MICRO-ORGANISMS INVOLVED IN IRON OXIDATION AND ACID MINE DRAINAGE FORMATION IN KWAZULU-NATAL AND THEIR CONTROL BY SOIL COVERS ON COAL WASTE DUMPS

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DECLARATION

I, the undersigned, declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature

Date

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SUMMARY

The biologically catalysed oxidation of pyrite in the outer layers of coal waste dumps leads to the formation of acid mine drainage. The oxidation of pyrite to ferric iron and sulphate is a complex process involving various abiotic and biologically catalysed reactions. Pyrite is abiotically oxidized by ferric iron, with the formation of thiosulphate and ferrous iron. Thiosulphate decomposes to form various inorganic sulphur compounds. Bacterial catalysis of pyrite oxidation is achieved by iron-oxidizing bacteria oxidizing ferrous iron to ferric iron. Bacteria that oxidize sulphur compounds assist the catalysis by oxidizing thiosulphate and its decomposition products. Heterotrophic organisms may play a role by consuming organic substances inhibitory to the lithotrophic bacteria.

Abiotic ecological factors, acid formation and populations of iron-oxidizing bacterial groups were studied in 10 differently constructed pilot scale coal waste dumps, as the second phase of a study which started in September 1993. Gas samples were withdrawn weekly from coal waste through permanently buried stainless steel probes, for analysis in the field using a portable oxygen/carbon dioxide meter. Samples of coal waste were extracted by auger for analysis of moisture, pH and microbial populations. The analyses of oxygen and pH can be recommended for the routine monitoring of rehabilitated waste dumps.

Covers of Avalon soil 0.3 or 0.5 m thick, were not adequate to prevent acidification. Coal waste covered with 0.7 m compacted beneath 0.3 m uncompacted Avalon soil, showed a slow pH decline, but reached approximately pH 3 in 1997. Covers of compacted Estcourt soil beneath uncompacted Avalon soil to a cover depth of 1 m were effective in preventing acidification and generally kept the coal waste anaerobic. However, all covers developed cracks during drought conditions in 1995, allowing aeration. Low pH of some samples from these dumps during 1995/1996 may have indicated the start of acidification.

Bacteria oxidizing high concentrations of ferrous iron and considered to be *Thiobacillus ferrooxidans,* were monitored routinely, but may not have been the dominant iron-oxidizer, as population counts using media with a lower ferrous iron concentration were higher. The majority of the latter organisms could also not oxidize sulphur, hence were not *T. ferrooxidans*. The

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populations of the high ferrous iron-oxidizing bacteria were affected by pH, tending to be high in acidified and low in non-acidified coal waste.

Investigations of microbial populations forming iron-oxidizing consortia in enrichment cultures from coal waste and acid drainage samples showed the presence of *T. ferrooxidans*, the heterotrophic bacterial genus *Acidiphilium*, fungi of the genus *Penicillium*, unidentified filamentous fungi, including *Cladophialophora*-like morphological types, and a yeast of the genus *Dipodascus*. In interaction studies, the *Penicillium* isolate had an inhibitory effect on *T. ferrooxidans* (subjected to organic compound stress), but the *Cladophialophora*-like fungi reduced inhibition by organics. Fungi have not previously been studied in detail as components of iron-oxidizing consortia, but the bacterial isolations agree with those elsewhere, indicating that appropriate conclusions from acid mine drainage research in other parts of the world can be applied in KwaZulu-Natal.

OPSOMMING

Die biologies gekataliseerde oksidasie van piriet in die buitenste lae van steenkoolafvalhope lei tot die vorming van suur mynafloopwater. Die oksidasie van piriet tot ferri-yster en sulfaat is 'n komplekse proses wat abiotiese en biologies gekataliseerde reaksies insluit. Piriet word abioties deur ferri-yster geoksideer, met die vrystelling van tiosulfaat en ferro-yster. Tiosulfaat verval om verskeie anorganiese swawelverbindings te vorm. Bakteriese katalise van pirietoksidasie word deur ysteroksiderende bakterieë wat ferro-yster na ferri-yster oksideer, bewerkstellig. Bakterieë wat swawelverbindings oksideer maak 'n bydrae tot die katalise deur tiosulfaat en vervalprodukte daarvan te oksideer. Heterotrofe organismes mag ook 'n rol speel deur organiese verbindings wat die litotrofe bakterieë mag inhibeer, te verbruik.

