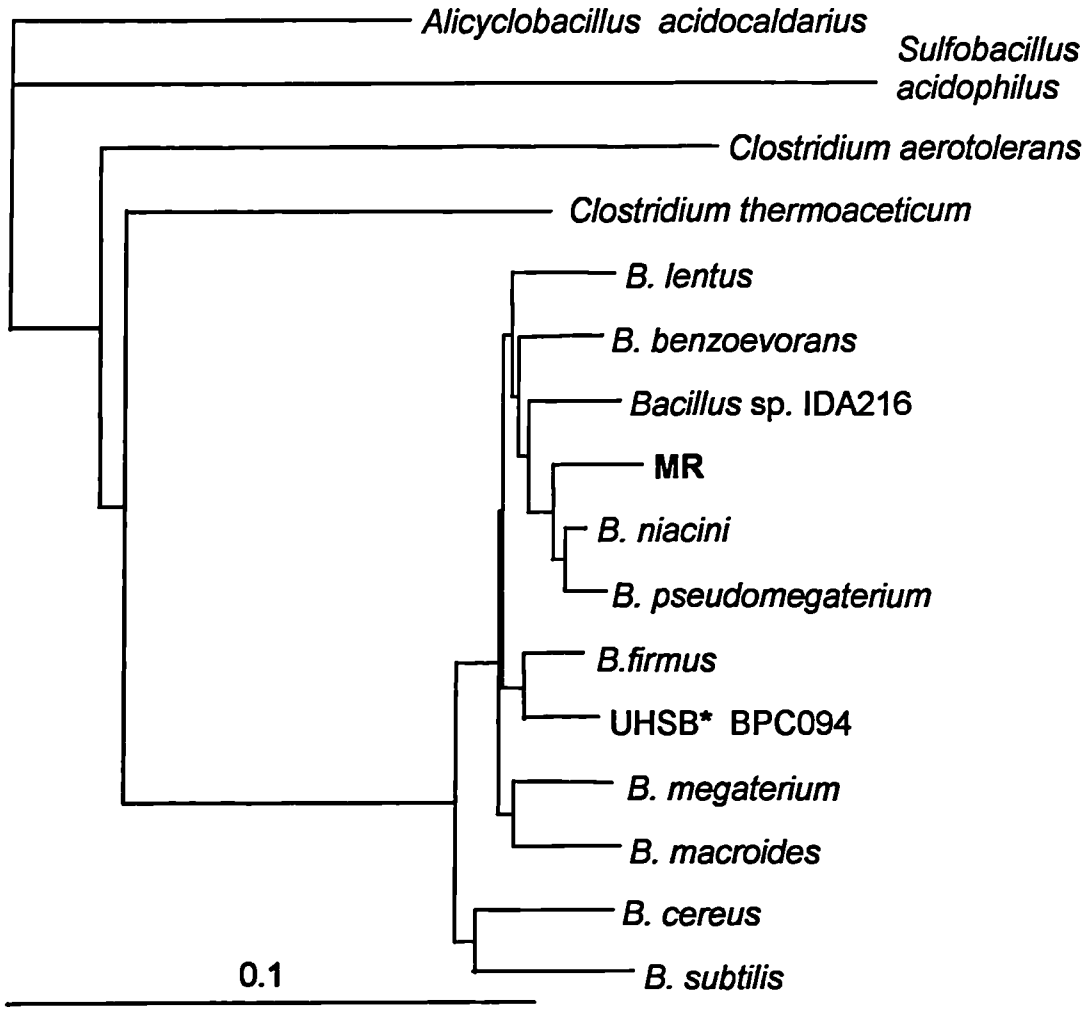


Figure 6.7. Phylogenetic tree showing relationships between isolate MR and related organisms, rooted with *Sulfobacillus acidophilus* as an outgroup. Branch lengths are proportional to the calculated evolutionary distances. The bar indicates 0.1 nucleotide substitutions per site. (*UHSB = Uncultured hydrocarbon seep bacterium.)

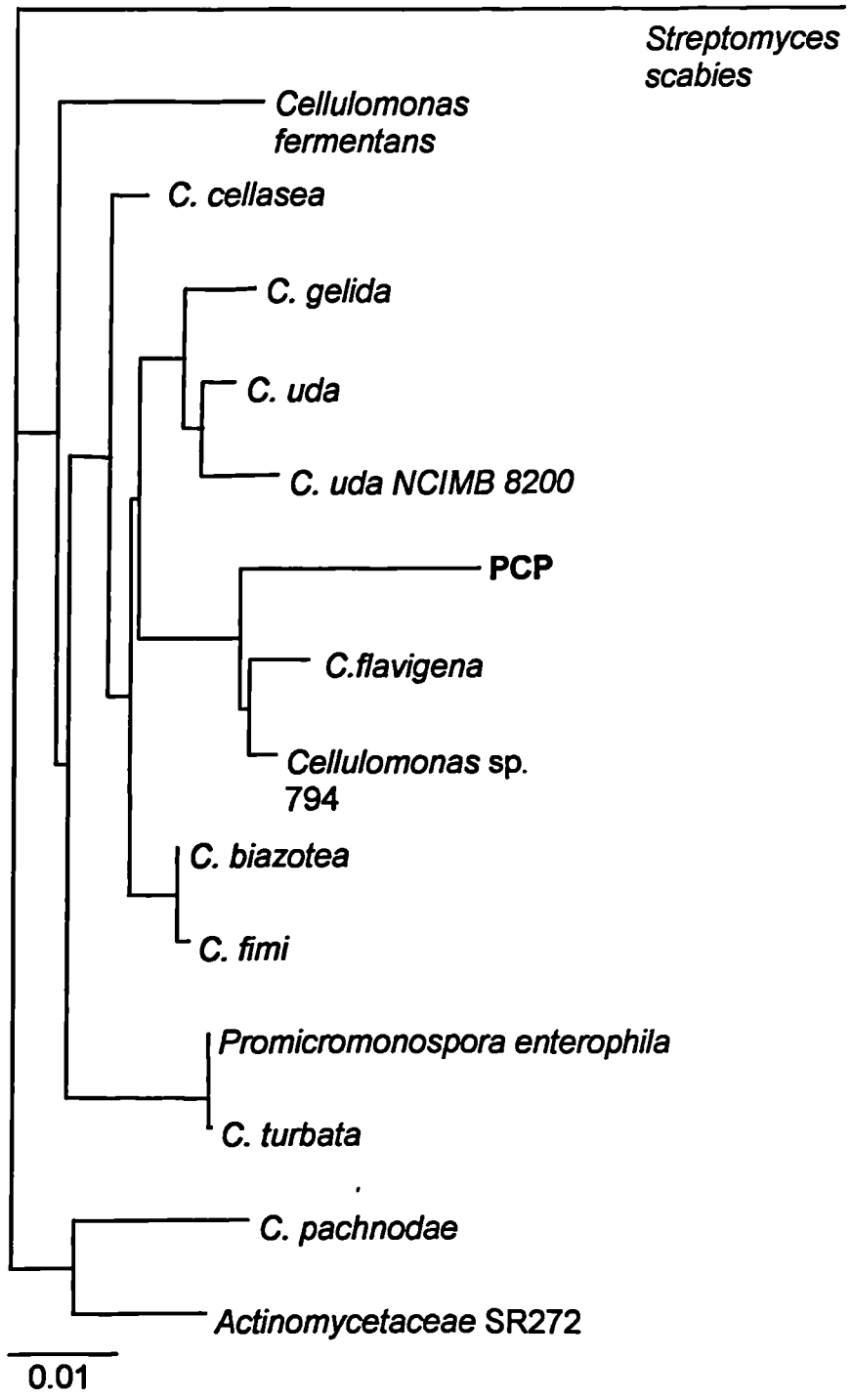


Figure 6.8 Phylogenetic tree showing relationships between isolate PCP and related organisms, rooted with *Streptomyces scabies* as an outgroup. Branch lengths are proportional to the calculated evolutionary distances. The bar represents 0.01 substitutions per site.

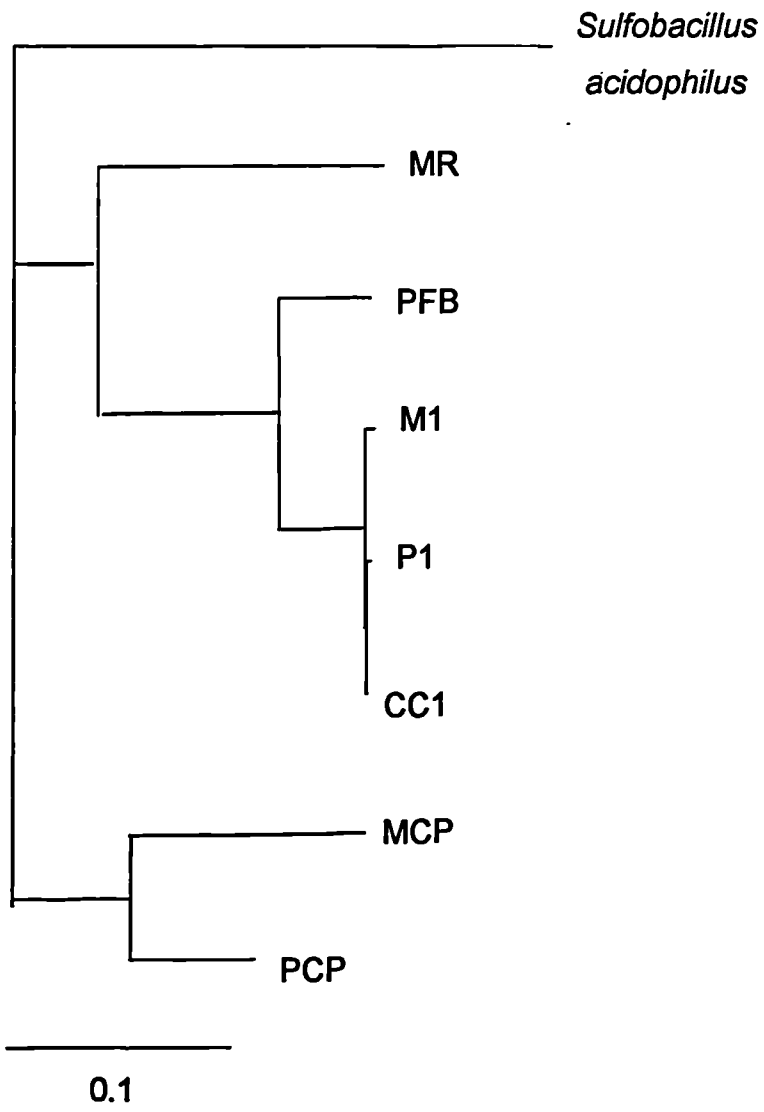


Figure 6.9 Phylogenetic tree to show relationships of all the isolates obtained from the acidophilic bioreactor cultures, using alignments of sequences of ~ 500 base pairs (as only partial DNA sequences were obtained for M1 and CC1). The tree is rooted using *Sulfobacillus acidophilus* as an outgroup. Branch lengths are proportional to the calculated evolutionary distances. Bar represents 0.1 substitutions per site.

6.5 Discussion and Conclusions

Seven novel isolates were obtained from the three mixed aSRB enrichment cultures of Parys, Cae Coch and Montserrat samples. Isolates that formed colonies that were black or black/brown coloured were selected for experimental purposes and characterised on the basis that they were likely to be acidophilic/acid tolerant sulphate reducers. Observations of colony and cell morphologies led to the initial conclusion that isolates P1, M1 and CC1 were very similar organisms: the sizes, morphologies and colours of their colonies on yeast extract overlay plates were identical, and their cellular characteristics were also very similar. Initially these isolates were thought to be similar to the Gram positive sulphate reducers of the genus *Desulfotomaculum*. In a previous study, Johnson *et al.* (1993) observed a similar, spore-forming *Desulfotomaculum*-like bacterium in a culture that reduced sulphate in growth medium initially poised (initially) at pH 2.9. Isolate PFB was also *Desulfotomaculum*-like in appearance, but was a considerably larger bacterium than P1, M1 and CC1, and colonies of PFB were often white rahter than black, which was not usually the case for the other isolates. Colonies of PFB tended to grow deeper into the agarose than other isolates. Isolates MCP and PCP also appeared to be very similar to each other from both colony (white with black centre) and cell (club-shaped rods/coccoid) morphology, although one contrasting feature was that PCP sometimes appeared as short rods which were not club-shaped. Cells of MCP were seen to aggregate in clusters and were either spherical or club-shaped. Isolate MR had the appearance of a classic bacillus; colonies of this isolate sometimes varied in colour, appearing usually as dark brown but being occasionally white.

Isolates P1, PFB, M1 and CC1 were all found to be most closely related phylogenetically to the genus *Desulfosporosinus* (Stackebrandt *et al.*, 1997). Bergey's Manual of Systematic Bacteriology (2nd ed.) lists

Desulfosporosinus to be in the family *Peptococcaceae* of the *Clostridia*, among the low G+C Gram positives. These isolates stained Gram positive, although Stackebrandt *et al.* (1997), in their description of the genus, state that *D. orientis* is a Gram-negative, curved rod that has a multilayered wall structure. It is difficult to explain this conflicting evidence, and may be due to the occasional ambiguity of the results obtained from Gram staining.

Due to the difficulty of obtaining and/or maintaining cultures of the aSRB and other isolates in liquid media (Chapter 4), it was not possible to use liquid cultures to test for substrate utilisation, as a "positive control" could not be guaranteed. Another experimental method was required, and use of solid media, where test substrates were incorporated into plates containing *Acidocella* WJB-3 in the gel underlayers proved to be successful. The rationale, which appeared to be correct, was that *Acidocella* WJB-3 would, as *Acidiphilium* SJH, 'detoxify' the solid media, thereby promoting growth of the aSRB but, unlike *Acidiphilium* SJH, *Acidocella* WJB-3 is unable to grow on most of the test substrates (including 'simple' sugars, such as glucose; Hallberg, 1998, unpublished data). In addition, *Acidocella* WJB-3 is an obligate aerobe, and would therefore not be active in the anaerobic systems used in these experiments. The substrate test revealed both some similarities and differences between the aSRB isolates. The patterns of growth of isolates P1 and M1 were generally the same, although M1 did not grow on medium amended with methanol or glycine, whilst P1 did. However, isolate P1 did not grow on glucose- and galactose-amended media, in contrast to isolate M1. Both P1 and M1 showed enhanced growth (relative to control plates) in hydrogen-containing jars, presumably by metabolising as a chemoautotroph,using hydrogen as electron donor, sulphate as electron acceptor, and carbon dioxide as carbon source. Klemps *et al.* (1985), in a study of *Desulfotomaculum* spp., found that, in contrast to other bacteria tested, *Desulfotomaculum orientis* (as it was then known), was also able to grow chemoautotrophically. This SRB was also able to grow in the absence of sulphate with a range of substrates,

forming acetate, leading to the conclusion that it should be considered a homoacetogenic bacterium (Klemps *et al.*, 1985). However, neither isolate P1 nor M1 grew on the solid medium containing acetic acid. It is possible this is a general feature of organic acids, rather than relating specifically to acetic acid. As suggested in section 4.1, acidophilic strains of SRB may not be able to utilise organic acids when present at concentrations used normally in growth media, due to the general toxicity of these compounds to acidophiles (Ingledew, 1982). However, some growth of all isolates (although not much greater than in controls) was observed on plates containing 0.5 mM pyruvic acid, and also (with the exception of PCP) 0.5 mM lactic acid. Only isolate MCP grew on plates containing acetic acid. Isolate PFB was noted to grow usually (on 0.02% yeast extract overlay plates) as black colonies, similar to those of isolates P1, M1, and CC1, but in the substrate test it grew as large white colonies, and all of the positive plates had a strong odour which was considered to be either propionic or butyric acid. Some bacteria which are relatively closely related to PFB (*Clostridium propionicum* and *C. butyricum)* ferment hexoses to propionate + acetate, and butyrate + acetate, respectively. The greatest amount of growth of isolate PFB was seen on medium containing mannitol (an sugar-alcohol with the formula $C_6H_8(OH)_6$), glucose or galactose, and some (less pronounced) growth was observed on ribose. This led to the conclusion that PFB is a facultative sulphate reducer which grows by fermentative metabolism in some circumstances, though further experimental work is required to confirm this. BLAST search results showed that PFB is more closely related to the sulphate reducer *Desulfosporosinus* strain S8 (97%), than are P1, M1, and CC1 to their most closely-related sequenced SRB, *Desulfosporosinus* Blif (95, 94 and 95% respectively). The energy requirements for the reduction of sulphate (section 1.7.4.1) may mean that PFB carries out fermentation preferentially and only uses sulphate to oxidise non-fermentable substrates.

Isolate PCP exhibited some growth on all the substrates tested apart from lactic and acetic acids. The most extensive growth of this isolate was seen on medium containing ribose, and, in this case, white colonies were seen rather than the characteristic white/black centred colonies seen on yeast extract plates. Figure 6.8 shows that PCP is most closely related to the genus *Cellulomonas*, clustering with *Cellulomonas flavigena* (type strain), and *Cellulomonas* sp. 794. Cellulomonads can be cellulolytic, e.g. in composting of pulp and paper mill primary solids (Atkinson *et al.*, 1997). They display both respiratory and fermentative metabolisms, and *C. flavigena* can also reduce nitrate (Bagnara *et al.*, 1985). The description of *C. humilata* (Collins and Pascual, 2000) bears some similarity to PCP: cells are predominantly filamentous and branched and have swollen ends, stain Gram positive, and after prolonged incubation they fragment into diptheroid or coccoid elements of varied size and shape. Mature colonies are small, opaque, smooth, entire and convex with a dark central region; rough colony variants occur occasionally. It is microaerobic to aerobic, with poor or no growth in anaerobic conditions (in contrast to PCP), requiring organic nitrogen to grow, and ferments sugars, producing lactic acid as a major end product.. It is not known whether or not isolate PCP can ferment sugars, although it is interesting to note that the best growth observed was on ribose (a fermentable sugar) medium. It is seems likely, in view of the phylogenetic data, that this isolate is not, as had been supposed, a sulphate reducing bacterium, and that the black colour of colony centres when the organism was grown on yeast extract overlay plates was due to the formation of sulphide from thiols (amino acids etc.) in yeast extract, rather than via reduction of sulphate.

Isolate MR also exhibited differences in growth patterns on different substrates. There was substantial growth (as white colonies) on mannitol-, glucose- and ribose-amended media, whilst the bacterium grew as dark brown colonies (as on yeast extract plates) on galactose, pyruvate, ethanol and hydrogen. *Bacillus spp.* are generally obligately or

facultatively aerobic (e.g. the related organisms *Bacillus megaterium* and *B. cereus*), and only one species is documented that can only grow anaerobically. *Bacillus infernus* is described as a strict anaerobe that grows on formate or lactate, with Fe (III), MnO_2, trimethylamine oxide or nitrate (reduced to nitrite) as electron acceptors, and it grows fermentatively on glucose (Boone *et al.*, 1995). The isolate MR may be a similar organism. Since the most extensive anaerobic growth of isolate MR was seen on glucose, ribose and mannitol plates, this might imply that it was fermenting these substrates. This isolate was found to be most closely related to *Bacillus niacini*, a nicotinate-metabolising mesophile isolated from soil.