Abiotiese ekologiese faktore, suurvorming en bevolkings ysteroksiderende bakterieë is in 10 verskillend gekonstrueerde loodsskaal steenkoolafvalhope bestudeer, as die tweede fase van 'n studie wat in September 1993 begin het. Gas monsters is weekliks uit die steenkoolafval onttrek deur vlekvrye staal peilers wat permanent daarin begrawe is, en met behulp van 'n draagbare suurstof/koolstofdioksiedanaliseerder in die veld ontleed. Monsters van die steenkoolafval is met behulp van 'n kleiboor vir die analise van vog, pH en mikrobepopulasies geneem. Die analise van suurstof en pH kan aanbeveel word vir die roetiene monitering van gerehabiliteerde afvalhope.

Bedekkings van 0.3 of 0.5 m Avalongrond was nie voldoende om suurvorming te verhoed nie. Steenkoolafval wat met 0.7 m gekompakteerde en 0.3 m ongekompakteerde Avalongrond bedek is, het 'n stadige pH-daling getoon, maar het in 1997 ongeveer pH 3 bereik. Bedekkings van gekompakteerde Estcourtgrond onder ongekompakteerde Avalongrond met 'n totale dikte van 1 m, was effektief in die voorkoming van suurvorming. Hulle het oor die algemeen die steenkoolafval anaerobies gehou, maar alle bedekings het tydens die droogte in 1995 krake ontwikkel, wat suurstof laat binnedring het. 'n Lae pH gedurende 1995/1996 by sommige monsters uit hierdie hope mag die begin van suurvorming aangedui het.

Bakterieë wat hoë konsentrasies ferro-yster oksideer en wat as *Thiobacillus ferrooxidans* beskou is, was moontlik nie die dominante ysteroksideerder nie, aangesien bevolkingstellings waar 'n medium met 'n laer konsentrasie ferro-yster gebruik is, hoër bevolkings getoon het. Die meerderheid van laasgenoemde organismes kon ook nie swawel benut nie en dus nie *T. ferrooxidans* was nie. Die

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bevolkings van die hoë ferro-ysteroksiderende bakterieë is deur pH beïnvloed, met 'n geneigdheid tot hoë bevolkings in suur en lae bevolkings in minder suur steenkoolafval.

Ondersoeke na die mikrobebevolkings wat in ysteroksiderende konsortia in verrykingskulture vanaf steenkoolafval- en suur mynafloopwatermonsters voorgekom het, het die teenwoordigheid van *T. ferrooxidans,* die heterotrofe bakteriegenus *Acidiphilium*, fungi van die genus *Penicillium*, ongeïdentifiseerde fungi, insluitend *Cladophialophora*-agtige tipes en 'n gis van die genus *Dipodascus* aangetoon. By interaksiestudies het die *Penicillium*-isolaat 'n inhiberende effek op *T. ferrooxidans* (onderworpe aan organiese verbindingstres) gehad, maar die *Cladophialophora*-agtige fungi het die inhibisie deur organiese verbindings verminder. Fungi is nog nie in detail as komponente van ysteroksiderende konsortia bestudeer nie, maar die isolasies van bakterieë stem saam met dié van elders wat aandui dat toepaslike gevolgtrekkings ten opsigte van suur mynafloopwaternavorsing vanaf ander dele van die wêreld ook in KwaZulu-Natal toegepas kan word.

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BIOGRAPHICAL SKETCH

Heinrich Mödinger was born in Stellenbosch on 23 April 1972. He matriculated at the Harrismith High School in 1990 as Dux pupil of his school. In 1991 he enrolled at the University of Stellenbosch and obtained the B.Sc. degree in 1993 and the B.Sc. Hons. in 1994. From 1993 to 1997 he was employed in the Microbial Ecology Laboratory of the Department of Microbiology at the University of Stellenbosch.