Isolate MCP did not display substantial growth on any of the substrates tested, but growth was only inhibited on media amended with either methanol or mannitol. This bacterium was found to be most closely related to *Luteococcus peritonei* CCUG 38120^T (Collins *et al.*, 2000), the cells of which are described as pleomorphic, non-pigmented Gram-positive rods which are facultatively anaerobic. Another of the organisms related to isolate MCP belongs to the genus *Propionibacterium* (Figure 6.9), which ferment lactate to propionate + acetate + CO_2. One other bacterium related to isolate MCP is *Tessarococcus bendigoniensis*. This isolate grows as cocci or clusters of cocci, and is a non-motile, facultative anaerobe, capable of reducing nitrate, with an optimum of 25°C (Maszenan *et al.*, 1999). It could be inferred from this information that, like MR, isolate MCP is possibly a nitrate reducer and/or fermenter.

There are no reports that any of the bacteria identified, from phylogenetic analyses, to be related to MCP, PCP, or MR have the capacity to reduce sulphate to sulphide. Whilst this might be due to them never having been examined for this ability, this would seem to be unlikely. It could be speculated that one or all of the isolates MCP, PCP and MR have some kind of syntrophic relationship with the aSRB in the mixed cultures. A

syntrophic reaction usually involves hydrogen gas being produced by one partner in the syntrophic relationship and being consumed by the other, and as such, syntrophy has also been called "interspecies H_2 transfer". The aSRB isolates P1 and M1 appear to be able to use H_2, which can be produced in a number of fermentation reactions, e.g. by the fermenter *Clostridium butyricum*, which, as mentioned above, ferments hexoses, resulting in the formation of butyrate, acetate, H_2, and CO_2. It is possible that the aSRB may be able to make use of such relationships in the mixed microbial community, particularly if exploiting them is more energetically favourable than utilising available electron donors directly. Another possibility is that whilst the isolates MCP, PCP and MR may be able to grow anaerobically (as seen in experiments with solid media incubated in anaerobic systems), but in a mixed culture containing aSRB, they may even be microaerophilic and "scavenge" oxygen which is present at low concentrations, creating more favourable conditions for the aSRB. However, further characterisation of the isolates would be required in order to substantiate any of these possibilities, and development of methods and suitable growth media for experimental purposes would be required.

Due to certain physiological, phenotypic and genetic differences between *Desulfotomaculum orientis* and other members of the genus, Stackebrandt *et al.* (1997) proposed that it should be removed, and proposed a new genus, *Desulfosporosinus*, for this organism. Stackebrandt *et al.* (1997) showed that *Desulfosporosinus orientis* strains clustered with species of *Desulfitobacterium*; these were collectively well separated from the other clusters of *Desulfotomaculum* species. Figure 6.3 and 6.4 show that this was confirmed for isolates PFB, P1, M1 and CC1, which all grouped with strains of *D. orientis* and species of *Desulfitobacterium*. Whilst only partial sequences were obtained for the isolates M1 and CC1, enough data were obtained to conclude that they are almost identical organisms (Figure 6.5). Phylogenetic affiliations allow reasonable inferences concerning the physiology of species. As all the aSRB isolates are most closely related to

D. orientis, it is not surprising that there appear to be some shared characteristics such as cell morphology and utilisation of some substrates, particularly in the cases of P1, M1 and CC1. Isolate PFB appears to be quite unrelated to the other three organisms as it showed low sequence identities with all of them, although it appeared to have marginally higher sequence relatedness to P1 than M1 and CC1. Despite the similarities in colony and cell morphology exhibited by M1 and P1, there were some clear differences in the substrates that they utilised. Isolate CC1 was not tested due to active cultures being unavailable at the time of the experiment, but it can be speculated that it may have similar growth characteristics to M1 and P1, as all three exhibited 99% sequence relatedness to each other. The sequences for the isolates '*Desulfosporosinus* sp. P1' and '*Desulfosporosinus* sp. PFB' were submitted to GenBank on the NCBI database and assigned the accession numbers AY007666 and AY007667 respectively.

The fact that all of the aSRB and putative sulphate reducers that have been isolated from the mixed cultures are Gram-positive may not be surprising, because in contrast to Gram-negative SRB, the Gram-positive, spore-forming SRB are more resistant to compromising environmental conditions such as pH and temperature shifts, elevated oxygen concentrations, and desiccation (Campbell and Postgate, 1965; Widdel, 1992a). In addition, they may be more tolerant of fluctuations in nutrient and sulphate availability. For example, Sass *et al.* (1997) reported that *Desulfotomaculum* spp. were dominant in the deeper layers of an oligotrophic lake, and Raskin *et al.* (1996) suggest that the presence of *Desulfotomaculum* spp. in the absence of sulphate may be explained by their ability to function as acetogens and/or fermenters.

Characterisation of the aSRB isolates was also hoped to include G + C content, identification of bisulphite reductases, presence/absence of desulfoviridin or cytochrome c_3, and fatty acid composition. These were

not carried out due to time constraints and the difficulty of growing pure cultures. However, the growth substrate test and sequencing of the 16S rRNA genes of the novel acidophilic isolates did, however, allow for identification and comparative investigation. This approach was a useful starting point for unknown species that have not previously been cultured or isolated for lack of suitable methodologies.

7. FINAL DISCUSSION AND CONCLUSIONS

Acid mine drainage effluents are one of the most widespread forms of pollution worldwide, and their effects can be acute and catastrophic (for example, the Los Frailes disaster in Southern Spain; section 1.3), or chronic, causing gradual depletion of flora and fauna. The effluents typically contain high concentrations of metals, some of which are more toxic to aquatic life at low pH; for example, dissolved aluminium prevented growth of salmonid fish at concentrations of >0.1 mg l^{-1} at pH <5.4 (Milner and Varallo, 1990). There are a number of methods of treatment available, (physical, chemical and biological, active and passive; section 1.4), but bioremediation (active biological treatment) is advantageous due to its potential for efficiency and low environmental impact. Neutrophilic SRB are currently employed in such biological treatment systems (e.g. Thiopaq®; section 1.5.2), but acidophilic/acid tolerant SRB, if found in acidic environments, have the potential to present a cost-effective alternative as they would be more tolerant of the low pH of acidic effluents.

Acidophilic SRB, i.e. SRB that have a growth optimum for growth of pH 4 or below, have not yet been described. This research programme aimed to isolate cultures of these bacteria from samples taken from three AMD environments at pH ~ 3, to examine their potential for bioremediation of AMD effluents, and also to investigate the nature of the bacteria, which were likely to be novel organisms. Enrichments of aSRB were made in bench-scale bioreactors with an upper limit of pH 4. In those enrichments in which sulphate reducing activity occurred, alkalinity generation was demonstrated to a lower pH limit of 1.7 – 2.5. This suggested that the SRB were either acidophilic or very acid-tolerant, as the pH optimum for growth of SRB is between pH 5 and 9 (Postgate, 1984), though they usually grow best over a relatively restricted pH range (7.2-7.8; MacFarlane and Gibson, 1991). Initial experiments demonstrated that the use of surfaces (glass beads) for attachment of the aSRB, as well as the use of sodium

sulphide as a reducing agent, promoted growth in liquid culture. One strain of aSRB was obtained from each of the AMD environments. Initial observations were that they were all spore-forming oval or curved rods. Three strains of acidophilic/acid-tolerant bacteria were isolated from the enrichment cultures which were not sulphate reducers (one from each AMD sample), growing as white colonies under anaerobic conditions. However, two of these strains grew on solid medium (with oxidation of ferrous iron in one case) under microaerobic conditions. This suggested that these bacteria were facultative anaerobes, which may have had important relationships with aSRB in the mixed cultures. For example, they might use residual oxygen for metabolic functions, or ferment substrates under anaerobic conditions, thereby providing more favourable conditions and/or substrates for the aSRB.

Liquid and solid media were developed and modified for growth of aSRB (Chapter 4). Mixed cultures grew in large (≥100 ml) batch cultures in filled, tightly capped containers. Pure cultures only grew in small (~20 ml) batch cultures which were incubated in anaerobic jars, and even this was not always reproducible. This again suggests that there may have been syntrophic relationships in the mixed cultures (for example, with non-SRB) that helped to promote sulphate reducing activity. It was recognised that physiological parameters such as substrate utilisation would need to be tested using a different method, i.e. solid medium (Chapter 6). However, other experiments were carried out with liquid cultures. It was demonstrated that the three pure aSRB strains required yeast extract (at a minimum concentration of 0.001 – 0.002%) to obtain significant growth (section 4.2.1.2). This experiment demonstrated utilisation of sulphate and glycerol with concomitant production of acetate, alkalinity and biomass. It was concluded that, like the closely related *Desulfotomaculum* species, strains M1, CC1 and P1 oxidised glycerol incompletely, producing acetic acid (sections 1.7.3 and 1.7.4.3). Addition of vitamins also promoted growth of these isolates in liquid cultures, indicating that they required one

or more growth factors, which might be perceived as a disadvantage in terms of their potential use in a bioremediation system. In ferrous iron-containing medium, cultures grew with a concomitant increase in pH to around neutral, and therefore other media were developed in an attempt to increase buffering capacity. A small number of aSRB cultures grew in zinc sulphate-containing medium, but only after prolonged incubation. This suggested that the aSRB strains are more likely to be acid-tolerant rather than acidophilic. Without a reliable method of growing aSRB, specific growth rates at different pH values could not be measured, and therefore the acidophily of the pure and/or mixed cultures could not be quantified.

Solid media based on overlay medium used to grow other acidophilic bacteria (Johnson *et al.*, 1987; Johnson *et al.*, 1993; Johnson, 1995a) were modified for growth and enumeration of aSRB and other associated bacteria from bioreactor cultures and environmental samples. 'Non-overlay' medium did not support the growth of aSRB from bioreactor cultures, although there was limited growth of some aSRB and other bacteria using inocula from AMD sediments. Overlay medium, using the acidophiles *Acidiphilium* SJH or *Acidocella* WJB-3 in the lower layer of a two-phase agarose medium was noticeably more effective for growth of aSRB (Chapter 4). The role of the *Acidiphilium* SJH in the medium was difficult to define: it was either acting as a 'detoxifier' or a 'substrate provider'. The irradiation of *Acidiphilium* SJH overlay medium with UV light (which disrupts DNA) before inoculation prevented the growth of aSRB. This suggested that *Acidiphilium* SJH was acting as a detoxifying agent in the medium, or that there were substrates being carried over from the liquid culture of *Acidiphilium* SJH which were accessed by the aSRB following diffusion through the gel. However, liquid cultures of aSRB could not be sustained in cell-free spent medium from *Acidiphilium* SJH (data not shown) which appears to rule out the "substrate provision" theory. Acid-washed (i.e. chemically 'detoxified') agarose medium did not promote growth of aSRB, though this may have been a less effective method than

"detoxification" by *Acidiphilium* SJH (the latter being continuous rather than a single event). The role of *Acidocella* WJB-3 in the medium was simpler to define, because it is an obligate aerobe, and therefore would not be active under anaerobic incubation. In addition, it is not able to utilise most of the substrates tested in these experiments, unlike *Acidiphilium* SJH. However, it is able to utilise pyruvic acid, which may be toxic to bacteria at low pH. The use of *Acidocella* WJB-3 in medium to test substrate utilisation in the isolates of aSRB and other bacteria (section 6.3) was therefore advantageous. Both types of overlay medium were found to be suitable for enumeration and assessment of diversity of aSRB and other bacteria from AMD sediments (section 4.3.5).

Phylogenetic analysis of the 16S rRNA sequences of four novel strains of aSRB (M1, CC1, P1, and PFB; Chapter 6) showed that all four were most closely related to a strain of the Gram-positive, spore-forming, incomplete-oxidiser *Desulfosporosinus*. However, PFB was different to the other three strains, morphologically, physiologically and phylogenetically. It grew as much larger cells, and when it was grown in pure culture, it formed white colonies which emitted a strong "organic acid" odour. However, in mixed culture it was often found as black colonies. It also appeared to grow on other substances such as certain sugars, and non-overlay medium, which the other aSRB strains were unable to do. Isolate PFB was most closely related to a different strain of *Desulfosporosinus* than the other aSRB. Isolates M1, CC1 and P1 were very similar to each other morphologically and phylogenetically, although there were some physiological differences (Table 6.2). The other isolates, MCP and PCP, which were very similar in terms of colony and cell morphology, but phylogenetically well-separated, probably had an important relationship in the bioreactor cultures, possibly relating to fermentation reactions and possibly syntrophy (section 6.5). Isolate MR was most closely related to *Bacillus niacini*, a nicotinate-metabolising mesophile isolated from soil whose closest relative is the only documented anaerobic bacillus, *Bacillus infernus*, an Fe (III)- and Mn

(IV)-reducing anaerobe from the deep terrestrial sub-surface (Boone, 1995). The fact that all the aSRB isolates are related to *Desulfosporosinus*, and therefore to *Desulfotomaculum* to a lesser extent, suggests that these organisms, like *Desulfotomaculum* spp. are very tolerant of dryness and oxic conditions (Widdel, 1992). Isolate MR is also related to a *Bacillus* sp. from an extreme environment. These findings are not surprising, as the sources of these organisms were all extreme environments in terms of acidity. These types of organisms could conceivably have a useful role in the bioremediation of AMD, in that changing or extreme conditions are less likely to have lethal effects.

The proposed microbiological process of using acidophilic/acid-tolerant sulphate reducing bacteria for bioremediation of acid mine drainage pollution is entirely feasible, based on the ability of the isolates and the mixed cultures to grow and reduce sulphate at acidic pH. Hard *et al.* (1997) concluded that two strains of facultatively methylotrophic sulphate reducing bacteria were suitable for bioremediation of acid mine water based on their ability to reduce sulphate at pH 4.0 and 4.5. Whilst there were problems with routine growth of pure cultures of aSRB at low pH (Chapter 4), immobilised mixed cultures reduced sulphate effectively at pH 4.0 and below in laboratory-scale batch and continuous mode bioreactor systems (Chapter 5). In batch bioreactor systems supplied with ethanol and yeast extract (0.002%), the Montserrat enrichment culture exhibited a maximum SRR at pH 4.0 (70 µmoles SO_4^{2-} reduced L^{-1} h^{-1}), whereas the Parys culture was able to reduce sulphate over a broader range of pH; although it exhibited a maximum SRR (58.5 µmoles SO_4^{2-} reduced L^{-1} h^{-1}) at pH 5.5, it was more active at pH <3.0 than the Montserrat culture (and was therefore the preferred candidate for continuous mode experiments).

In the experimental runs in which ethanol concentrations were measured, a much smaller amount of ethanol than expected (given the amount of sulphate reduced) was depleted in both systems. This is difficult to

explain, because if the ethanol was being utilised in fermentation reactions by other organisms in the mixed culture, more of the ethanol would have disappeared. As it was difficult to detect ethanol using the available HPLC system, glycerol was used in continuous culture experiments. In these experiments, various residence times (16.5, 33 and 66 h) and pH were tested, and the highest sulphate conversion rate (1 g SO_4^{2-} reduced L^{-1} day^{-1}) was found at an HRT of 16.5 h at pH 4 and 30°C. The SRA in this experiment was 23.4 µmoles sulphate reduced L^{-1} h^{-1} * µmole ATP. However, lower residence times were not tested, and it is very likely that the system could achieve higher sulphate conversion rates and SRA than was demonstrated in this investigation. The production of acetate due to the incomplete oxidation of glycerol may be a limiting factor in the system. Higher conversion rates may be possible if an inexpensive substrate is found that could be completely oxidised. Experiments with acetate were set up to test whether the system could be adapted to use acetate, whether by the aSRB or other bacteria in the mixed culture; a maximum SRA of 0.29 µmoles sulphate reduced L^{-1} h^{-1} * µmole ATP at an HRT of 66 h, was obtained. The performance of the system was therefore poor when compared with glycerol as electron donor.

The investigation into which substrates promoted growth of the pure aSRB showed that mannitol, methanol, ethanol and hydrogen were utilised by the Parys P1 isolate (section 6.3.2), which was a key organism in the mixed culture used in the continuous mode experiments. Further experiments with immobilised cultures using these substrates would inform as to whether their use in bioreactor systems would be likely to improve performance. The cost of obtaining these substrates cheaply, for example, in industrial by-products (glycerol is a major component of distillery stillage) may be more important in a long-term treatment process for treating AMD than optimisation of a system in which a more expensive, high-grade substrate is provided.

A system that could operate at low pH over a long period and with minimal maintenance would probably be more valuable than one with optimised conversion rates. Cost-benefit analysis would be required to confirm this, but in general, industry is reluctant to invest heavily in new reactor design (J. Boonstra, pers. comm.). Systems are already established that can treat acidic wastewaters using hydrogen and carbon dioxide and operated at neutral (6-8) pH (van Houten *et al.*, 1994; van Houten *et al.*, 1995; van Houten and Lettinga, 1996). These laboratory-scale gas-lift reactors can treat artificial influents with a pH of 2.0 without lowering the pH of the system (van Houten *et al.*, 1995), achieving very high sulphate conversions (30 g SO_4^{2-} L^{-1} day^{-1}). It has not been established whether such high conversion rates would be possible – or indeed necessary - with acidophilic/acid-tolerant strains of SRB in a low pH reactor for an AMD treatment system. This would depend on defining and measuring the factors that determine scale-up of the process, which was beyond the scope of the current research programme.

Whilst it has not been unequivocally demonstrated that the SRB studied in this research programme are truly acidophilic, it has been shown that they are very acid-tolerant compared with the majority of SRB previously studied, and that the different cultures showed varying degrees of acid tolerance. The SRB that exist in naturally acidic environments, such as Parys Mountain, may have adapted to be considerably more acid-tolerant than those that exist in more favourably buffered environments. Therefore, they are indeed suitable target organisms for bioremediation of AMD and other acidic effluents. However, further investigations and method development are necessary to determine the extent of their acidophily and the options for their use in a treatment process.

REFERENCES

1. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1994). *Molecular Biology of the Cell*, 3rd edition, Garland Publishing, Inc., New York and London.
2. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Research*, **25**, 3389-3402.
3. Amman, R.I., Ludwig, W., and Schliefer, K. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, no. 1, 143-169.
4. Atkinson, T.; Cairns, S.; Cowan, D.A.; Danson, M.J.; Hough, D.W.; Johnson, D.B.; Norris, P.R.; Raven, N.; Robson, R.; Robinson, C. and Sharp, R.J. (2000) "A microbiological survey of Montserrat island hydrothermal biotopes" (in press).
5. Atkinson, C.F., Jones, D.D., Gauthier, J.J. (1997) Microbial activities during composting of pulp and paper-mill primary solids. *World Journal of Microbiology and Biotechnology*, **13**, no. 5, 519-525.
6. Atlas, R.M., and Bartha, R. (1993) *Microbial Ecology: Fundamentals and Applications*, 3rd edition. Benjamin/Cummings Publishing Company, Inc., CA, USA.
7. Badziong, W. Thauer, R.K., and Zeikus, J.G. (1978) Isolation of *Desulfovibrio* growing on hydrogen plus sulfate as the sole energy source. *Archives of Microbiology*, **116**, 41-49.
8. Badziong, W., and Thauer, R.K. (1980) Vectorial electron transport in Desulfovibrio vulgaris (Marburg) growing on hydrogen plus sulphate as sole energy source. *Archives of Microbiology*, **125**, 167-174.
9. Bagnara, C., Toci, R., Gaudin, C., and Belaich, J.P. (1985) Isolation and chacterisation of a cellulolytic microorganism, *Cellulomonas fermentans* sp. nov. *International Journal of Systematic Bacteriology*, **35**, 502-507.