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GENERAL INTRODUCTION

Drainage from coal mines is typically high in acidity (as sulphuric acid), iron and sulphates (Kleinmann, 1979). Sulphuric acid and ferric iron, resulting from the chemical and biological oxidation of pyrite, enter the drainage and runoff areas surrounding coal mine waste dumps. Pollution from coal mine drainage water causes great concern and is difficult to treat. The rate-limiting step for the oxidation of pyrite is the oxidation of ferrous to ferric iron, which then oxidizes pyrite (Hutchins *et al.,* 1986; Lundgren and Silver, 1980; Moses *et al.*, 1987; Mustin *et al.*, 1992; Sand *et al.*, 1995). *Thiobacillus ferrooxidans* and other iron-oxidizing bacteria growing in the aerobic outer layers of coal waste dumps play a major role in the formation of acid drainage (Kleinmann and Crerar, 1979; Kleinmann *et al.*, 1981). As *T. ferrooxidans* grows as an aerobic micro-organism when it oxidizes iron (Kelly and Harrison, 1989), anaerobic conditions can be expected to inhibit iron oxidation by this organism, thereby reducing the production of acid drainage.

Many of the older coal waste dumps in South Africa are producers of acid drainage. (Director General: Water Affairs, 1987-88; Henzen and Pieterse, 1978; Kemp, 1962) Recent developments in dump construction and rehabilitation techniques have the aim of counteracting both acid drainage and spontaneous combustion of the coal waste by reducing access of air to the dumps and the flow of water through and from the dumps. Dump compaction is one such technique; covering dumps with soil which is vegetated or with a clay cap and vegetated soil are other techniques.

The effects of these dump construction and rehabilitation techniques on acid drainage production have to be assessed. Hydrological and chemical studies of the dumps are important, but studies of the occurrence of iron-oxidizing bacteria in the dumps, particularly of population sizes, may most rapidly give an evaluation of the success of different dump construction techniques in limiting acid drainage and also indicate where problems still exist, i.e. where construction or rehabilitation procedures are inadequate to prevent bacterial development and do not block the bacterial reaction(s) in the production of acid drainage. Attention needs to be focussed on the outer layers of dumps for ease of sampling and because of the limited penetration of oxygen into dumps which are not even covered or compacted. From reports elsewhere (Dugan, 1975; Erickson, 1985; Good *et al.*, 1970; Goodman *et al.*, 1983) conditions may become anaerobic at depths from as shallow as 30 cm or less to several m, with fluctuation due to dump 'breathing'.

Norris and Kelly (1982) reviewed evidence of the possibility that acid formation might be caused not only by *T. ferrooxidans*, but also by several other bacterial species found in pyritic materials undergoing acidification. Although *T. ferrooxidans* was confirmed as the most

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important iron-oxidizing microorganism in the mesophilic temperature range, roles of the iron-oxidizing *Leptospirillum ferrooxidans* and the sulphur-oxidizing *Thiobacillus thiooxidans* were sometimes indicated (see also Wichlacz and Unz, 1981). Furthermore, the strictly lithotrophic iron-oxidizing bacteria of the species *T. ferrooxidans* live in close association with heterotrophic bacteria in their environment. Many of these heterotrophic bacteria have been placed in the genus *Acidiphilium* (Harrison, 1981; 1984, 1989; Johnson and Kelso, 1983; Wichlacz *et al.,* 1986). These bacteria consume organic molecules that are inhibitory to the lithotrophs, thereby reducing the inhibition and enhancing the growth of the lithotrophs (Harrison, 1984). Moderately thermophilic, mixotrophic or facultatively chemolithotrophic iron-oxidizing bacteria have been isolated and studied by Norris and Barr (1985) and Ghauri and Johnson (1991), while Johnson *et al.* (1992) and Pronk and Johnson (1992) have isolated and studied mesophilic heterotrophic iron-oxidizing bacteria from acid mine drainage. No studies have previously been conducted on the consortia of microorganisms involved in acid mine drainage production in northern KwaZulu-Natal or even in other parts of South Africa.