10. Bak, F., and Cypionka, H. (1987) A novel type of energy metabolism involving fermentation of inorganic sulphur compounds. *Nature*, **326**, 891-892.
11. Banks, D., Younger, P.L., Amesen, R., Iversen, E.R., and Banks, S.B. (1997) Mine-water chemistry: the good, the bad and the ugly. Environmental Geology, **32** (3), 157-174.
12. Barnes, L.J., Janssen, F.J., Sherren, J., Versteegh, J.H., Koch, R.O., and Scheeren, P.J.H. (1991) A new process for the microbial removal of sulphate and heavy metals from contaminated waters extracted by a geohydrological control system. *Chemical Engineering Research and Design*, **69**, 184-186.
13. Baronofsky, J.J., Wilhelmus, J., Schreurs, A., and Kasket, E.R. (1984) Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. *Applied and Environmental Microbiology*, **48**, no. 6, 1134-1139.
14. Barton, L.L., and Tomei, F.A. (1995) Characteristics and activities of sulfate-reducing bacteria, In: Barton, L.L. (ed.) Sulfate-reducing bacteria (pp 151-184). Biotechnology Handbooks, no. 8 (Atkinson, T., and Sherwood, R.F., series eds.), Plenum Press, New York.
15. Bass, C.J., Webb, J.S., Sanders, P.F., and Lappin-Scott, H.M. (1996) Influence of surfaces on sulphidogenic bacteria. *Biofouling*, **10 (1-3)**, 95-109.
16. Bass, C.J., Sanders, P.F., and Lappin-Scott, H.M. (1998) Study of biofilms of sulfidogens from North Sea oil production facilities using continuous-flow apparatus. *Geomicrobiology Journal*, **15**, 101-120.
17. Bassler, B.L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current Opinion in Microbiology*, **2**, 582-587.
18. Beech, I.B., Graylarde, C.C., Smith, J.J., and Gessey, G.G. (1991) Extracellular polysaccharide from Desulfovibrio desulfuricans and Pseudomonas fluorescens in the presence of stainless steel. *Applied Microbiology and Biotechnology*, **35**, 45.

19. Beech, I.B., Cheung, C.W.S., Johnson, D.B., and Smith, J.R. (1996) Comparative studies of bacterial biofilms on steel surfaces using atomic force microscopy and environmental scanning electron microscopy. *Biofouling*, **10**, 65-77.

20. Benner, S.G., Blowes, D.W., and Ptacek, C.J. (1997) A full-scale porous reactive wall for prevention of acid mine drainage. *Ground Water Monitoring and Remediation*, 1997 (Fall), 99-107.

21. Benner, S.G., Blowes, D.W., and Ptacek, C.J. (2000) Long term performance of the Nickel Rim reactive barrierL: a summary. In: ICARD 2000 (International Conference on Acid Rock Drainage), Volume II, 1221-1225. Society for Mining, Metallurgy, and Exploration, Inc. (SME), CO, USA. ISBN 0-87335-182-7.

22. Beveridge, T.J., Makin, S.A., Kadurugamuwa, J.L., and Li, Z. (1997) Interactions between biofilms and the environment. *FEMS Microbiology Reviews*, **20**, 291-303.

23. Bond, P.L., and Banfield, J.F. (2001) Design and performance of rRNA targeted oligonucleotide probes for in situ detection and phylogenetic identification of microorganisms inhabiting acid mine drainage environments. *Microbial Ecology*, **41**, 149-161.

24. Boone, D.R., Liu, Y., Zhao, Z.J., Balkwill, D.L., Drake, G.R., Stevens, T.O., and Aldrich, H.C. (1995) *Bacillus infernus* sp. nov., an Fe (III)- and Mn (IV)-reducing anaerobe from the deep terrestrial sub-surface. *International Journal of Systematic Bacteriology*, **45**, 441-448.

25. Boonstra, J., van Lier, R., Janssen, G., Dijkman, H., and Buisman, C.J.N. (1999). In: Amils, R., and Ballester, A. (eds.) Biohydrometallurgy and the environemnt toward the mining of the 21st century. Process metallurgy 9A: Amsterdam: Elsevier. 645-656.

26. Booth, G.H. (1964) Sulphur bacteria in relation to corrosion. *Journal of Applied Bacteriology*, **27**, 174-181.

27. Bories, A., Raynal, J., and Jover, J.P. (1982). Fixed film reactor with plastic media for methane fermentation of distilleries. In: Strub, A.,

Chartier, P., and Schleser, G. (eds.) *Energy from biomass*, 567-571. London: Applied Science Publisher.

28. Boult,, S., Collins, D.N., White, K.N. and Curtis, C.D. (1994). Metal transport in a stream polluted by acid mine drainage – the Afon Goch, Anglesey, UK. *Environmental Pollution*, **84**, 279-284.
29. Boulegue, J., and Michard, G. (1979) Sulfur speciation and redox processes in reducing environments. In: Jenne, E.D. (ed.) *Chemical Modeling in Aqueous Systems*, ACS Symposium Series, 93, 25-50. American Chemical Society, Washington, DC.
30. Bowden, G.H.W., and Hamilton, I.R. (1998) Survival of oral bacteria. *Critical Reviews in Oral Biological Medicine*, **9**, 54-85.
31. Brazier, J.S., and Hall, V. (1994) A simple evaluation of the Anaero*Gen*™ system for the growth of clinically significant anaerobic bacteria. *Letters in Applied Microbiology*, **18**, 56-58.
32. Brock, T.D. (1997) Biology of Microorganisms. Madigan, M., Martinko, J., and Parker, J. (eds), Eighth (International) edition, Prentice-Hall International Inc., USA.
33. Brodie, G.A., Britt, C.R., Tomaszewski, T.M., and Taylor, H.N. (1993) Anoxic limestone drains to enhance performance of aerobic acid drainage treatment wetlands: Experience of the Tennessee Valley Authority. In: Moshiri, G.A. (Ed.) *Constructed Wetlands for Water Quality Improvement*. Lewis Publishers, Boca Raton, 129-138.
34. Brown, D.S., and Reed, S.C. (1994) Inventory of constructed wetlands in the United States. *Water Science and Technology*, **29**, no 4., 309-318.
35. Bryers, J. (ed.) (2000) *Biofilms II: Process Analysis and Applications*. Wiley-Liss, Inc.
36. Buisman, C., Post, R., Yspeert, P., Geraats, G., and Lettinga, G. (1989) Biotechnological process for sulphide removal with sulphur reclamation, *Acta Biotechnology*, **9**, 255-267.

37. Buisman, C., Yspeert, P., Geraats, G., and Lettinga, G. (1990) Optimization of sulfur production in a biotechnological sulfide-removing reactor. *Biotechnology and Bioengineering*, **35**, 50-56.

38. Buisman, C.J.N., Boonstra, J., Krol, J.P., and Dijkman, H (1996) Biotechnological removal of sulfate and heavy metals from waste water. In: *Int. Conference Advanced Waste Water Treatment*, Amsterdam.

39. Campbell, L.L., and Postgate, J.R. (1965) Classification of the spore-forming sulphate reducing bacteria. *Bacteriological Reviews*, **29**, 359-363.

40. Canty, M. (2000) Innovative in situ treatment of acid mine drainge using sulphate-reducing bacteria. In: ICARD 2000 (International Conference on Acid Rock Drainage), Volume II, 1139-1147. Society for Mining, Metallurgy, and Exploration, Inc. (SME), CO, USA. ISBN 0-87335-182-7.

41. Castro, J.M.; Wielinga, B.W.; Gannon, J.E, and Moore, J.N. (1999) Stimulation of sulfate-reducing bacteria in lake water from a former open-pit mine through addition of organic wastes. *Water Environment Research*, **71**, no.2, 218-223.

42. Christensen, Bjorn; Laake, Morten, and Lien, Torliev. (1996) Treatment of acid mine water by sulfate-reducing bacteria: results from a bench scale experiment. *Water Research*, **30**, no.7, 1617-1624. Pergamon.

43. Christensen, H., Hansen, M., and Sørensen, J. (1999) Counting and size classification of active soil bacteria by fluorescence in situ hybridisation with an rRNA oligonucleotide probe. *Applied and Environmental Microbiology*, **65**, no.4, 1753-1761.

44. Clarke, L.B. (1995) Coal mining and water quality. International Energy Agency (IEA) Coal Research, London, July 1995. ISBN 92-9029-255-5.

45. Collins, M.D., and Pascual, C. (2000) Reclassification of Actinomyces humiferus (Gledhill and Casida) as Cellulomonas humilata nom. corrig., comb, nov. *International Journal of Systematic and Evolutionary Microbiology*, **50**, no.2, 661-663.

46. Collins, D., Lawson, P.A., Nikolaitchouk, N., and Falsen, E. (2000) *Luteococcus peritonei* sp. nov., isolated from the human peritoneum. *International Journal of Systematic and Evolutionary Microbiology*, **1**, 179-181.

47. Connell, W.E., and Patrick, W.H. Jr. (1968) Sulphate reduction in soil: effect of redox potential and pH. *Science*, **159**, 86-87.

48. Cook, R.B.; Kelly, C.A.; Schindler, D.W., and Turner, M.A. (1986) Mechanisms of hydrogen ion neutralization in an experimentally acidified lake. *Limnology and Oceanography*, **31**, (1), 134-148.

49. Cord-Ruwisch, R. (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *Journal of Microbiological Methods*, **4**, 33-36.

50. Cord-Ruwisch, R., and Garcia, J.L. (1985) Isolation and characterization of an anerobic benzoate-degrading spore-forming sulfate-reducing bacterium, *Desulfotomaculum sapomandens* sp. nov. *FEMS Microbiology Letters*, **29**, 325-330.

51. Couthino, C.M.L.M., Magalhaes, F.C., Araujo-Jorge, T.C. (1994) Morphology of the surface coat and extracellular matrix of sulphidogenic biofilms enriched in sulphate reducing bacteria involved in biocorrosion processes in the offshore oil extraction industry off Brazil's Atlantic coast. *Journal of General and Applied Microbiology*, **40**, 227-241.

52. Cypionka, H. (1995) Solute transport and cell energetics. In: Barton, L.L. (ed.) Sulfate-reducing bacteria (pp 151-184). Biotechnology Handbooks, no. 8 (Atkinson, T., and Sherwood, R.F., series eds.), Plenum Press, New York.

53. Cypionka, H. (2000) Oxygen respiration by Desulfovibrio species. *Annual Review of Microbiology*, **54**, 827-848.

54. Davison, J. (1993) Successful acid mine drainage and heavy metal site bioremediation. In: Moshiri, G.A. (Ed.), Constructed Wetlands for Water Quality Improvement. Lewis Publishers, Boca Raton, FL., 167-170.

55. Day, S.J. (1994) Evaluation of acid generating rock and acid consuming rock mixing to prevent acid rock drainage. In: Proceedings of the International Land Reclamation and Mine Drainage Conference on the Abatement of Acidic Drainage (Volume 2: Mine drainage). Pittsburgh, PA, USA, 24-29, April 1994. US Department of the Interior, Bureau of Mines Special Publication SP06B-94, 77-86 (Apr 1994).

56. Delaquis, P.J., Caldwell, D.E., Lawrence, J.R. and McCurdy, A.R. (1989) Detachment of *Pseudomonas fluorescens* from biofilms in response to nutrient stress. *Microbial Ecology*, **18**, 199-210.

57. De Vegt, A.L., and Buisman, C.J.N. (1995) Full Scale Biological Treatment of Heavy Metal Contaminated Groundwater. In: Hager, J.P., Mishra, B., Davidson, C.F., and Litz, J.L. (eds.) Treatment and Minimisation of Heavy Metal-containing Wastes. The Minerals, Metals and Materials Society.

58. De Vegt, Andre L.; Krol, Johannes P.; and Buisman, Cees J. (1997). Biological sulfate removal and metal recovery from mine waters. In: Verachtert, H., and Verstraete, W. (eds), 'Environmental Biotechnology' - Proceedings of the International Symposium I, Technologish Insituut, Belgium.

59. De Vegt, A.L., and Buisman, C.J. (1996) Sulfur compounds and heavy metal removal. In: Warren, Garry W (ed., 1995) EPD Congress, The Minerals, Metals and Materials Society.

60. Devereux, R., Delaney, M., Widdel, F., and Stahl, D.A. (1989) Natural relationships among sulphate reducing eubacteria. *Journal of Bacteriology*, **171**, 6689-6695.

61. Devereux, R., He, S.-H., Doyle, C.A., Orkland, S., Stahl, D.A., LeGall, J., *et al.* (1990) Diversity and origin of Desulfovibrio species: phylogenetic definition of a family. *Journal of Bacteriology,* **172**, 3609-3619.

62. Devereux, R., and Stahl, D.A. (1993) Phylogeny of Sulfate-Reducing Bacteria and a perspective for analyzing their natural communities. In: In: Odom, J.M., and Singleton, R. (eds) *The sulfate-reducing bacteria:*

contemporary perspectives. Brock/Springer Series, Springer-Verlag, New York. 131-160.

63. Diaz, M.A., Monhemius, A.J., and Narayanan, A. (1997). Consecutive hydroxide-sulphide precipitation treatment of acid rock drainage. In: Fourth International Conference on Acid Rock Drainage (Volume III), Vancouver, May 1997.

64. Dilling, W., and Cypionka, H. (1990) Aerobic respiration in sulfate-reducing bacteria. *FEMS Microbiology Letters*, **71**, 123-138.

65. Dugan, P.R., Macmillan, C.B., and Pfister, R.M. (1970a) Aerobic heterotrophic bacteria indigenous to to pH 2.8 acid mine water: Microscopic examination of acid streamers. *Journal of Bacteriology*, **101**, 982-988.

66. Dugan, P.R.,Macmillan, C.B., and Pfister, R.M. (1970b) Aerobic heterotrophic bacteria indigenous to to pH 2.8 acid mine water: Predominant slime-producing bacteria in acid streamers. *Journal of Bacteriology*, **101**, 973-981.

67. Dugan, P.R. (1987) Prevention of formation of acid mine drainage from high-sulfur coal refuse by inhibition of iron-oxidising and sulfur-oxidising bacteria. 2. Inhibition in run of mine refuse under simulated field conditions. *Biotechnology and Bioengineering*, **29**, 49-54.

68. Dugan, P.R., and Apel, W.A. (1983) Bacteria and acidic drainage from coal refuse: inhibition by sodium lauryl sulphate and sodium benzoate. *Applied and Environmental Microbiology*, **46**, no.1, 279-282.

69. Dunbabin, J.S., and Bowmer, K.H. (1992) Potential use of constructed wetlands for treatment of industrial wastewaters containing metals. *The Science of the Total Environment*, **11**, 151-168.

70. du Preez, L.A., Odendaal, J.P., Maree, J.P., and Pónsonby, M. (1992) Biological removal of sulphate from industrial effluents using producer gas as energy source. *Environmental Technology*, **13**, 875-882.

71. Dvorak, D.H., Hedin, R.S., Edenborn, H.M., and Gustafson, S.L. (1991) Treatment of metal-contaminated water using bacterial sulfate reduction: results from pilot-scale reactors. In: *Proceedings of the 2nd*

International Conference on Abatement of Acidic Drainage, Montreal, Quebec, September 16-18, 1991, 301-314,

72. Dvorak, D.H., Hedin, R.S., Edenborn, H.M., and McIntire, P.E. (1992) Treatment of metal-contaminated water using bacterial sulfate reduction: results from pilot-scale reactors. *Biotechnology and Bioengineering*, **40**, 609-616.

73. Ehrenreich, A. and Widdel, F. (1994) Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Applied and Environmental Microbiology*, **60**, 4517-4526.

74. Elliot, P., Ragusa, S., Catcheside, D. (1998) Growth of sulfate-reducing bacteria under acidic conditions in an upflow anaerobic bioreactor as a treatment system for acid mine drainage. *Water Reseach*, **32**, no. 10, 3724-3730.

75. Enviromine Technical (1998). *Tailings dam failure in Spain.* http://www.infomine.com/ftp/pub/list_archives/enviromine_technical/1775.html

76. Evangelou, V.P. (1995) *Pyrite Oxidation and its Control*, CRC Press, New York, USA.

77. Evangelou, V.P., and Zhang, Y.L. (1995) A review: pyrite oxidation mechanisms and acid mine drainage prevention. *Critical Reviews in Environmental Science and Technology*, **25**, no. 2, 141-199. CRC Press, New York, USA.

78. Evans, L.V. (ed.) (2000) *Biofilms: Recent Advances for Their Study and Control.* Harwood Academic Publishers, London.

79. Fauque, G., LeGall, J., and Barton, L.L. (1991) Sulfate-reducing and sulfur-reducing bacteria. In: Shively, J.M., and Barton, L.L. (eds) *Variations in Autotrophic Life*, 271-337. Academic Press Limited, London.

80. Fauque, G.D. (1995) Ecology of sulfate-reducing bacteria. In: Barton, L.L. (ed) *Sulfate-reducing bacteria*, 217-241. Plenum Press, New York.

81. Feasby, D.G., Blanchette, M., and Tremblay, G. (1991) The mine environment neutral program. In: Second International Conference on

the Abatement of Acidic Drainage, v.**1**. 1-26. Ottawa: MEND. CANMET.

82. Feltenstein, J. (1993) PHYLIP -Phylogeny Inference Package (version 3.5c), modified from Feltenstein, J. (1989) PHYLIP- Phylogeny Inference Package (version 3.2). *Cladistics*, **5**, 164-166.

83. Fischer, S.G., and Lerman, L.S. (1979) Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell*, **16**, 191-200.

84. Flemming, H-C., Wingender, J., Mayer, C., Körstgens,V., and Borchard, W. (2000) Cohesiveness in biofilm matrix polymers. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms*. Fifty-ninth Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

85. Fowler, V.J., Widdel, F., Pfennig, N., Woese, C.R., and Stackebrandt, E. (1986) Phylogenetic relationships of sulphate- and sulphur reducing eubacteria. *Systematic and Applied Microbiology*, **8**, 32-41.

86. Fox, G., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J., Zablen, L.B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Luehrsen, K.R., Chen, K.N., and Woese, C.R. (1980) Phylogeny of prokaryotes. *Science*, **209**, 457-463.

87. Fukui, M., and Takii, S. (1990a) Colony formation of free-living and particle-associated sulphate reducing bacteria. *FEMS Microbiology and Ecology*, **73**, 85-90.