Kemp (1962) reported serious pollution of rivers in northern KwaZulu-Natal as a result of acid mine drainage from coal mining operations in that area. Recently, acting on recommendations of Report WP E-87 of the Director General: Water Affairs (1987-88), the Department of Water Affairs and Forestry has started to rehabilitate old coal waste dumps under its jurisdiction. The strategy being followed is to collect all the coal waste of a mine into a well-defined dump, which is then covered with a layer of clay followed by topsoil to give a total cover thickness of 1 m. A suitable vegetation cover, for example, grass, is established on the topsoil. This rehabilitation technique appears superficially to be highly successful, but is expensive, costing in the region of R2 000 000 per dump. Before the present investigation, scientific assessment of the inhibition of acid-producing microorganisms by the covers and whether similar inhibition could be achieved by a thinner, cheaper cover has been lacking. Also the hydrology of dumps under covers of different types under the conditions of northern KwaZulu-Natal has not been determined. Pilot scale coal waste dumps without and with different covers were constructed by the Department of Water Affairs and Forestry for the microbiological investigations described in this thesis, as well as a parallel hydrological investigation conducted by Wates, Meiring and Barnard (1993, 1995a,b) as project K5/575 of the Water Research Commission.

The objectives of this study were:

(i) Comparative quantitative and qualitative studies of iron-oxidizing bacterial populations (for example, *Thiobacillus ferrooxidans*), which could catalyse acid drainage production in the outer layers of coal waste dumps of different construction (different rehabilitation techniques), namely, non-compacted (control) and compacted dumps, dumps without and with clay and/or soil caps, vegetated and non-vegetated dumps.

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(ii) From the results of (i), identification of dump construction or rehabilitation techniques which most successfully limit populations of acid drainage-producing bacteria.

(iii) From the results of (i), and accompanying studies of ecological determinants in the dump, identification of ecological factors in the variously constructed dumps which cause acid drainage-producing bacteria to flourish or to be suppressed.

(iv) From (ii) and (iii), an assessment of the success of present construction and rehabilitation techniques for coal waste dumps in inhibiting or limiting the development of iron-oxidizing bacteria, thereby inhibiting or reducing the production of acid mine drainage.

(v) Determination of the main microbial species or groups involved in the production of acidity in coal waste dumps and the drainage water therefrom in northern Kwazulu-Natal, including the possible role of consortia.

To attain these objectives the research was divided into two distinct experimental parts, Experimental Part 1 and Experimental Part 2 .

In Experimental Part 1 the effects of different dump construction techniques on abiotic ecological factors and bacteria causing acid mine drainage were investigated. The study was conducted using the pilot scale dumps constructed by the Department of Water Affairs and Forestry.

The abiotic ecological determinants, rainfall and moisture in the coal waste, oxygen and carbon dioxide concentrations of the atmosphere in the coal waste and the pH of the coal waste were studied. The last four determinations were made in the coal waste to a depth of 15-30 cm in covered pilot scale dumps (mini-dumps or cells) or between depths of 15 and 30 cm in uncovered pilot scale dumps (outer layer of the coal waste). The present investigation followed that of Cleghorn (1997) to give studies extending over 3- or 4-year periods.

Monitoring of bacterial population sizes in samples from the outer layer of the coal waste in the pilot scale dumps in the present study included populations of the following bacteria:

(i) Acidophilic chemolithotrophic bacteria oxidizing high concentrations of ferrous iron (assumed during the planning of the experiment to be *T. ferrooxidans*).

(ii) Acidophilic bacteria oxidizing high concentrations of ferrous iron at relatively high temperature and low pH.

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(iii) Acidophilic bacteria oxidizing moderate concentrations of ferrous iron.

(iv) Acidophilic bacteria oxidizing moderate concentrations of ferrous iron and sulphur ($S°$).

(v) Acidophilic bacteria oxidizing moderate concentrations of ferrous iron, sulphur and thiosulphate.

The group under (i) was monitored in every sample over a 3-year period, and the groups under (ii)-(v) over shorter periods, which usually included at least three samplings. When the significance or not of a particular group was established, another group was investigated.

As nothing was known of the identity of microorganisms involved in acid mine drainage formation in the Klip River Coal Field of northern Kwazulu-Natal and as the bacterial groups investigated under Experimental Part 1 of this investigation were not studied as far as establishing their identity, the following studies were undertaken in Experimental Part 2:

(i) Development of stable iron-oxidizing enrichment cultures, especially in the selective medium for acidophilic chemolithotrophic high ferrous iron-oxidizing bacteria, from coal waste samples from mine dumps in the Klip River Coal Field. Attempted isolation of the iron-oxidizing bacteria from these cultures. Isolation and identification of heterotrophic microorganisms (bacteria and fungi) growing in association with the iron-oxidizing bacteria in the enrichment cultures (microbial consortia).

(ii) Development of stable iron-oxidizing cultures in the selective medium for acidophilic chemolithotrophic high ferrous iron-oxidizing bacteria, from acid mine drainage water from mine dumps in the Klip River Coal Field. Isolation and identification of iron-oxidizing bacteria from these cultures. Isolation and identification of heterotrophic microorganisms (bacteria and fungi, including yeasts) growing in association with the iron-oxidizing bacteria in the enrichment cultures.

(iii) A study of the effects of heterotrophic fungi isolated under (i) and (ii) on growth and iron-oxidation by *T. ferrooxidans*.

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LITERATURE REVIEW

CHEMICAL, MICROBIOLOGICAL AND ECOLOGICAL ASPECTS OF PYRITE OXIDATION IN RELATION TO ACID MINE DRAINAGE FORMATION

Introduction

Coal and other minerals are enclosed in geological formations of a reduced nature, and are often associated with pyrite. When mining activities expose pyrite to oxidizing agents such as molecular oxygen (O_2) and ferric iron, a complex oxidation process occurs. This process is a combination of auto-oxidation and bacterially catalysed oxidation reactions (Atlas and Bartha, 1993; Bos *et al.*, 1994; Mustin *et al.*, 1992). The phenomena involved in the process include oxidation-reduction processes, as well as solid-solution equilibria, involving ions and intermediate sulphur-containing compounds. Furthermore, the semiconducting properties of pyrite also play a role.

The oxidation of sulphidic minerals, such as pyrite, is central to a number of environmentally and economically important issues. These include the formation of acid mine drainage, the leaching of gold, copper, uranium and other minerals from ore and the biogeochemical cycling of sulphur, carbon, oxygen, iron and other metals (Hutchins *et al.*, 1986, Lundgren and Silver, 1980; Moses *et al.*,1987; Mustin *et al.*, 1992). An understanding of the mechanisms of pyrite oxidation is needed to understand these processes.

Various bacterial groups catalyse the oxidation of pyrite. These organisms can directly catalyse pyrite oxidation by the oxidation of ferrous iron to ferric iron which can then act as an oxidant in pyrite oxidation. Other organisms indirectly catalyse pyrite oxidation, either by stimulating the growth of iron-oxidizing organisms or by oxidizing intermediary sulphur compounds formed during the oxidation of pyrite (Harrison, 1984; Johnson, 1995a; Sand et al., 1995; Schippers et al., 1996).

In this review pyrite oxidation will be described as both an abiotic and a biologically catalysed process. The classical view of biological pyrite oxidation will be evaluated critically, taking into account recent observations and alternative hypotheses. This review supports the model of bacterial pyrite oxidation proposed by Sand *et al.* (1995), based on the concept that the oxidation of pyrite is a chemical process driven by the concurrent reduction of ferric iron to ferrous iron. Bacterial catalysis of this process is achieved by the lithotrophic bacteria, *Thiobacillus ferrooxidans* and *Leptospirillum*

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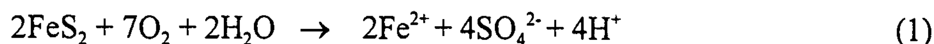
ferrooxidans, regenerating ferric iron during their energy metabolism and concentrating ferric iron in their extracellular polymer matrix (Gehrke *et al.*, 1995). Further, the oxidation of thiosulphate, a major intermediate product of pyrite oxidation, is also driven by the reduction of ferric iron in abiotic processes, as well as by enzymatic catalysis by *T. ferrooxidans* and other *Thiobacillus* (Pronk *et al.*, 1990; Schippers *et al.*, 1996). The mechanism of pyrite oxidation and the involvement of the energy metabolism of the lithothrophic bacteria therein will therefore be described as a cyclical process dependent on the ferric:ferrous iron redox couple. This review will also focus on the characteristics of the most important organisms hitherto identified that directly or indirectly catalyse the formation of acid mine drainage. An understanding of these organisms can assist in understanding acid mine drainage formation and in the development of strategies for the isolation, enumeration and identification of the organisms involved in the microbial ecology of the process.