88. Fukui, M., and Takii, S. (1990b) Survival of sulphate reducing bacteria in oxic surface sediment of a seawater lake. *FEMS Microbiology and Ecology*, **73**, 317-322.

89. Fukui, M., and Takii, S. (1996) Microdistribution of sulphate reducing bacteria in sediments of a hypertrophic lake and their response to the addition of organic matter. *Ecological Research*, **11**, 257-267.

90. Fytas, K., Bousquet, P., and Evangelou, V. (2000) Silicate coating technology to prevent acid mine drainage. In: ICARD 2000 (International Conference on Acid Rock Drainage), Volume I, 87-95. Society for Mining, Metallurgy, and Exploration, Inc. (SME), CO, USA. ISBN 0-87335-182-7.

91. Gazea, B., Adam, K., and Kontopoulos, A. (1996) A review of passive systems for the treatment of acid mine drainage. *Minerals Engineering*, **9**, no.1, 23-42.

92. Gibson, G.R., Cummings, J.H., and MacFarlane, G.T. (1991) Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis. *FEMS Microbiology and Ecology*, **86**, 103-112.

93. Goto, K., and Omura, T. (2000) Application of the partial 16S rRNA sequence as an index for rapid identification of species in the genus *Bacillus*. *Journal of General and Applied Microbiology*, **46**, 1-8.

94. Greenberg, E.P. (2000) Acyl-homoserine lactone quorum sensing in bacteria. *Journal of Microbiology*, **38**, no.3, 117-121.

95. Gyure, R.A., Konopka, A., Brooks, A., and Doemel, W. (1987) Algal and bacterial activities in acidic (pH 3) strip mine lakes. *Applied and Environmental Microbiology*, **53**, no.9, 2069-2076.

96. Gyure, R.A., Konopka, A., Brooks, A., and Doemel, W. (1990) Microbial sulfate reduction in acidic (pH 3) strip-mine lakes. *FEMS Microbiology Ecology*, **73**, 193-202.

97. Häggblom, M.M., Knight, V.K., and Kerkhof, L.J. (2000) Anaerobic decomposition of halogenated aromatic compounds. *Environmental Pollution*, **107**, 199-207.

98. Hallberg. K.B., Sen, A.M., Lloyd, J.R., and Johnson, D.B. (in preparation). Phylogeny of acidophilic sulphate reducing bacteria.

99. Hallberg, K.B., Kolmert, Å.K., Johnson, D.B., and Williams, P.A. (1999) A novel metabolic phenotype among acidophilic bacteria: aromatic degradation and the potential use of these organisms for the treatment of wastewater containing organic and inorganic pollutants. In: Amils,

R., and Ballester, A. (eds.) *Biohydrometallurgy and the environment toward the mining of the 21st century*; Proceedings of the International Biohydrometallurgy Symposium Process metallurgy 9A: Amsterdam: Elsevier. 719-728.

100. Hamilton, W.A. (1985) Sulfate-reducing bacteria and anaerobic corrosion. *Annual Reviews of Microbiology*, **39**, 195-217.

101. Hamilton, W.A., and Lee, W. (1985) Biocorrosion. In: Barton, L.L. (ed.) *Sulfate-reducing bacteria* (pp 243-264). Biotechnology Handbooks, no. 8 (Atkinson, T., and Sherwood, R.F., series eds.), Plenum Press, New York.

102. Hamilton, W.A. (1994) Biocorrosion: the action of sulphate reducing bacteria. In: Ratledge, C. (ed.) *Biochemistry of Microbial Degradation*, chapter 17. Kluwer Academic Publishers, Dordrecht.

103. Hamilton, W.A. (1998) Sulfate-reducing bacteria: physiology determines their environmental impact. *Geomicrobiology Journal*, **15**, 19-28.

104. Hamilton, W.A (1998) Bioenergetics of sulphate-reducing bacteria in relation to their environmental impact. *Biodegradation*, **9**, 201-212.

105. Hammack, R.W., Dvorak, D.H., and Edenborn, H.M. (1993). The use of biogenic hydrogen sulfide to selectively recover copper and zinc from severely contaminated mine drainage. In: Biohydrometallurgical technologies, I. Bioleaching processes. The Minerals, Metals and Materials Society, Warrendale, PA., 631-640.

106. Hammack, R.W., Edenborn, H.M., and Dvorak, D.H. (1994a) Treatment of water from an open-pit copper mine using biogenic sulfide and limestone: a feasibility study. *Water Research*, **28**, no.11, 2321-2329.

107. Hammack, R.W., Dvorak, D.H., and Edenborn, H.M. (1994b) Bench-scale test to selectively recover metals from metal mine drainage using biogenic H_2S. *Proceedings of the Third International Conference on the Abatement of Acidic Drainage, Pittsburgh, PA, April 1994.*

108. Hansen, T.A. (1993) Carbon metabolism of sulfate-reducing bacteria. In: Odom, J.M., and Singleton, R. (eds) The sulfate-reducing bacteria: contemporary perspectives. Springer-Verlag, New York. 21-40.

109. Hansen, T.A. (1994) Metabolism of sulfate-reducing prokaryotes. *Antonie van Leeuwenhoek*, **66**, 165-185.

110. Hao, O., Chen, J., Huang, L., and Buglass, R. (1996) Sulphate-reducing bacteria. *Critical Reviews in Environmental Science and Technology*, **26**, no. 1, 155-187.

111. Hard, B.C., Friedrich, S., and Babel, F.W. (1997) Bioremediation of acid mine water using facultatively methylotrophic metal-tolerant sulfate-reducing bacteria. *Microbiological Research*, **152**, 65-73.

112. Head, I.M., Saunders, J.R., and Pickup, R.W. (1998) Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology*, **35**, 1-21.

113. Hellawell, J.M. (1986) Biological indicators of freshwater pollution and environmental management. Applied Science Publishers, London.

114. Herlihy, A.T., and Mills, A.L. (1985). Sulfate reduction in freshwater sediments receiving acid mine drainage. *Applied and Environmental Microbiology*, **49**, 179-186.

115. Herlihy, A.T., Mills, A.L., Hornberger, G.M., and Bruckner, A.E. (1987) The importance of sediment sulphate reduction to the sulfate budget of an impoundment receiving acid mine drainage. *Water Resources Research* **23** (2), 287-292.

116. Hristova, K.R., Mau, M., Zheng, D., Aminov, R.I., Mackie, R.I., Gaskins, H.R., and Raskin, L. (2000) *Desulfotomaculum* genus- and subgenus-specific 16S rRNA hybridisation probes for environmental studies. *Environmental Microbiology*, **2**, no.2, 143-159.

117. Hulschoff Pol, L.W., Lens, P.N.L., Stams, A.J.M., and Lettinga, G. (1998) Anaerobic treatment of sulphate-rich wastewaters. *Biodegradation*, **9**, 213-224.

118. Hiligsmann, S., Jacques, P., and Thonart, P. (1998) Isolation of highly performant sulfate reducers from sulfate-rich environments. *Biodegradation*, **9**, 285-292.

119. Huntsman, B.E., Solch, J.G., and Porter, M.D. (1978) Utilisation of *Sphagnum* species dominated bog for coal acid mine drainage abatement. Geological Society Am. (91st Annual Meeting) Abstracts, Toronto, Ontario, Canada, 322.

120. Imhof, A., and Heinzer, I. (1996) Continuous monitoring of oxygen concentrations in several systems for cultivation of anaerobic bacteria. *Journal of Clinical Microbiology*, **34**, no. 7, 1646-1648.

121. Ingledew, W.J. (1982) *Thiobacillus ferrooxidans*: the bioenergetics of an acidophilic chemolithotroph. *Biochimica Biophysica Acta*, **683**, 89-117.

122. Jack, T.R., and Westlake, D.W.S. (1995) Control in Industrial Settings. In: Barton, L.L. (ed.) *Sulfate-reducing bacteria* (pp 265-292). Biotechnology Handbooks, no. 8 (Atkinson, T., and Sherwood, R.F., series eds.), Plenum Press, New York.

123. Jenkins, D.A., and Johnson, D.B. (1993) Abandoned metal mines: a unique mineralogical and microbiological resource. *Journal of the Russell Society*, **5**, vol 1, 40-44.

124. Johnson, D.B., Kelso, W.I., and Jenkins, A. (1979). Bacterial streamer growth in a disused pyrite mine. *Environmental Pollution*, **18**, 107-118.

125. Johnson, D.B., and Kelso, W.I. (1981) Extracellular polymers of acid streamers from pyritic mines. *Environmental Pollution (Series A)*, **24**, 291-301.

126. Johnson, D.B., MacVicar, J.H.M., Rolfe, S. (1987) A new solid medium for the isolation and enumeration of *Thiobacillus ferrooxidans* and acidophilic heterotrophic bacteria. *Journal of Microbiological Methods*, **7**, 9-18.

127. Johnson, D.B., and McGinness, S. (1991) Ferric iron reduction by acidophilic heterotrophic bacteria. *Applied and Environmental Microbiology*, **57**, 207-211.

128. Johnson, D.B., Ghauri, M. Afzal, and Said, M.F. (1992) Isolation and characterisation of an acidophilic, heterotrophic bacterium capable of oxidising ferrous iron. *Applied and Environmental Microbiology*, **58**, no.5, 1423-1428.

129. Johnson, D.B., Ghauri, M.A. and McGinness, S. (1993) Biogeochemical cycling of iron and sulphur in leaching environments. *FEMS Microbiology Reviews*, **11**, 63-70.

130. Johnson, D.B. (1995a) Selective solid media for isolating and enumerating acidophilic bacteria. *Journal of Microbiological Methods*, **23**, 205-218.

131. Johnson, D.B. (1995b) Acidophilic microbial communities: candidates for bioremediation of acidic mine effluents. *International Biodeterioration and Biodegradation*, 41-58.

132. Johnson, D.B., and Bridge, T.A.M. (1997) The role of microbial dissimilatory reduction processes in the bioremediation of metal-rich, acidic waste waters. In: Verachtert, H., and Verstraete, W. (eds), 'Environmental Biotechnology' - Proceedings of the International Symposium I, Technologish Insituut, Belgium.

133. Johnson, D.B. (1998) Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbiology Ecology*, **27**, 307-317.

134. Johnson, D.B. (1999) Importance of microbial ecology in the development of new mineral technologies. In: Amils, R., and Ballester, A. (eds.) *Biohydrometallurgy and the environment toward the mining of the 21st century*; Proceedings of the International Biohydrometallurgy Symposium Process metallurgy 9A: Amsterdam: Elsevier. 645-656.

135. Johnson, D.B. (2000) Biological removal of sulphurous compounds from inorganic wastewaters. In: Lens, P. and Hulshoff-Pol, L. (eds.) Environmental technologies to treat sulphur pollution: principles and engineering. London: International Association on Water Quality.

136. Johnson, D.B., Dziurla, M., and Kolmert, Å. (2000) Novel approaches for bioremediation of acidic, metal-rich effluents using indigenous bacteria. In: ICARD 2000 (International Conference on Acid Rock Drainage), Volume II, 1209-1217. Society for Mining, Metallurgy, and Exploration, Inc. (SME), CO, USA. ISBN 0-87335-182-7.

137. Kalin, M., Cairns, J., and McCready, R. (1991). Ecological engineering methods for acid mine drainage treatment of coal wastes. *Resources, Conservation and Recycling*, **5**, 265-275.

138. Kelly, D. (1984) *Complete A Level Chemistry*. Bell and Hyman, London, U.K.

139. Kepler, D.A., and McCleary, E.C. (1994) Successive Alkalinity Producing Systems (SAPS) for the treatment of acidic mine drainage. In: Proc. Of the 1994 International Land Reclamation and Mine Drainage Conference, Pittsburgh, PA, U.S. Bureau of Mines SP GA-94, 195-204.

140. Kim, S.D.; Kilbane (II), J.J., and Cha, D.K. (1999) Prevention of acid mine drainage by sulfate reducing bacteria: organic substrate addition to mine waste piles. *Environmental Engineering Science*, **16**, no.2,139-145.

141. King, D.L., Simmler, J.J., Decker, C.S., and Ogg, C.W. (1974) Acid strip mine lake recovery. *Journal of Water Pollution Control Federation*, **46**, no. 10, 2301-2315.

142. Klemps, R., Cypionka, H., Widdel, F., and Pfennig, N. (1985) Growth with hydrogen, and further physiological characteristics of *Desulfotomaculum* species. *Archives of Microbiology*, **143**, 203-208.

143. Knapp, R.B., and Faison, B.D. (1997) A bioengineering system for *in situ* bioremediation of contaminated groundwater. *Journal of Industrial Microbiology and Biotechnology*, **18**, 189-197.

144. Kolenbrander, P.E., Andersen, R.N., Kazmerzak, K.M., and Palmer Jr., R.J. (2000) Coaggregation and coadhesion in oral biofilms. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms*. Fifty-ninth

Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

145. Kolmert, Å., and Johnson, D.B. (1999) Remediation of acidic wastewaters using immobilised acidophilic sulphate-reducing bacteria. In press, and also In: Kolmert, Å. (1999) Sulfate-Reducing Bacteria in Bioremediation Processes. Licentiate Thesis, Lund University, Sweden.

146. Kolmert, Å., Henrysson, T., Hallberg, R., and Mattiasson, B. (1997) Optimization of sulphide production in an anaerobic continuous biofilm process with sulphate-reducing bacteria. *Biotechnology Letters,* **19**, no.10, 971-975.

147. Kolmert, Å. (1999) Sulfate-Reducing Bacteria in Bioremediation Processes, Licentiate Thesis. Lund University, Sweden.

148. Kolmert, Å., Wikström, P., and Hallberg, K. (2000) A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures. *Journal of Microbiological Methods*, in press.

149. Kremer, D.R., Veenhuis, M., Fauque, G., Peck, H.D., LeGall, J., Lampreia, J., Moura, J.J.G., and Hansen, T.A. (1988) Immunocytochemical localisation of APS reductase and bisulfite reductase in three *Desulfovibrio* species. *Archives of Microbiology*, **150**, 296-301.

150. Laanbroek, H.J., and Geerligs, H.J. (1983) Influence of clay particles (illite) on substrate utilisation by sulphate reducing bacteria. *Archives of Microbiology*, **134**, 161-163.

151. Lackey, J.B. (1938). The flora and fauna of surface water polluted by acid mine drainage. Public Health Report, Washington, **53**, 1499-1507.

152. Lamb, H.M., Dodds-Smith, M., and Gusek., J. (1998) Development of a long-term strategy for the treatment of acid mine drainage at Wheal Jane. In: Geller, W., Klapper, H., and Salomomns, W. (eds.) *Acidic Mining Lakes*, pp 335-346. Springer Verlag, Berlin.

153. Lawrence, J.R., Korber, D.R., Wolfaardt, G.M., and Caldwell, D.E. (1995) Bacterial behavioural strategies at interfaces. *Advances in Microbial Ecology*, **14**, 1-75.

154. Ledin, M., and Pedersen, K. (1996) The environmental impact of mine wastes – Roles of microorganisms and their significance in treatment of mine wastes. *Earth Science Reviews*, **41**, 67-108.

155. Lee, W., and Characklis, W.G. (1993) Corrosion of mild steel under anaerobic biofilm. *Corrosion*, **49**, 186-199.

156. Lettinga, G., van Velzen, A., Hobma, S., de Zeeuw, W., and Klapwijk, A. (1986) Use of the Upflow Sludge Blanket reactor concept for biological wastewater treatment, especially for anaerobic treatment (UASB). *Biotechnology and Bioengineering*, **22**, 699-734.

157. Lide, D.R. (1998) *Handbook of Chemitry and Physics*. CRC (Chemical Rubber Company) Press, Boca Raton, Florida, U.S.A.

158. Lloyd, R. (1992) Pollution and Freshwater Fish. Fishing News Books.

159. Loeb, G.I., and Niehof, R.A. (1977) Absorption of an organic film at the platinum seawater interface. *Journal of Marine Research*, **35**, 283-291.

160. Lovley, D.R. (1991) Disimilatory Fe(III) and Mn(IV) reduction. *Microbiology Reviews*, **55**, 259-287.

161. Lovley, D.R., and Phillips, E.J.P. (1987) Rapid assay for microbially reducible ferric iron in aquatic sediments. *Applied and Environmental Microbiology*, **53**, 1536-1540.

162. Lowry, O.H., Rosenburgh, N.J., Farr, A.L., and Randall, R.J. (1951) protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.

163. Lyew, D., Knowles, R., and Sheppard, J.D. (1994) The biological treatment of acid mine drainage under continous flow conditions in a reactor. *Trans. Institution of Chemical Engineers*, **72 (B)**, 42-47.

164. Lyew, D., and Sheppard, J.D. (1997) Effects of physical parameters of a gravel bed on the activity of sulphate-reducing bacteria in the

presence of acid mine drainage. *Journal of Chemical Technology and Biotechnology*, **70**, 223-230.

165. MacFarlane, G.T., and Gibson, G.R. (1991) Sulphate-reducing bacteria. In: Levett, P.N. (ed.) *Anaerobic microbiology: a practical approach.* In: Rickworth, D., and Hames, B.D. (series eds.) The Practical Approach Series. IRL Press, at Oxford University Press, U.K.

166. Manz, W., Eisenbrecher, M., Neu, T.R., and Szewzyk, U. (1998) Abundance and spatial organisation of Gram-negative sulphate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiology Ecology*, **25**, 43-61.

167. Maree, J.P., and Strydom, W.F. (1985) Biological sulphate removal from industrial effluent in an upflow packed bed reactor. *Water Research*, **21**, 141-146.

168. Maree, J.P., Gerber, A., McLaren, A.R., and Hill, E. (1987) Biological treatment of mining effluents. *Environmental Technology Letters*, **8**, 53-64.

169. Marquis, R.E. (1995) Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. *Journal of Industrial Microbiology*, **15**, 198-207.

170. Marschall, C., Frenzel, P., and Cypionka, H. (1993) Influence of oxygen on sulfate reduction and growth of sulfate-reducing bacteria. *Archives of Microbiology*, **159**, no 2, 168-173.

171. Marsh, P.D., and Bowden, G.H. (2000) Microbial community interactions in biofilms. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms.* Fifty-ninth Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

172. Marshall, K.C. (1985) Bacterial adhesion in oligotrophic habitats. *Microbiological Science*, **2**, 323-326.

173. Maszenan, A.M., Seviour, R.J., Patel, B.K.C., Schumann, P., and Rees, G.N. (1999) *Tessarococcus bendigoniensis* gen. nov., sp. nov., a gram-positive coccus occurring in regular packages or tetrads, isolated from activated sludge biomass. *International Journal of Systematic Bacteriology*, **49**, 459-468.

174. Matsuda, H., Fuijama, Y., Andoh, A., Ushijama, T., Kajinami, T., and Bamba, T. (2000) Characterisation of antibody responses against rectal mucosa-associated bacterial flora in patients with ulcerative colitis. *Journal of Gastroenterology and Hepatology*, **15**, 61-68.