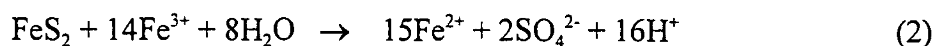
Chemistry of Pyrite Oxidation

Early models of pyrite oxidation

The solubility of pyrite is very low, but it is chemically unstable in aqueous environments containing molecular oxygen or ferric iron as oxidizing agents. The overall reactions for the oxidation of pyrite by these oxidizing agents are as follows (Moses *et al.*, 1987):



$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+ \quad (1)$$



$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (2)$$

At and above neutral pH, oxidation by atmospheric or dissolved oxygen (reaction 1) occurs spontaneously and rapidly, but this reaction slows down dramatically below pH 4,5 (Kleinmann *et al.*,1981). Moses *et al.* (1987) indicated that in terms of macro-reaction rates ferric iron is the preferred oxidizing agent (as opposed to dissolved oxygen) in solutions between pH 2 and pH 9. However, the availability of ferric iron for oxidation is limited by its low solubility at pH higher than 3.5 (Sand *et al.*, 1995).

Although pyrite oxidation can occur abiotically, micro-organisms (bacteria) play an important role in catalysing the process, especially in low pH environments. The resulting high rates of pyrite oxidation are significant in the formation of acid mine drainage and other biogeochemical phenomena already mentioned (Atlas and Bartha, 1993; Bos *et al.* 1994; Kleinmann and Crerar, 1979; Kleinmann *et al.*,

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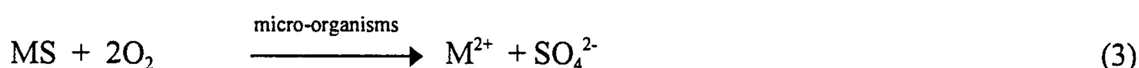
1981; Lundgren and Silver, 1980; Silverman, 1967). Historically the mechanisms by which micro-organisms catalyse the oxidation of pyrite and other sulphide minerals were considered to be via the so-called *direct* mechanism, involving direct enzymatic attack on the pyrite, or the *indirect* mechanism, where inorganic products of microbial metabolism acted as reagents (Ewart and Hughes, 1991; Lundgren and Silver, 1980; Sand *et al.,* 1995; Silverman, 1967). These mechanisms were believed to run alongside each other, and to act synergistically.

Direct mechanism. The postulated direct mechanism for the oxidation of sulphide minerals was seen as a purely enzymatic process that does not entail the use of ferric iron as oxidizing agent (Sand *et al.*, 1995). It involves physical contact between the bacteria and the sulphide mineral surface. The bacteria then oxidize both the metal and the sulphur moieties of the sulphide mineral enzymatically, using molecular oxygen (O_2) as electron acceptor. The reaction therefore can be represented as:



$$MS + 2O_2 \xrightarrow{\text{micro-organisms}} M^{2+} + SO_4^{2-} \qquad (3)$$

In experiments using iron-free synthetic cobalt and nickel sulphides and washed cells of *Thiobacillus ferrooxidans* the bacteria consumed oxygen and solubilized the metals as sulphate, suggesting that the direct mechanism was involved (Duncan *et al.*, 1967; Rickard and Vanselow, 1978).

Indirect mechanism. Ferric iron is the main oxidizing agent of pyrite and other sulphidic minerals in acid environments. At pH lower than 4.5, the reduction of ferric iron to ferrous iron by pyrite is more rapid than the reoxidation of ferrous iron to ferric iron by dissolved oxygen (Evangelou and Zhang, 1995 and references therein). To maintain high rates of pyrite oxidation in acidic environments, a mechanism is necessary for ferric iron to be regenerated rapidly. The oxidation of ferrous iron by iron-oxidizing bacteria (such as *T. ferrooxidans*, *L. ferrooxidans*, or acidophilic heterotrophic iron-oxidizing bacteria) is considered to be the main catalytic function of bacteria involved in pyrite oxidation (Pronk and Johnson, 1992; Silverman, 1967).

The indirect mechanism proposed the following steps in the oxidation of pyrite (Evangelou and Zhang, 1995):

(i) The pyrite is oxidized chemically by Fe^{3+} according to reactions 4 and 5, or 2, which is the sum of these reactions or an overall reaction not specifying the production of sulphur as an intermediate.

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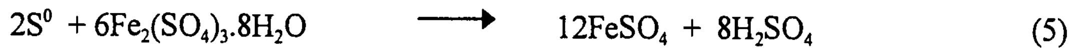
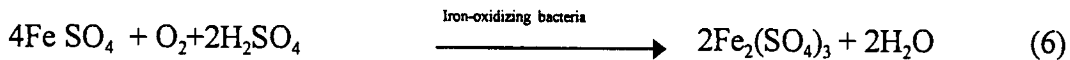
$$FeS_2 + Fe_2(SO_4)_3 \longrightarrow 3FeSO_4 + 2S^0 \quad (4)$$



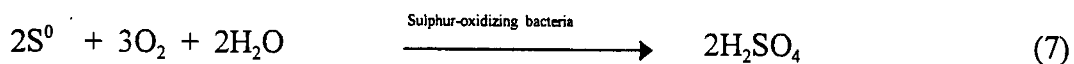
$$2S^0 + 6Fe_2(SO_4)_3.8H_2O \longrightarrow 12FeSO_4 + 8H_2SO_4 \quad (5)$$

(ii) Chemolithotrophic iron-oxidizing bacteria, such as *T. ferrooxidans* and *L. ferrooxidans,* then regenerate the ferric ions (reaction 6), thereby causing the reactions to continue (Evangelou and Zhang, 1995).



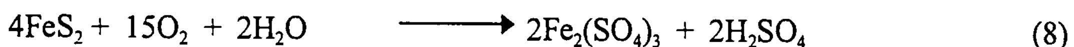
$$4Fe\,SO_4 + O_2 + 2H_2SO_4 \xrightarrow{\text{Iron-oxidizing bacteria}} 2Fe_2(SO_4)_3 + 2H_2O \quad (6)$$

(iii) Micro-organisms capable of oxidizing sulphur, such as members of the genus *Thiobacillus*, could oxidize the sulphur (S^0) formed in reaction 4 to sulphuric acid (reaction 7), thereby regenerating the acid consumed in reaction 6 (Ewart and Hughes, 1991; Evangelou and Zhang, 1995; Lundgren. and Silver, 1980; Sand *et al.*, 1995).



$$2S^0 + 3O_2 + 2H_2O \xrightarrow{\text{Sulphur-oxidizing bacteria}} 2H_2SO_4 \quad (7)$$

Reaction 8, which is derrived from reactions 4, 6 and 7, is an overall reaction for the microbially mediated oxidation of pyrite. It would lead to the dissolution of pyrite and the formation of acid mine drainage.



$$4FeS_2 + 15O_2 + 2H_2O \longrightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4 \quad (8)$$

However, the ferric sulphate could react with water to form insoluble ferric hydroxide ('yellow boy') and sulphuric acid (Atlas and Bartha. 1993).

Criticism of early models. These early models of microbially mediated oxidation of pyrite in acidic mineral environments explain most experimental observations and give a broad overview of the possible abiotic and biotic processes involved in the oxidation of pyrite. However, the schemes are a gross simplification, as they consider only very superficially the sulphur chemistry of pyrite oxidation. They pay very little attention to the role of the extracellular organic matrices produced by these bacteria, the enzymatic systems and metabolism of pyrite-oxidizing bacteria, the various phases of attack (as can be observed in batch culture experiments), the role of non-attached and attached cells during the oxidation process and the role that impurities and electrochemical dissolution sites of

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natural pyrite play in the interaction of the bacteria and pyrite. The chemical reactions (reactions 4 to 8) are stoichiometric descriptions of the process, but have very little mechanistic meaning. Furthermore, it is extremely doubtful whether the direct mechanism, as it is understood in the classical model, exists at all. The main reasons for doubting this mechanism of attack are as follows:

(i) Most of the sulphate oxygen derived from pyrite oxidation in acid mine drainage field settings as well as in laboratory experiments is derived from water and not dissolved molecular oxygen as the direct mechanism would imply (Taylor *et al.*, 1984 a, b).