175. Mayer, C., Moritz, R., Kirschner, C., Borchard, W., Maibaum, R., Wingender, J., and Flemming, H-C. (1999) The role of intermolecular interactions: studies on model systems for bacterial biofilms. *International Journal of Biological Macromolecules*, **26**, 3-16.

176. McCoy,

177. McGinness, S., and Johnson, D.B. (1993) Seasonal variations in the microbiology and chemistry of an acid mine drainage stream. *The Science of the Total Environment*, **132**, 27-41.

178. McGinness, S., Brown, M.M.E., Atkinson, K., and Webb, J.S. (1996) Constructed Wetlands: A Flawed Concept? In: *Metals, Minerals and the Environment (II)*, IMM, Prague, 1996.

179. McIntyre, P.E., Edenborne, H.M., and Hammack, R.W. (1990) Incorporation of bacterial sulphate-reduction into constructed wetlands for the treatment of acid and metal mine drainage. In: Skousen, J., Scencindiver, J., and Samuel, D. *Proceedings of the 1990 Mining and Reclamation Conference*, 409-415, Morgantown, WV: West Virginia University Publications.

180. Milner, N.J., and Varallo, P.V. (1990) Effects of acidification on fish and fisheries in Wales. In: Edwards, R.W. (ed.) *Acid Waters in Wales*, 121-143. Kluwer Academic Publishers, Dordrecht, The Netherlands.

181. Mitchell, P., Potter, C., and Watkins, M. (2000) Treatment of acid rock drainage: field demonstration of silica micro encapsulation technology and comparison with an existing caustic soda-based

system. In: *ICARD 2000 (International Conference on Acid Rock Drainage)*, Volume II, 1035-1043. Society for Mining, Metallurgy, and Exploration, Inc. (SME), CO, USA. ISBN 0-87335-182-7.

182. Misken, Katharine A., and Figueroa, Linda A. (1993) Suspended culture of sulfate reducing bacteria for the remediation of acid mine drainage. *Abstracts of the Anerican Chemical Society*, **205** (2).

183. Möller, D., Schauder, R., Fuchs, G., and Thauer, R.K. (1987) Acetate oxidation to CO_2 via a citric acid cycle involving an ATP-citrate lyase: a mechanism for the synthesis of ATP via substrate level phosphorylation in *Desulfobacter postgatei* growing on acetate and sulphate. *Archives of Microbiology*, **148**, 202-207.

184. Monhemius, A.J. (1977) "Precipitation diagrams for metal hydroxides, sulphides, arsenates and phosphates". *Transactions of the Institution of Mining and Metallurgy*, Section C, **86**, C202-C206.

185. Moore, G.F., Dunsmore, B.C., Jones, S.M., Smejkal, C.W., Jass, J., Stoodley, P., and Lappin-Scott, H.M. (2000) Microbial detachment from biofilms. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms*. Fifty-ninth Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

186. Moriwaki, Hisamitsu, and Matsushima, Eiji (1997). Utilization of sulfate-reducing bacteria technology for treatment of acid mine drainage. *Metal Mining Agency of Japan (MMAJ)*

187. Myers, R.J. (1986) The new low value for the second dissociation constant for H_2S: its history, its best value, and its impact on the teaching of sulfide equilibria. *Journal of Chemistry Education*, **63**, no. 8, 687-690.

188. Nanninga, H.J., and Gottschal, J.C. (1987) Properties of *Desulfovibrio carbinolicus*, sp. nov., and other sulfate-reducing bacteria isolated from an anerobic-purification plant. *Applied and Environmental Microbiology*, **51**, 572-579.

189. National Rivers Authority, Welsh Region, Caernarfon, Wales, U.K. (1994). Thomas, I.P.: Internal Report: Mawddach Environmental Impact Assessment.

190. Nogrady, N., Barrow, P.A., and Nagy, B. (2000) "Quorum sensing" signal mechanisms of bacteria. *Magyar Allatorvosok Lapja*, **122,** no. 11, 682-688.

191. Nordstrom, D.K., Jenne, E.A., and Ball, J.W. (1979) Redox equilibria of iron in acid mine waters. In: Jenne, E.D. (ed.) *Chemical Modeling in Aqueous Systems*, ACS Symposium Series, 93, 51-79. American Chemical Society, Washington, DC.

192. Nordstrom, D.K., Alpers, C.N., Ptacek, C.J., and Blowes, D.W. (2000) Negative pH and extremely acidic mine waters from Iron Mountain, California. *Environmental Science and Technology*, **34**, 254-258.

193. Norris, P.A., and Ingledew, W.J. (1992) Acidophilic bacteria: adaptations and applications. In: Herbert, R.A., and Sharp, R.P. (eds.) *Molecular Biology and Biotechnology of Extremophiles*, Blackie, Glasgow, U.K., 115-142.

194. Norris, P.R., and Johnson, D.B. (1998). Acidophilic microorganisms. In: Horikoshi, K., and Grant, W.D. (eds.) *Extremophiles: Microbial Life in Extreme Environments.* From: Mitchell, R. (series ed.) Wiley Series in Ecological and Applied Microbiology. Wiley-Liss Inc., New York, U.S.A.

195. Odom, J.M., and Peck, H.D. Jr. (1981) Hydrogen cycling as a general mechanism for energy coupling in the sulphate reducing bacteria, *Desulfovibrio* sp. *FEMS Microbiology Letters*, **12**, 47-50.

196. O'Flaherty, V., Mahony, T., O'Kennedy, R., and Colleran, E. (1998) Effect of pH on growth kinetics and sulfide toxicity thresholds of a range of methanogenic, syntrophic and sulfate-reducing bacteria. Process Biochemistry 33, (5), 555-569.

197. Okabe, S., Satoh, H., Itoh, T., and Watanabe, Y. (1999) Microbial ecology of sulphate-reducing bacteria in wastewater biofilms analysed

by microelectrodes and fish (fluorescent in situ hybridisation) technique. *Water Science and Technology*, **39**, no.7, 41-47.

198. Olem, H. (1991) Mitigation Strategies. In: *Liming Acidic Surface Waters*. Lewis, Chelsea, Michigan.

199. Pankhania, I.P. (1988) Hydrogen metabolism in sulphate reducing bacteria and its role in anaerobic corrosion. *Biofouling*, **1**, 27-47.

200. Pesez, M., and Bartos, J. (1974) *Colorimetric and Fluorimetric Analysis of Organic Compounds and Drugs*. Marcel Dekker, Inc. New York. In: Schwartz, Morton K (ed) Clinical and Biochemical Analysis, 1.

201. Pfennig, N., Widdel., F., and Trüper, H.G. (1981) The dissimilatory sulfate-reducing bacteria. In: Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., and Schlegel, H.G. (eds.) *The Prokaryotes: A handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*. 2nd ed., Vol 1, 926-940.

202. Pitcher, M.C.L., Beatty, E.R., and Cummings, J.H. (2000) The contribution of sulphate-reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut*, **46**, 64-72.

203. Pitzer, K.S. (1991) Correction. *Journal of Physical Chemistry US*, **17**, 6746.

204. Postgate, J.R. (1979) *The sulphate-reducing bacteria*. 1st ed., Cambridge University Press, Cambridge, England.

205. Postgate, J.R. (1984) *The sulphate-reducing bacteria*. 2nd ed., Cambridge University Press, Cambridge, England.

206. Pourbaix, M. (1966) *Atlas of electrochemical equilibria in aqueous solutions*. Pergamon Press.

207. Power, M.E., Araujo, J.C., van der Meer, J.R., Harms, H., and Wanner, O. (1999) Monitoring sulphate-reducing bacteria in heterotrophic biofilms. *Water Science and Technology*, **39**, no.7, 49-56.

208. Pronk, J.T. (1991) *Physiology of the acidophilic thiobacilli*. PhD Thesis, University of Delft.

209. Pronk, J.T., and Johnson, D.B. (1992) Oxidation and reduction of iron by acidophilic bacteria. *Geomicrobiology Journal*, **10**, 153-171.

210. Qatabi, A-I., Cayol, J.L., and Garcia, J-L. (1989) 1,2- and 1,3-propanediol degardation by Desulfovibrio alcoholovorans sp. nov., in pure culture or through interspecies transfer. In: *FEMS Symposium: Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogen transfer.* New York: Plenum Publishing Corporation.

211. Qatabi, A-I., Bories, A., and Garcia, J-L. (1991) Sulfate reduction and anaerobic glycerol degradation by a mixed microbial culture. *Current Microbiology*, **22**, 47-52.

212. Rainey, F.A. Weiss, N., and Stackebrandt, E. (1995) Phylogenetic analysis of the genera Cellulomonas, Promicromonospora, and Jonesia, and proposal to exclude the genus Jonesia from the family Cellulomonadaceae. *International Journal of Systematic Bacteriology*, **45 (4)**, 649-652.

213. Rand, M.C., Greenburg, A.E., and Taros, M.J. (1975) In: Standard Methods for the Examination of Waste Water. 14th edition, 496-498. U.S.S. American Public Health Association.

214. Raskin, L., Rittmann, B.E., and Stahl, D.A. (1996) Competition and coexistence of sulphate-reducing and methanogenic populations in anaerobic biofilms. *Applied and Environmental Microbiology*, **62**, no.10, 3847-3857.

215. Reddy, K.R., and D'Angelo, E.M. (1997) Biogeochemical indicators to evaluate pollutant removal efficiency in constructed wetlands. *Water Science and Technology*, **35**, no.5, 1-10.

216. Reddy, K.R., and D'Angelo, E.M. (1997) Biogeochemical indicators to evaluate pollutant removal efficiency in constructed wetlands. *Water Science Technology*, **35**, no.5, 1-10.

217. Reis, M.A.M., Lemos, P.C., Almeida, J.S., and Carrondo, M.J.T (1990) Influence of produced acetic acid on growth of sulfate reducing bacteria. *Biotechnology Letters*, **12**, no.2, 145-148.

218. Reynolds, J.S., Machemer, S.D., Updegraff, D.M., and Wildeman, T.R. (1997) Potentiometric titration method for determining rates of

sulfate reduction in a constructed wetland. *Geomicrobiology Journal*, **14**, 65-79.

219. Rhodes, S.H., Kelley, B.C., Peck, P.C., Longford, D.H., and Guerin, T.F. (1994) The implementation of bioremediation technology in the Australian mining industry. In: Biomine '94

220. Rittmann, B.E. (1992) Innovations in biological processes for pollution control. In: Environmental Microbiology, 265-286. Wiley-Liss.

221. Robertson, W.J., Franzman, P.D., and Mee, B.J. (2000) Spore-forming, *Desulfosporosinus*-like sulphate-reducing bacteria from a shallow aquifer contaminated with gasoline. *Journal of Applied Microbiology*, **88**, 248-259.

222. Rohwerder, T., Schippers, A., and Sand, W. (1997) Microcalorimetric quantification of bioleaching. In: Proceedings of the XX International Mineral Processing Congress, Aachen, Vol. 4, Solid-liquid Separation, Hydro- and Biohydrometallurgy. Hoberg, H., and von Blottnitz, H. (eds), Gesellschaft für Bergbau, Metallurgie, Rohstoff- und Umwelttechnik (GMDB), Clausthal-Zellerfeld, Germany, 475-484.

223. Rudd, J.W.M., Kelly, C.A. and Furutani, A. (1986) The role of sulfate reduction in long-term accumulation of organic and inorganic sulfur in lake sediments. *Limnology and Oceanography*, **31**, 1281-1291.

224. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science, **230**, 1350-1356.

225. Saitou, N., and Nei, M. (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolutionary*, **4**, 406-425.

226. Sanders, P.F., Lappin-Scott, H.M., and Bass, C.J. (1994) Corrosion implications of thermophilic sulphate-reducing bacteria in oil reservoirs. *Proceedings of UK Corrosion '94*, Bournemouth, U.K: **4**, 116-127.

227. Santegoeds, C.M., Ferdelman, T.G., Muyzer, G., and de Beer, D. (1998) Structural and functional dynamics of sulphate-reducing populations in bacterial biofilms. *Applied and Environmental Microbiology*, **64**, no.10, 3731-3739.

228. Sass, H., Cypionka, H., and Babenzien, H.-D. (1997) Vretical distribution of sulphate reducing bacteria at the oxic-anoxic interface in sediments of the oligotrophic Lake Stechlin. *FEMS Microbiology Ecology*, **22**, 245-255.

229. Saunders, S.E., and Burke, J.F. (1990) Rapid isolation of miniprep DNA for double strand sequencing. *Nucleic Acids Research* **18**, 4948.

230. Satake, K. (1977). Microbial sulphate reduction in a volcanic lake having pH 1.8 to 2.0. *Japanese Journal of Limnology*, **38**, 33-35.

231. Scheeren, P.J.H., Koch, R.O., and Buisman, C.J.N. (1993). Geohydrological containment system and microbial water treatment plant for metal-contaminated groundwater at Budelco. Proceedings of the International Symposium- World Zinc '93, Hobart, 10-13 October, 1993. 373-383.

232. Scheeren, P.J.H., Koch, R.O., Buisman, C.J.N., Barnes, L.J., and Versteegh, J.H. (1991) New biological treatment plant for heavy metal-contaminated groundwater. In: European Metals Conference '91: *Non-ferrous metallurgy - present and future*, 403- 416 (London, New York: Elsevier, 1991)

233. Schippers, A., Jozsa, P., Gehrke, T., Rohwerder, T., and Sand, W. (2000) Bacterial metal sulfide degradation – pathways, inhibition measures, and monitoring. In: ICARD 2000 (International Conference on Acid Rock Drainage), Volume I, 75-82. Society for Mining, Metallurgy, and Exploration, Inc. (SME), CO, USA. ISBN 0-87335-182-7.

234. Sen, A.M., and Johnson, D.B. (1999). Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage. In: In: Amils, R., and Ballester, A. (eds.) *Biohydrometallurgy and the environment toward the mining of the 21st century*, Proceedings of the

International Biohydrometallurgy Symposium, 709-718. Process metallurgy 9A: Amsterdam: Elsevier.

235. Silva, G., Oliveira, S., Gomes, C.M., Pacheco, I., Liu, M.Y., Xavier, A.V., Teixeira, M., LeGall, J.. and Rodrigues-Pousada, C. (1999) *Desulfovibrio gigas* neelaredoxin - a novel superoxide dismutase integrated in a putative oxygen sensory operon of an anaerobe. *European Journal of Biochemistry*, **259,** 1-2, 235-243.

236. Singleton, R. Jr. (1993) The sulphate-reducing bacteria: an overview. In: Odom, J.M., and Singleton, R. (eds) *The sulfate-reducing bacteria: contemporary perspectives.* Brock/Springer Series, Springer-Verlag, New York. 1-20.

237. SME (Society for Mining, Metallurgy, and Exploration Inc.) 2000. Proceedings from the Fifth International Conference on Acid Rock Drainage (ICARD), Volumes I & II, CO, USA.

238. Somlev, V., and Tishkov, S. (1994) Anaerobic corrosion and bacterial sulfate reduction: application for the purification of wastewater. *Geomicrobiology Journal*, **12**, 53-60.

239. Speich, N., and Trüper, H.G. (1988) Adenylylsulphate reductase in a dissimilatory sulphate-reducing archaebacterium. *Journal of General Microbiology*, **134**, 1419-1425.

240. Spratt, H.G., Morgan, M.D., and Good, R.E. (1987) Sulphate reduction in peat from a New Jersey Pinelands cedar swamp. *Applied and Environmental Microbiology*, **53**, 1406-1411.

241. Stackebrandt, E., Stahl, D.A., and Devereux, R. (1995) Taxonomic relationships. In: Barton, L.L. (ed) *Sulfate-reducing bacteria*, 49-87. Plenum Press, New York.

242. Stackebrandt, E., Sproer, C., Rainey, F.A., Burghardt, J., Päuker, O., and Hippe, H. (1997) Phylogenetic analysis of the genus *Desulfotomaculum*: evidence for the misclassification of *Desulfotomaculum guttoideum* and description of *Desulfotomaculum orientis* as *Desulfosporosinus orientis* gen. nov., comb. nov. *International Journal of Systematic Bacteriology*, **47**, no.4, 1134-1139.

243. Stetter, K.O., Lauerer, G., Thomm, M., and Neuner, A. (1987) Isolation of extremely thermophilic sulfate reducers: evidence for a novel branch of Archaebacteria. *Science*, **236**, 822-824.

244. Stober, J.T, O'Connor, J.T., and Brazos, B.J. (1997) Winter and spring evaluations of a wetland for tertiary wastewater treatment. *Water Environment Research*, **69**, no. 5, 961-968.

245. Stoodley, P., Hall-Stoodley, L., Boyle, J.D., Jørgensen, F., and Lappin-Scott, H.M. (2000) Environmental and genetic factors influencing biofilm structure. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms.* Fifty-ninth Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

246. Stookey, L.L. (1970) Ferrozine – a new spectrophotometric reagent for iron. *Analytical Chemistry*, **42**, 779-781.

247. Szewzyk, R., and Pfennig, N. (1990) Competition for ethanol between sulphate reducing and fermenting bacteria. *Archives of Microbiology*, 153, 470-477.

248. Tarutis, W.J., and Unz, R.F. (1995) Iron and manganese release in coal mine drainage wetland microcosms. *Water Science and Technology*, **32**, no. 3, 187-192.

249. Temple, K.L., and Koehler, W.A. (1954). Drainage from bituminous coal mines. Engineering Experimental Station, University of West Virginia, Morgantown, Research Bulletin, no.25.

250. Thamdrup, B., Finster, K., Hansen, J.W., and Bak, F. (1993) Bacterial disproportionation of elemental sulphur coupled to chemical reduction of iron or manganese. *Applied and Environmental Microbiology*, **59**, 101-108.

251. Thauer, R.K., Jungermann, K., and Decker, K. (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriology Reviews*, **41**, 100-180.

252. Thauer, R.K. (1989) Energy metabolism of sulphate reducing bacteria. In: Schlegel, H.G., and Bowien, B. (eds.) *Autotrophic Bacteria*, 397-413. Science Tech Publishers, Madison, USA.

253. Thauer, R.K., and Kunow, J. (1995) Sulfate-Reducing Archaea. In: Barton, L.L. (ed.) Sulfate-reducing bacteria (pp 151-184). Biotechnology Handbooks, no. 8 (Atkinson, T., and Sherwood, R.F., series eds.), Plenum Press, New York.

254. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.

255. Torma, A. E. (1986) Biohydrogeology as an emerging technology. *Biotechnology and Bioengineering*, **16**, 49-63.

256. Tuovinen, O.H., and Kelly, D.P. (1973) Studies on the growth of Thiobacillus ferrooxidans I. Use of membrane filters and ferrous iron agar to determine viable numbers, and comparison with $^{14}CO_2$ – fixation and iron oxidation as measures of growth. *Arch. Mikro biol.*, **88**, 285-298.

257. Tuttle, J.H., Dugan, P.R., MacMillan, C.B., and Randles, C.I. (1969a) Microbial dissimilatory sulfur cycle in acid mine water. *Journal of Bacteriology*, **97**, 594-602.

258. Tuttle, John H; Dugan, Patrick R.; and Randles, Chester I. (1969b) Microbial sulphate reduction and its potential ability as an acid mine drainage pollution abatement procedure. *Applied Microbiology*, **97**, no.2, 594-604.

259. Tyrell, W.R., Mulligan, D.R., Sly, L.I., and Bell, L.C. (1997). Trialing wetlands to treat coal mining wastewaters in a low rainfall, high evaporation environment. *Water Science and Technology*, **35**, no.5, 293-299.

260. Utkin, I., Woese, C., and Wiegel, J. (1994) Isolation and characterisation of *Desulfitobacterium dehalogenans*, gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates

chlorophenolic compounds. *International Journal of Systematic Bacteriology*, **44**, no.4, 612-619.

261. Unz, R.F., and Dietz, J.M. (1986) Biological applications in the treatment of acidic mine drainages. *Biotechnology and Bioengineering Symposium*, **16**, 163-170.

262. van Houten, R.T., Hulschoff Pol, L.W., Lettinga, G. (1994) Biological sulphate reduction using gas-lift reactors fed with hydrogen and carbon dioxide as energy and carbon source. *Biotechnology and Bioengineering*, **44**, 586-594.

263. van Houten, R.T.; Oude Elferink, S.J.W.H.; van Hamel, S.E.; Hulshoff Pol, L.W.; and Lettinga, G. (1995) Sulphate reduction by aggregates of sulphate-reducing bacteria and homo-acetogenic bacteria in a lab-scale gas-lift reactor. *Bioresource Technology*, **54**, 73-79.

264. van Houten, R.T., and Lettinga, G. (1995) Treatment of acid mine drainage with sulphate-reducing bacteria using synthesis gas as energy and carbon source. Med. Fac. Landbouww., University of Gent, 60/4b, 2693-2700.

265. van Houten, R.T., and Lettinga, G. (1996) Biological sulphate reduction with synthesis gas: microbiology and technology. In: Wijffels, R.H., Buitelaar, R.M., Bucke, C., and Tramper, J. (eds.) *Immobilized Cells: Basics and Applications*. Elsevier Science B.V.

266. Videla, H.A. (2000) An overview of mechanisms by which sulphate-reducing bacteria influence corrosion of steel in marine environments. *Biofouling*, **15**, 1-3, 37-47.

267. Vile, M.A., and Wieder, R.K. (1993) Alkalinity generation by Fe(III) reduction versus sulfate reduction in wetlands constructed for acid mine drainage treatment. *Water, Air and Soil Pollution*, **69**, 425-441.

268. Wakao, Norio; Takahashi, Taeko; Sakurai, Yonekichi; and Shiota, Hideo (1979) A treatment of acid mine water using sulfate-reducing bacteria. *Journal of Fermentation Technology*, **57**, no.5, 445-452.

269. Webb, J.S., McGinness, S., and Lappin-Scott, H.M. (1998) Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. *Journal of Applied Microbiology*, 84, 240-248.

270. White, C., and Gadd, G.M. (1996) A comparison of carbon/energy and complex nitrogen sources for bacterial sulphate-reduction: potential applications to bioprecipitation of toxic metals as sulphides. *Journal of Industrial Microbiology*, **17**, 116-123.

271. Widdel, F. (1986) Sulphate reducing bacteria and their ecological niches. In: Barnes, E.M. and Mead, G.C. (eds.) *Anaerobic bacteria in habitats other than man.* Blackwell Scientific Publications, UK.

272. Widdel, F. (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In: Zehnder, J.B. (ed.), *Biology of Anaerobic Microorganisms.* Wiley Series in Ecological and Applied Microbiology, Wiley, NY, 469-585.

273. Widdel, F. (1992a) The genus *Desulfotomaculum.* In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds) *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, Vol. IV, 2nd edn., 1792-1799. New York: Springer-Verlag.

274. Widdel, F. (1992b) The genus *Thermodesulfobacterium.* In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds) *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications,* Vol. IV, 2nd edn., 3390-3392. New York: Springer-Verlag.

275. Widdel, F. and Bak, F. (1992) Gram-negative mesophilic sulphate reducing bacteria. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds) *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications,* Vol. IV, 2nd edn., 3352-3378. New York: Springer-Verlag.

276. Widdel, F., and Hansen, T. (1992) The dissimilatory sulphate- and sulphur-reducing bacteria. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H. (eds) *The Prokaryotes: A Handbook on*

the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, Vol. IV, 2nd edn., 583 – 624. New York: Springer-Verlag.

277. Wieder, R.K. (1993) Ion input-output budgets for five wetlands constructed for acid coal mine drainage treatment. *Water, Air and Soil Pollution*, **71**, 231-270.

278. Wieder, R.K., and Lang, G.E. (1982) Modification of acid mine drainage in a freshwater wetland. In: McDonald, B.R. (ed.) *Proceedings of the Symposium on Wetlands of the Unglatiated Appalachian Region*, West Virginia University, Morgantown, WV.

279. Wieder, R.K., and Lang, G.E. (1984) Influence of wetlands and coal mining on stream water chemistry. *Water, Air and Soil Pollution*, **23**, 381-396.

280. Wildlife News (1999) http://web.ukonline.co.uk/k.heath/wildlife/article/donana.htm

281. Wildeman, T., and Cevaal, J.N. (1994) Constructed wetlands use natural processes to treat acid mine drainage. *The Hazardous Waste Consultant*, July/August 1994, 1.24-1.28.

282. Williams, P., and Stewart, G.S.A.B. (1994) Cell density dependent control of gene expression in bacteria-implications for biofilm development and control. In: Wimpenny, J.W.T., Nichols, W., Stickler, D., and Lappin-Scott, H.M. (eds.): *Bacterial Biofilms and their Control in Medicine and Industry*, pp 9-12. Cardiff: Bioline.

283. Willis, A.T. (1969) Techniques for the study of anaerobic, spore-forming bacteria. In: Norris, J.R., and Ribbons, D.W. (eds) (1969) *Methods in Microbiology*, Vol. 3B.

284. Wilson, K. (1987) Preparation of genomic DNA from bacteria. In: Ausubel, F.M., Brent, R., Kingston, R.E. , Moore, D.D., Seidman, J.A. and Struhl, K. (eds.) *Current Protocols in Molecular Biology*, p. 2.4.1. – 2.5.4. New York: Green and Wiley Interscience.

285. Wimpenny, J. (2000) An overview of biofilms as functional communities. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms.*

Fifty-ninth Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

286. Wingender, J., and Flemming, H-C. (1999) Autoaggregation in flocs and biofilms. In: Winter, J. (ed.) *Biotechnology*, vol.8, 63-86. Weinheim:VCH.

287. Wingender, J., Neu, T., and Flemming, H-C. (1999) What are extracellular polymeric substances? In: Wingender, J., Neu, T., and Flemming, H-C. (eds.) *Microbial Extracellular Polymeric Substances*, 1-19. Heidelberg, Berlin and New York: Springer.

288. Woese, C.R., Achenbach, L., Rouvière, P., and Mandelco, L. (1991) Archaeal phylogeny: reexamination of the phylogenetic position of Archaeoglobus fulgidus in light of ceratin composition-induced artifacts. *Systematic and Applied Microbiology*, **14**, 364-371.

289. Wolfaardt, G.M., and Cloete, T.E. (1992) The effect of some environmental parameters on surface colonization by microorganisms. *Water Research*, **26**, no. 4, 527-537.

290. Wolfaardt, G.M., Korber, D.R., Karthikeyan, S., and Caldwell, D.E. (2000) Biodegradation by biofilm communities. In: Allison, D.G., Gilbert, P., Lappin-Scott, H.M., and Wilson, M. (eds.) *Community structure and co-operation in biofilms*. Fifty-ninth Symposium of the Society for General Microbiology, September 2000, University of Exeter. Cambridge University Press.

291. Younger, P.L. (1997) The longevity of minewater pollution: a basis for decision-making. *The Science of the Total Environment*, **194/195**, 457-466.

292. Zavilgelsky, G.B., and Manukhov, I.V. (2001) Quorum sensing, or how bacteria "talk" to each other. *Molecular Biology*, **35**, no. 2, 224-232.

293. Zehnder, A.J.B., and Wuhrmann, K. (1976) Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. *Science*, **194**, 1165-1166.

294. Ziemkiewicz, P.F., Skousen, J.G., and Lovett, R. (1994) Open limestone channels for treating acid mine drainage: A new look at an old idea. *Green Lands*, **24**, 36-41. West Virginia Mining Reclamation Association, Charleston.

295. Ziemkiewicz, P.F., Skousen, J.G., Brant, D.L., Sterner, P.L., and Lovett, R.J. (1997) Acid mine drainage treatment with armored limestone in open limestone channels. *Journal of Environmental Quality*, **26**, 1017-1024

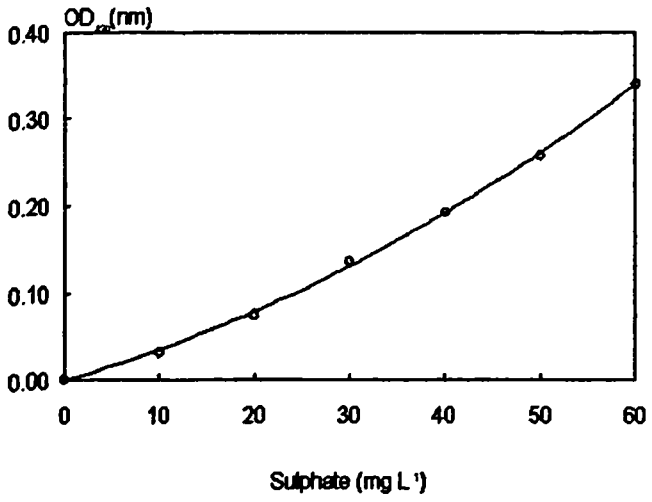
296. Zinkevich, V., and Beech, I.B. (2000) Screening of sulfate-reducing bacteria in colonoscopy samples from healthy and colitic human gut mucosa. *FEMS Microbiology Ecology*, **34**, 147-155.

297. Zobell, C. (1958) Ecology of sulphate reducing bacteria. *Producers Mon. Penn. Oil Prod. Ass.*, **22**, 12-29.

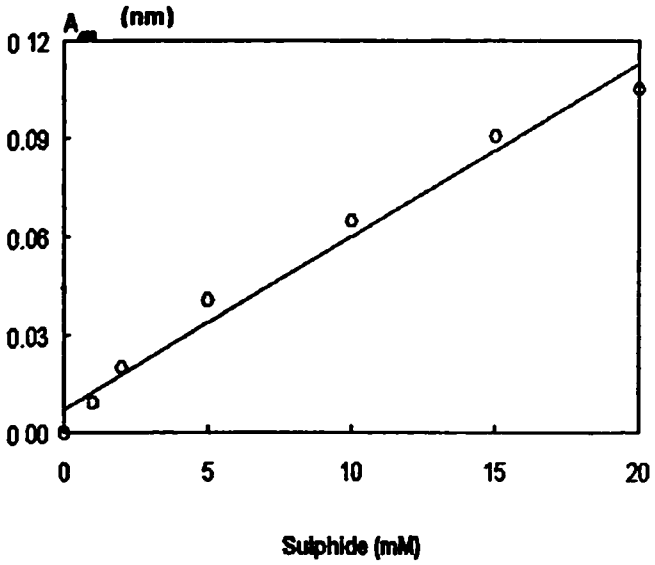
APPENDIX 1.1

Nordstrom *et al.* (2000) claim that the mine waters at this site are the most acidic known, and contain very high levels of dissolved metals and sulphate. In this study it was demonstrated that pH can be defined and measured below zero, and that effluents with such a low pH exist in the environment. According to the authors, there are a number of limitations to measurements of very low pH, such as the range of standard pH buffers, and interferences with the reversible response of the glass H^+ - sensitive membrane electrode. This study documents methods of calibration and measurements, which are recommended for use in ultra-acidic waters. Theroretical pH values were calculated using the Pitzer model (Pitzer, 1991).

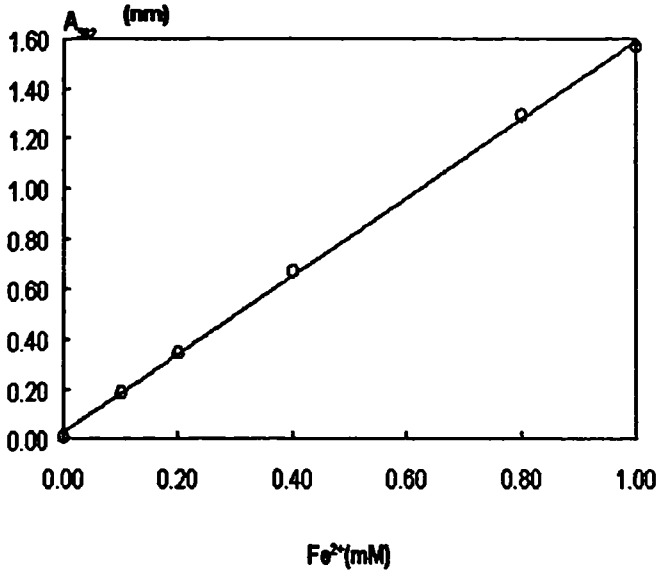
APPENDIX 2.1



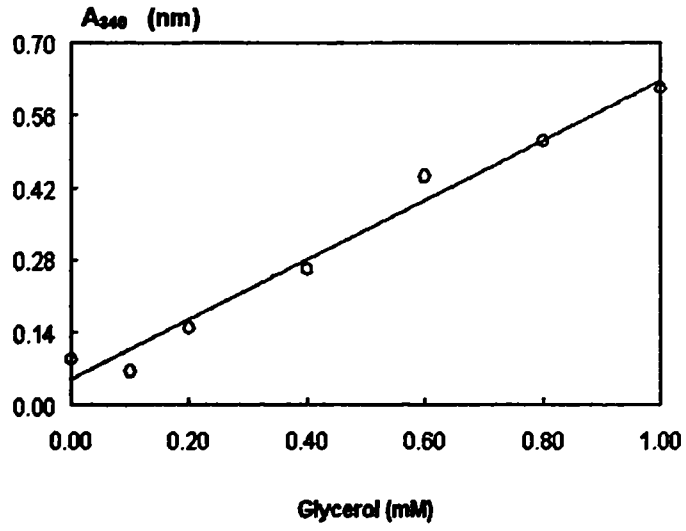
2.1(a). A typical standard curve for the assay of sulphate ion using the turbidimetric method (modified from Rand *et al.*, 1975). The equation of the polynomial curve is -0.003082 + SQRT ((9.4 x 10^{-6})-(1.72 x 10^{-4})*(0.00003-OD_{420}))/(8.6 x 10^{-5}). Rval = 0.9996.



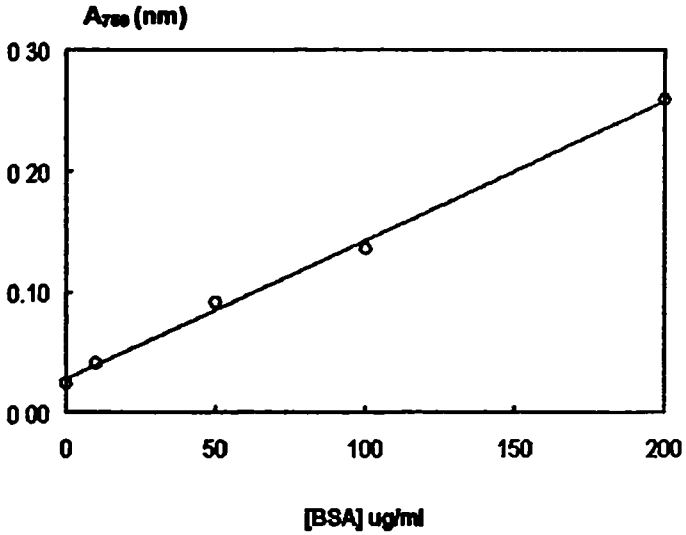
2.1(b). A typical standard curve for sulphide using the copper sulphate assay devised by Cord-Ruwisch (1985). The equation of the fitted line is y = (0.005274*x) + 0.07379. Rval = 0.989



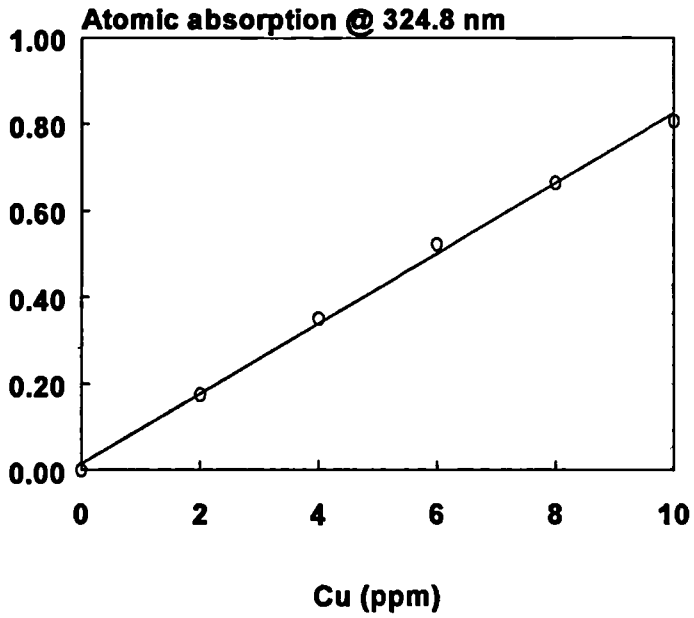
2.1 (c). A typical standard curve for the assay of ferrous iron using ferrozine. The equation of the fitted line is y = (1.560427*x) + 0.027672. Rval = 0.999



2.1 (d). A typical standard curve obtained by running glycerol standards using the colorimetric assay for 1,2-diols. The equation of the fitted line is y = (0.576246*x) + 0.049305. Rval = 0.988



2.1 (e). A typical standard curve for an assay for protein (Lowry *et al.*, 1951). Standards of bovine serum albumin (BSA) were made and absorbance at 750 nm determined. The equation of the fitted line is y = (0.0011*x) + 0.031027. Rval = 0.998



2.1 (f). A typical standard curve for the measurement of copper ions using AAS. Values shown are means of three readings taken for each standard solution of 2, 4, 6, 8 and 10 ppm. The equation of the fitted line is y = (0.081143* x)+ 0.014286. Rval = 0.998.

APPENDIX 5.1

Properties of Poraver glass beads: Estimation of density

380 ml of Poraver (size 5-6 mm) were weighed wet and dry.

Wet weight = 229.7 g
Dry weight = 54.27 g
∴ Volume of water in the pores of the Poraver = 174 ml (= 174 g).

500 ml of water was added to the Poraver in a large measuring cylinder and allowed to settle.

The following was then measured:

Total volume = 720 ml
∴ Volume of Poraver (solid volume) = 220 ml

So: Total water volume = 500 + 174 = 674 ml
Total volume including Poraver = 720 ml
∴ Solid volume of Poraver = 46 ml

Therefore, if the volume of Poraver = 380 ml, and
mass = 54 g

Density = Mass / Volume
∴ **(Apparent) Density = 0.14 g/cm^3**

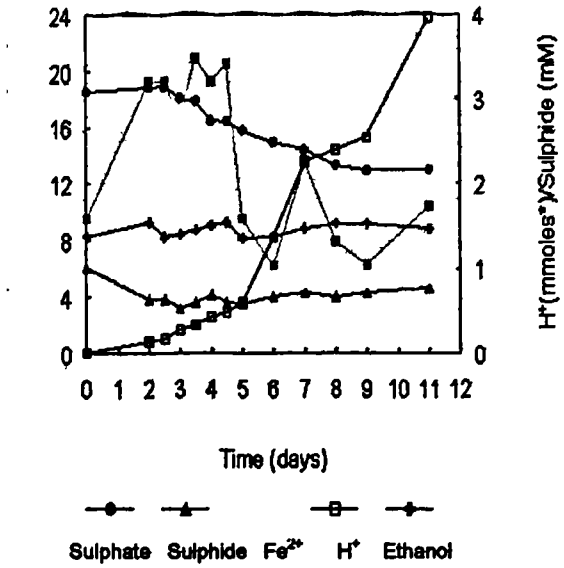
However, the porosity of the Poraver needs to be estimated so that the true density of the material can be calculated.

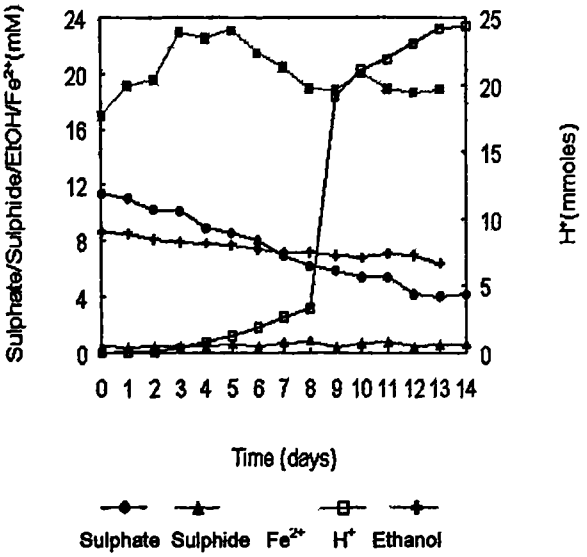
Porosity: in a 380 ml sample of Poraver, solid volume is 46 ml, therefore 334 ml is air. Pore space = 334 x 100/380 = 87.89
∴ **Pore space = ~ 88 %**

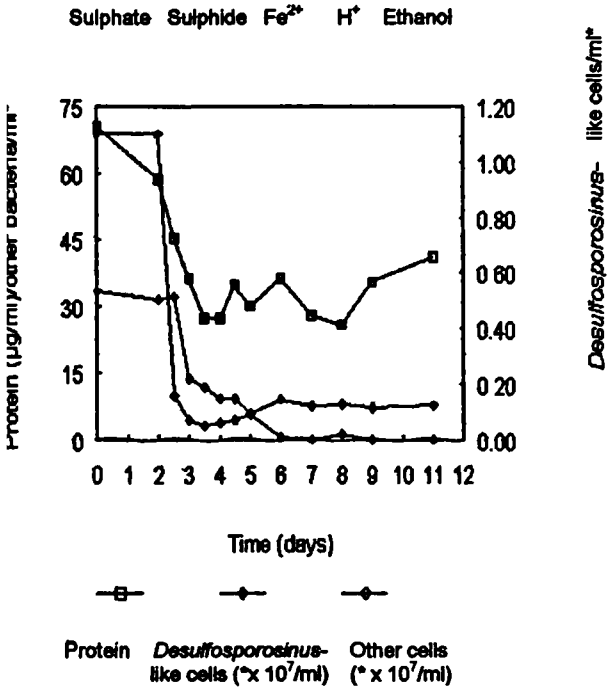
The actual volume of the Poraver is not 380 ml but 46 ml. Therefore the **actual density of the Poraver = 54/46 = 1.17 g/cm^3**

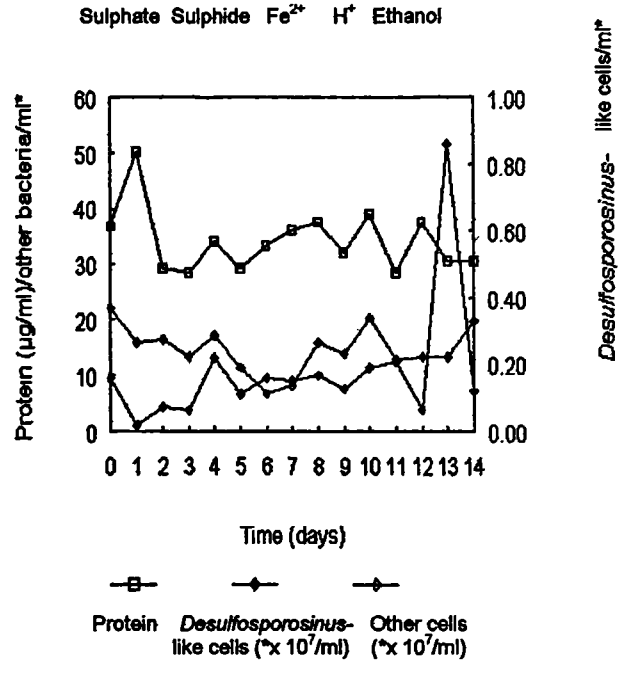
As this figure is greater than 1, this explains how it is still possible for Poraver beads to sink despite their apparent low density, and the fact that they are ~ 88% air.

APPENDIX 5.2



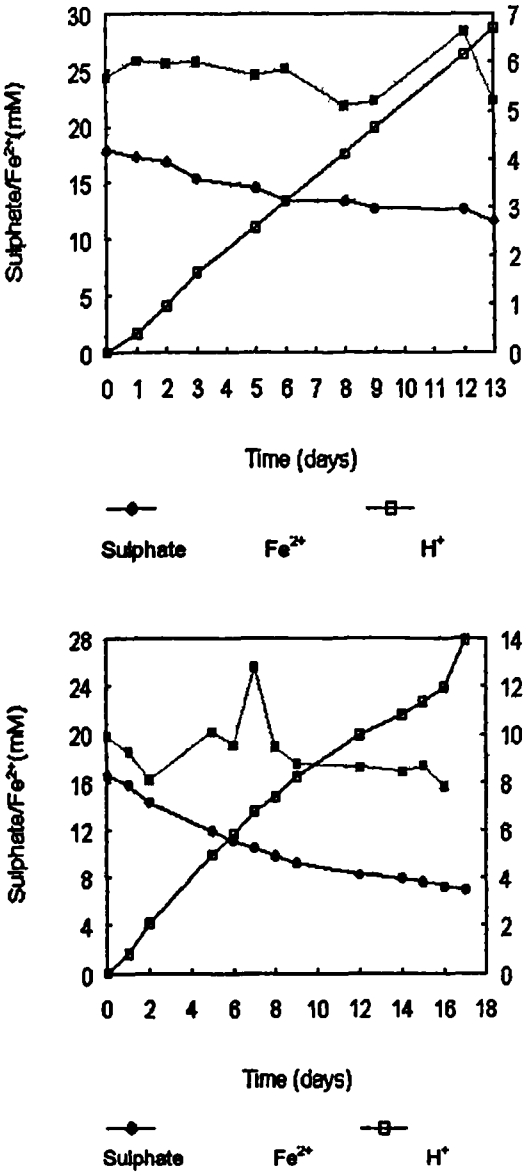




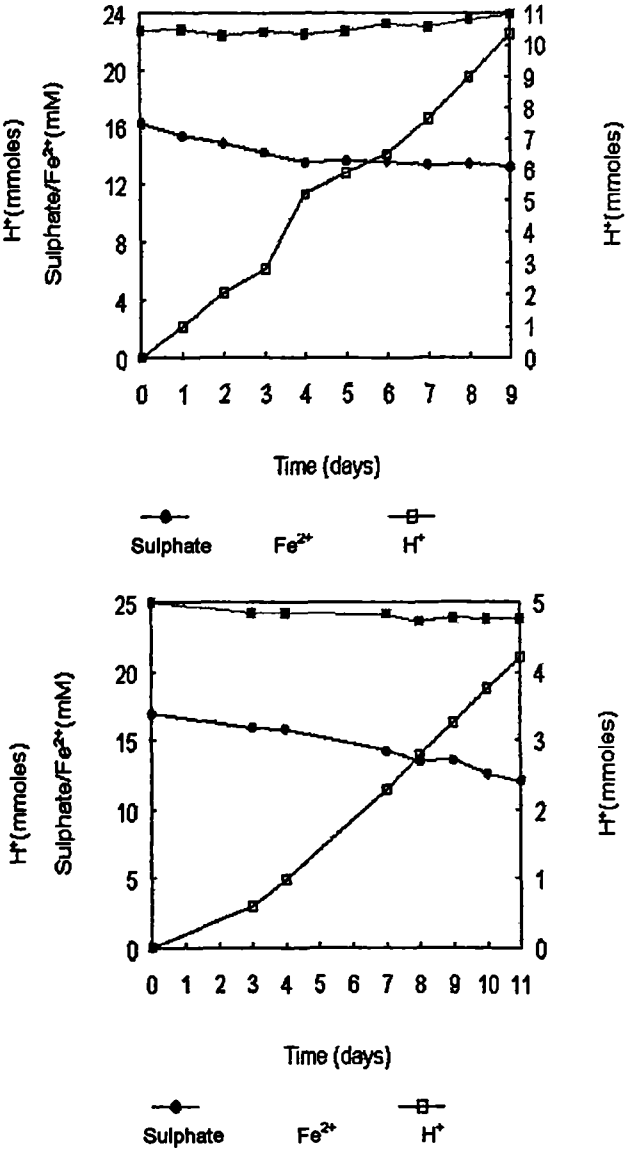


Chemical and microbiological data for the mixed culture system (Parys) run in batch mode, pH 3.5 (without OFN sparging). H^+ is expressed in mmoles required for pH maintenance (cumulative).

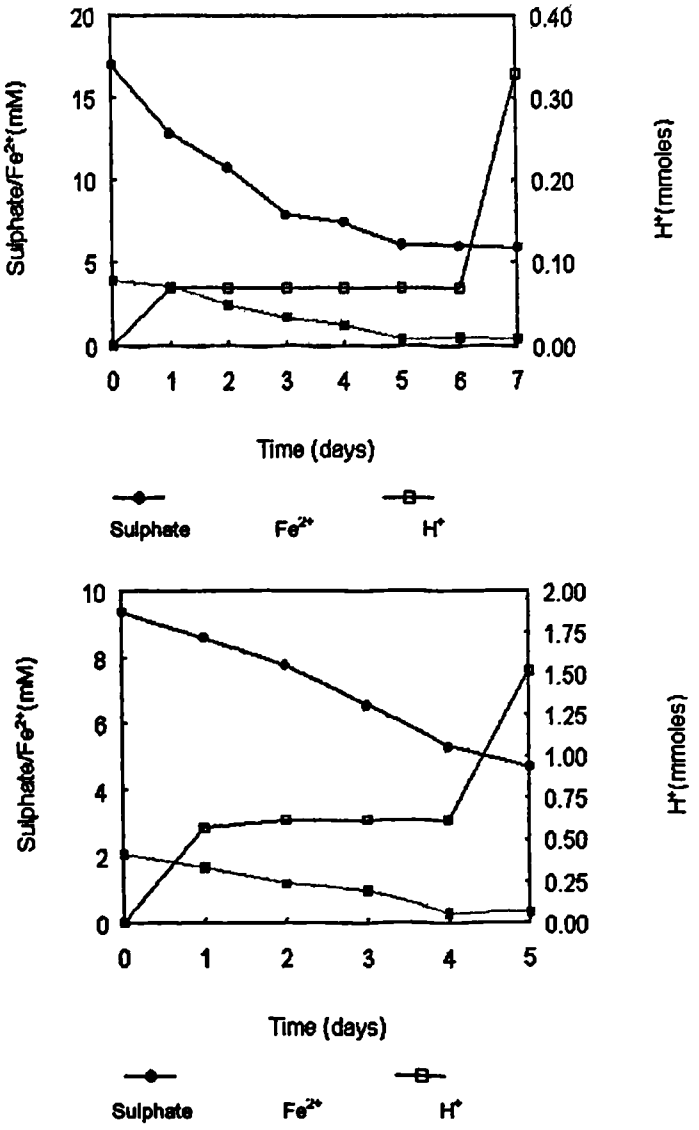
Chemical and microbiological data for the mixed culture system (Parys), pH 3.5 (with sparging). H^+ is expressed in mmoles required for pH maintenance (cumulative). (N.B. For y-axis labels refer to graphs on left).



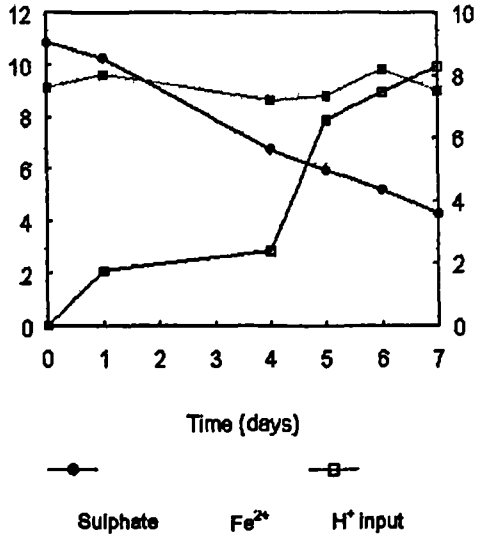
Chemical data for the mixed culture system (Parys), top: pH 3.5, with sparging; bottom, pH 3.0. H^+ is expressed in mmoles required for pH maintenance (cumulative)



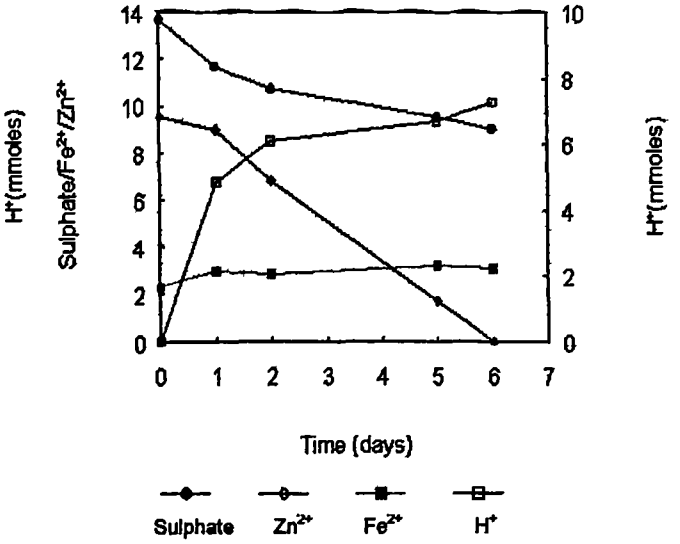
Chemical data for the mixed culture system (Parys), top: pH 2.5; bottom: pH 4.5. H^+ is expressed in mmoles required for pH maintenance (cumulative)

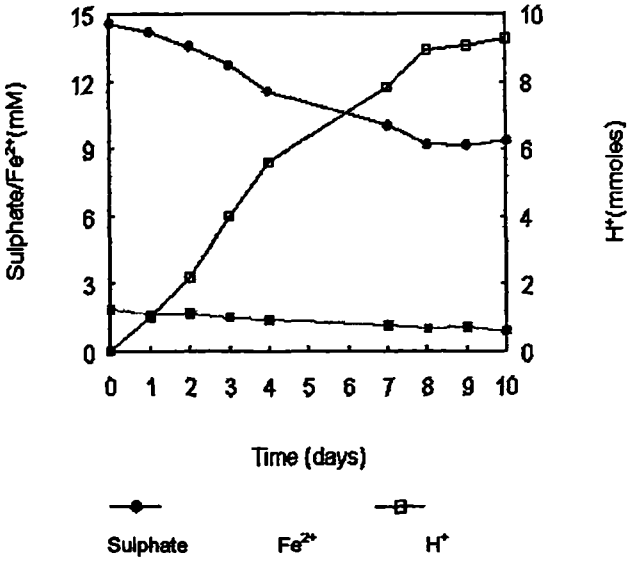


Chemical data for the mixed culture system (Parys), top: pH pH 5.5; bottom, pH 5.5 and low [Fe^{2+}]. H^+ is expressed in mmoles required for pH maintenance (cumulative)

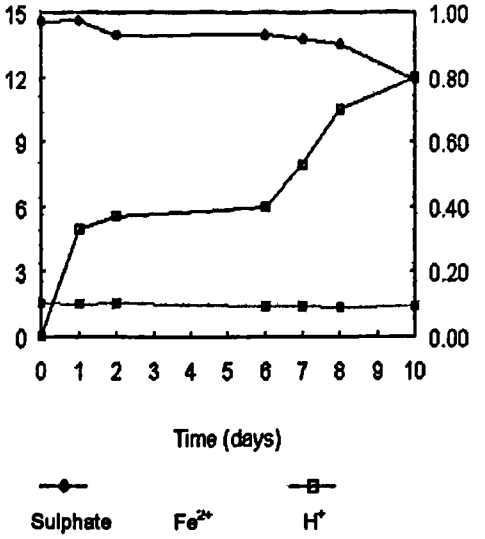


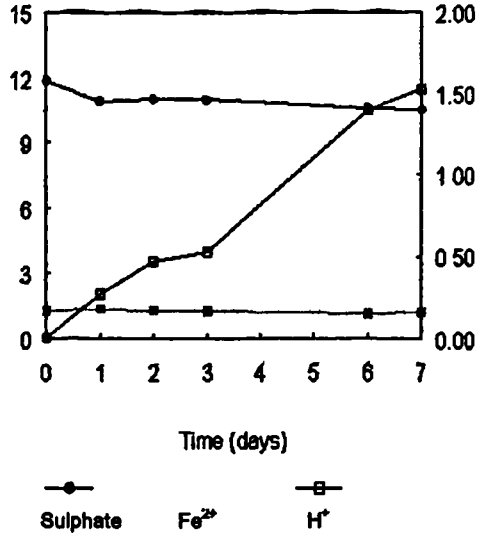
Chemical data for the mixed culture system (Montserrat) run in batch mode, pH 4.0. H^+ is expressed in mmoles required for pH maintenance (cumulative).



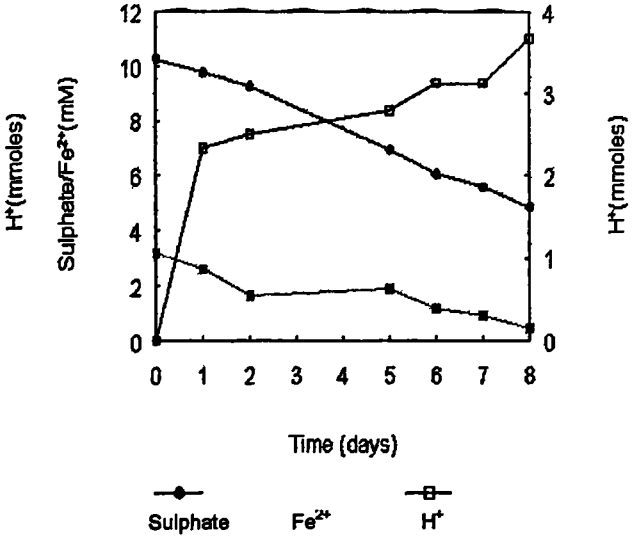


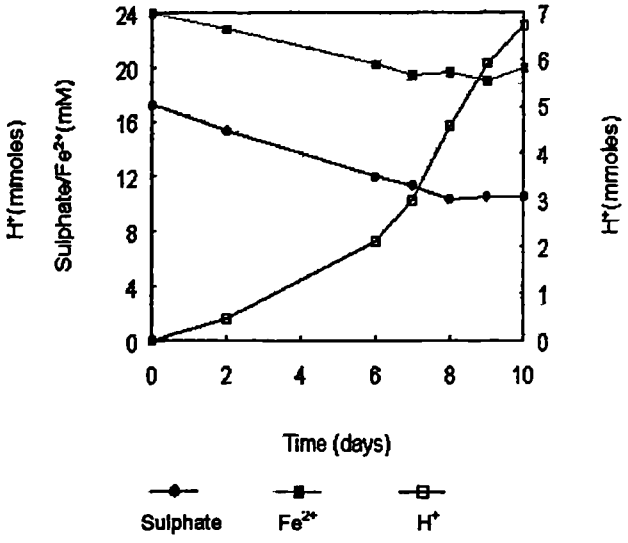
Chemical data for the mixed culture system (Montserrat) run in batch mode, top: pH 4.0 with medium containing $ZnSO_4$; bottom: pH 4.0 and low $[Fe^{2+}]$. H^+ is expressed in mmoles required for pH maintenance (cumulative).



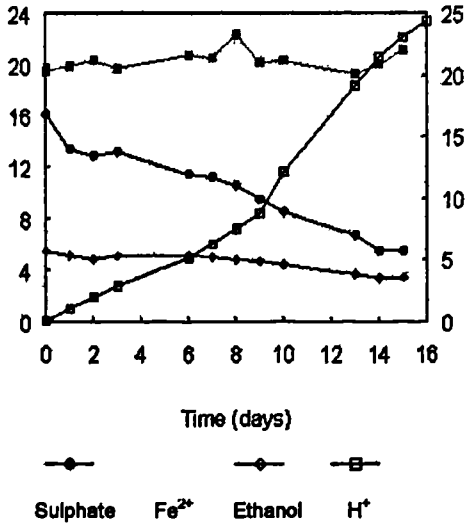


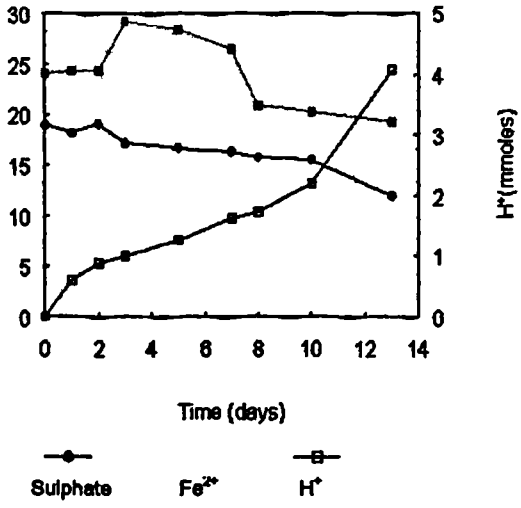
Chemical data for the mixed culture system (Montserrat) run in batch mode, top: pH 4.0, low [Fe^{2+}], 20°C; bottom, pH 4.0, low [Fe^{2+}], 25°C. H^+ is expressed in mmoles required for pH maintenance (cumulative).



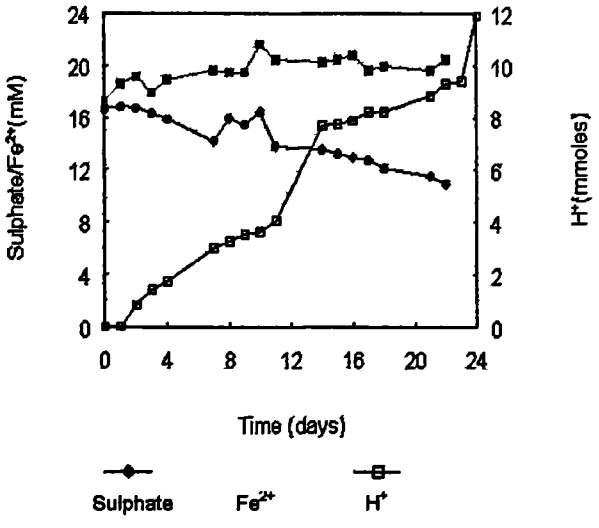


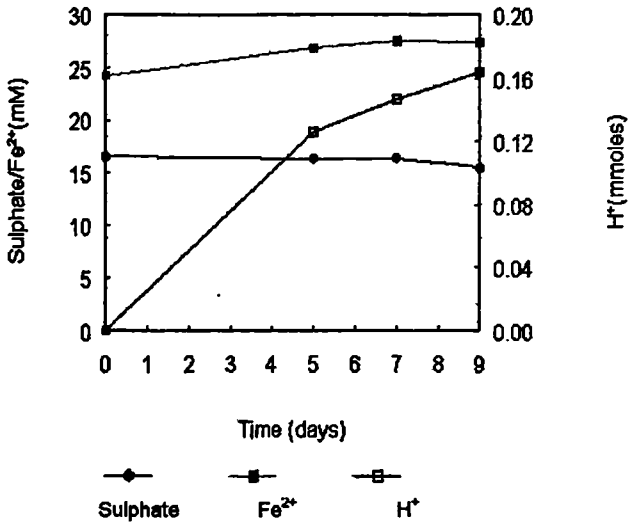
Chemical data for the mixed culture system (Montserrat) run in batch mode, top: pH 5.5; bottom: pH 4.5. H^+ is expressed in mmoles required for pH maintenance (cumulative).





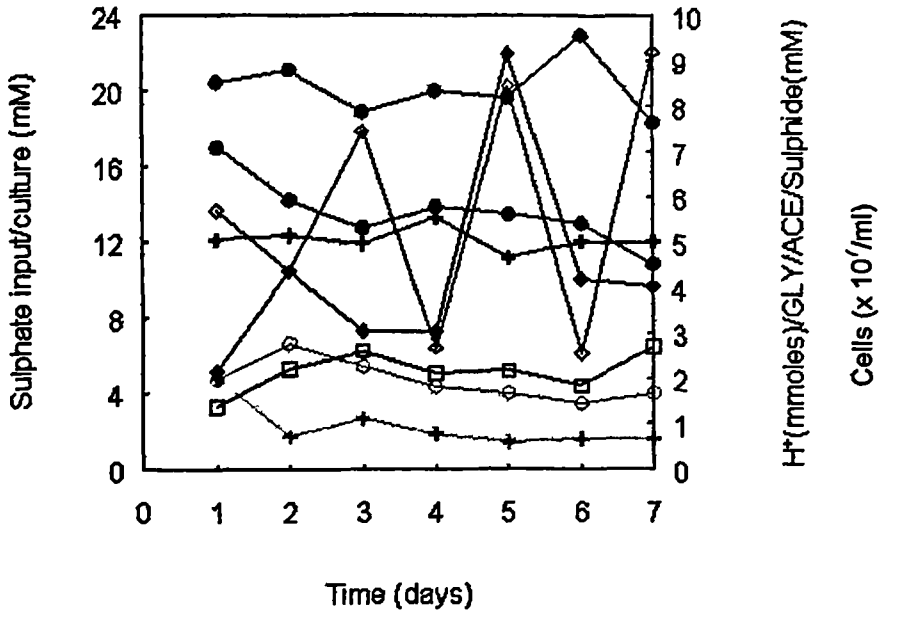
Chemical data for the mixed culture system (Montserrat) run in batch mode, top: pH 3.5; bottom: also pH 3.5, four months later. H^+ is expressed in mmoles required for pH maintenance (cumulative).

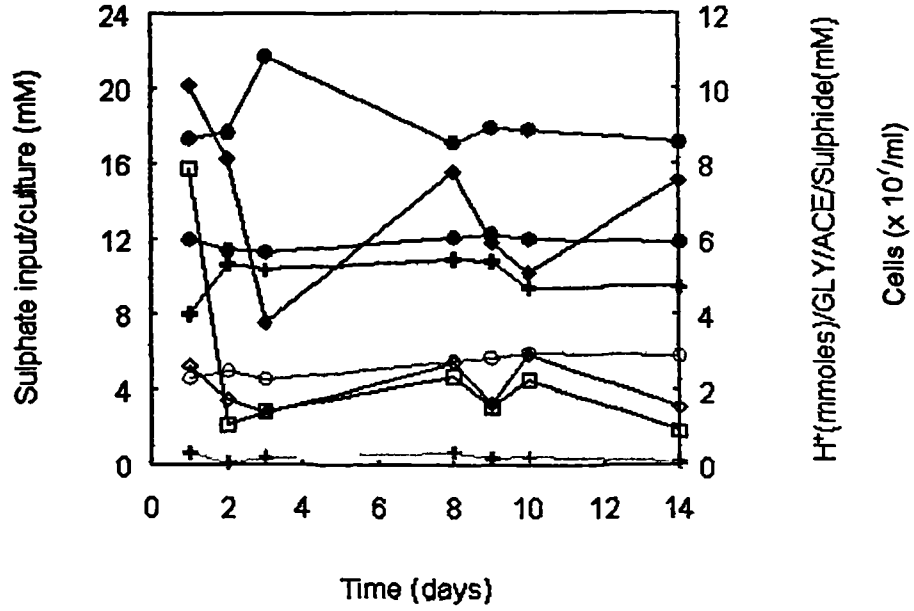




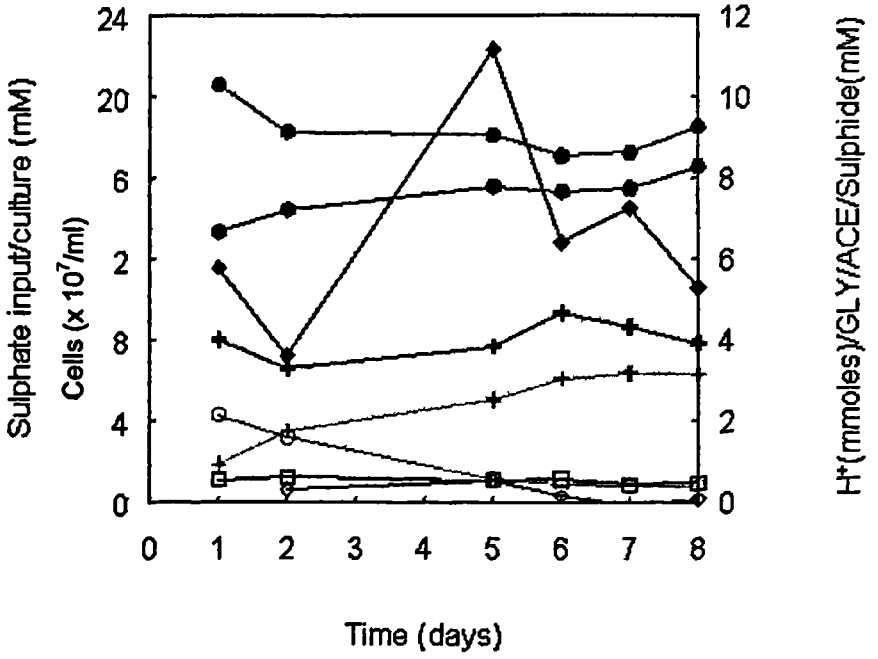
Chemical data for the mixed culture system (Montserrat) run in batch mode, top: pH 3.0; bottom: pH 2.5. H^+ is expressed in mmoles required for pH maintenance (cumulative).

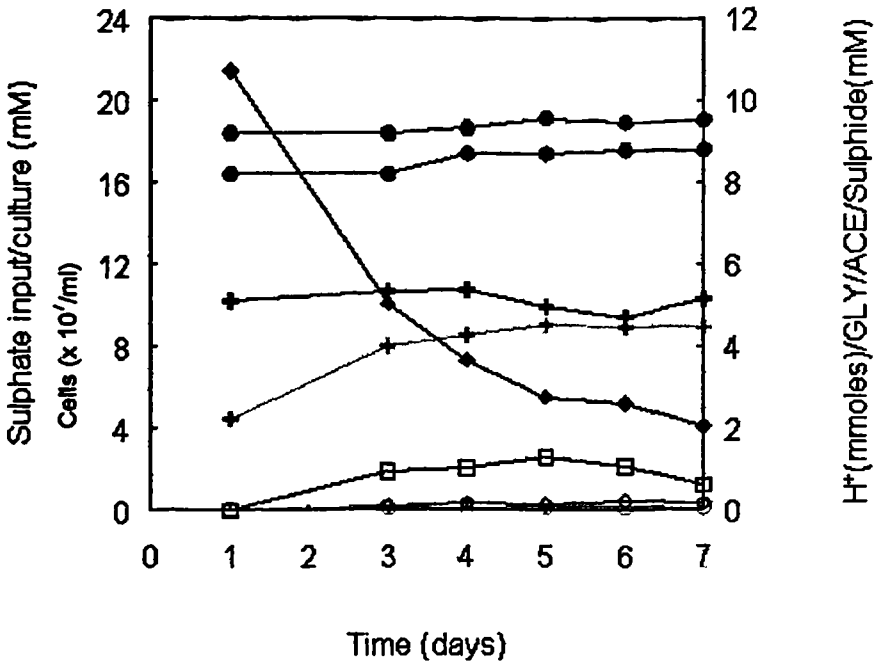
APPENDIX 5.3



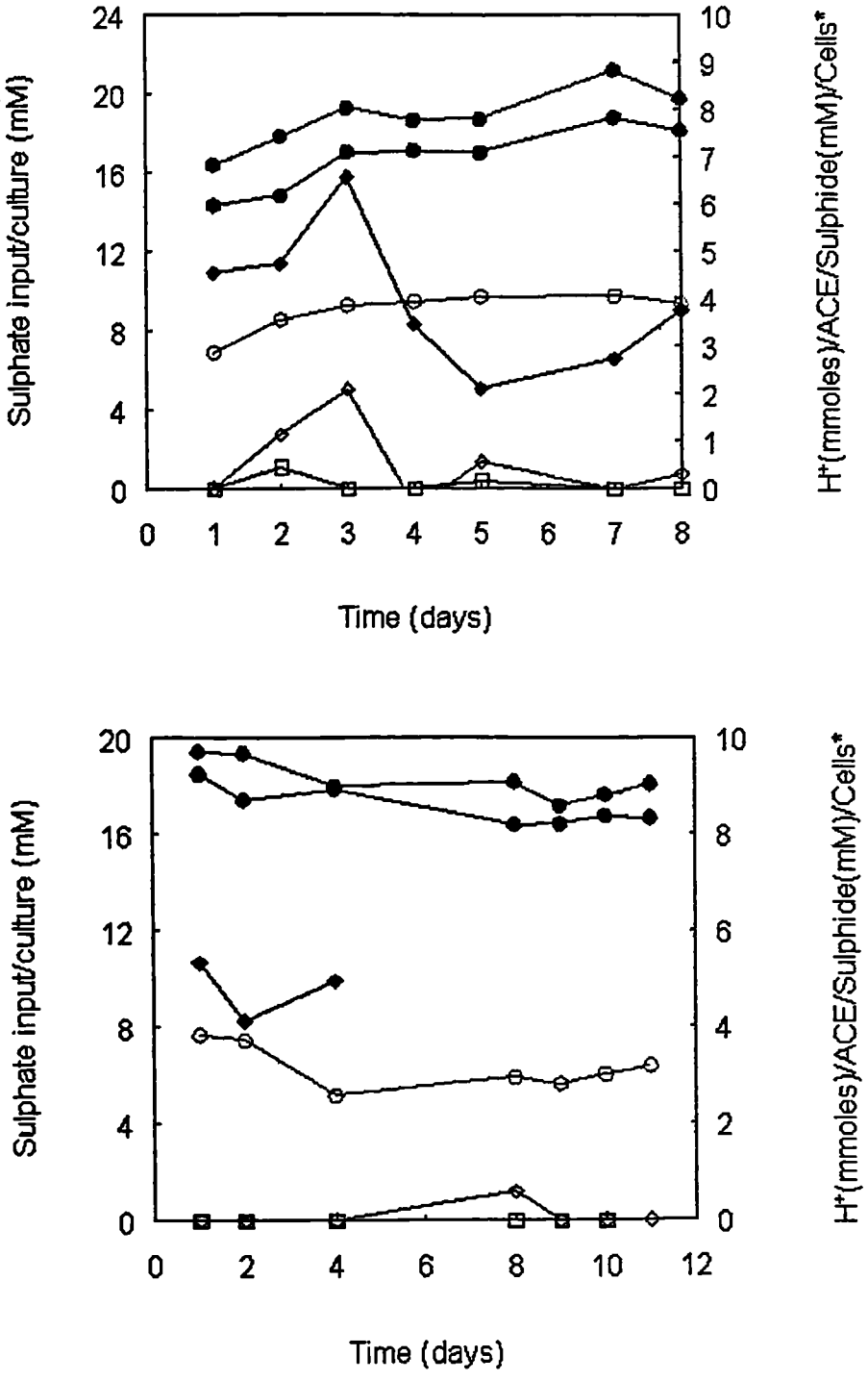


Chemical and microbiological data for mixed culture (Parys) run (continuous mode); top: pH = 4.0, HRT= 33 h; bottom: pH = 3.5, HRT= 66 h. Key: ● [sulphate] (inflow); ● = [sulphate] (culture vessel); ✚ = [glycerol] (inflow); ✚ = [glycerol] (culture vessel); O = [acetate] (culture vessel); ◆ = cell nos. (x 10^7/ml); ◇ = [dissolved sulphide]; □ = H^+ addition (total in mmoles). GLY= glycerol; ACE = acetate.





Chemical and microbiological data for mixed culture (Parys) run (continuous mode); top: pH = 3.0, HRT= 66 h; bottom: pH = 2.5, HRT= 66 h. Key: ● [sulphate] (inflow); ● = [sulphate] (culture vessel); ✚ = [glycerol] (inflow); ✚ = [glycerol] (culture vessel); ○ = [acetate] (culture vessel); ◆ = cell nos. (x 10^7/ml); ◇ = [dissolved sulphide]; □ = H^+ addition (total in mmoles). GLY= glycerol; ACE = acetate.



Chemical and microbiological data for mixed culture (Parys) run (continuous mode); top: pH = 4.0, HRT= 33 h, bottom: pH = 4.8, HRT= 66 h. Key: ● [sulphate] (inflow); ● = [sulphate] (culture vessel); O = [acetate] (culture vessel); ◆ = *cell nos. (x 10^7/ml); ◇ = [dissolved sulphide]; □ = H^+ addition (total in mmoles). ACE = acetate.