(ii) Electron microscopic studies have revealed that the dissolution and primary oxidation of pyrite, by adhered cells of *T. ferrooxidans*, occurs in a reaction space formed by extracelluar polymers and not on the cell membrane surface as the direct mechanism implies (Rodriguez-Leiva and Tributsch, 1988; Rojas *et al.*, 1995).

(iv) Experiments using iron-free metal sulphides and washed cells of *T. ferrooxidans,* which indicated the presence of a ferric iron-independent (direct) mechanism (Duncan *et al.*, 1967; Rickard and Vanselow, 1978), are not valid for the oxidation of pyrite (and most naturally occurring sulphidic minerals) where iron is present (Sand *et al.*, 1995).

(v) Only the iron-oxidizing bacteria *T. ferrooxidans* and *L. ferrooxidans* can grow on pyrite as sole source of energy (Hallmann *et al.*, 1992; Kelly and Harrison, 1989). This suggests that the oxidation of iron, and therefore the production of ferric ions, is central to the oxidation of pyrite.

(vi) Certain enzymes involved in sulphur metabolism by *T. ferrooxidans* are linked to the ferric:ferrous iron redox couple and are strongly inhibited by ferrous iron (Sugio *et al.*, 1990).

(vii) Thiosulphate is the first intermediary sulphur compound in both biotic and abiotic pyrite oxidation (Schippers *et al.*, 1996).

For these reasons it has become necessary to take a closer look at the mechanism of pyrite oxidation, both by abiotic and microbially mediated processes.

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Abiotic pyrite oxidation

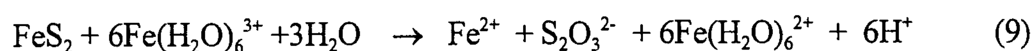
Models explaining the mechanism of the oxidative attack on pyrite, need to take into account the following observations (Lowson, 1982; Luther, 1987; Moses *et al.* , 1987; Sand *et al.*, 1995):

(i) Ferric iron (as $Fe(H_2O)_6^{3+}$) is apparently the oxidizing agent.

(ii) Sulphur and sulphoxy anions, such as sulphite (SO_3^{2-}), thiosulphate ($S_2O_3^{2-}$) and polythionates ($S_nO_6^{2-}$) appear to be formed as intermediates.

Using molecular orbital theory, Luther (1987) deduced that dissolved ferric iron (ferric hexahydrate) was a suitable oxidizing agent for the attack on pyrite. This was supported by the observation that water, and not dissolved oxygen, was the source of sulphate-oxygen in laboratory as well as acid mine drainage field settings (Taylor *et al.*, 1984 a,b). It could therefore be proposed that reaction 2, but with the ferric ion as the hexahydrate ($Fe(H_2O)_6^{3+}$), is the most important reaction involved in the oxidation of pyrite in acid mine drainage environments.

Luther (1987) and Moses *et al.* (1987) independently proposed similar mechanisms for the oxidation of pyrite using ferric hexahydrate as oxidizing agent, with thiosulphate as the first intermediate sulphoxy anion. Schippers *et al.* (1996) used silver ions to prove conclusively that thiosulphate is the first intermediary in the oxidation of pyrite in both biotic and abiotic processes. A schematic summary of the mechanism of pyrite oxidation as proposed by Luther (1987) is given in Fig. 1. The sum of the reactions for the first step in the oxidation of pyrite by ferric iron can therefore be written as follows:



$$FeS_2 + 6Fe(H_2O)_6^{3+} + 3H_2O \rightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_2O)_6^{2+} + 6H^+ \qquad (9)$$

Moses *et al.* (1987) postulated a mechanism involving the addition of two hydroxy groups followed by the removal of water to add each of the oxygens to the one pyrite sulphur atom. It is not clear how the addition of the last two hydroxy-ions to supply the third oxygen of the thiosulphate would occur.

Brown and Jurinak (1989) found that the oxidation of pyrite was enhanced by an increase in pH. They proposed that the enhancement of pyrite oxidation by hydroxy ions (OH^-) may be through an inner-

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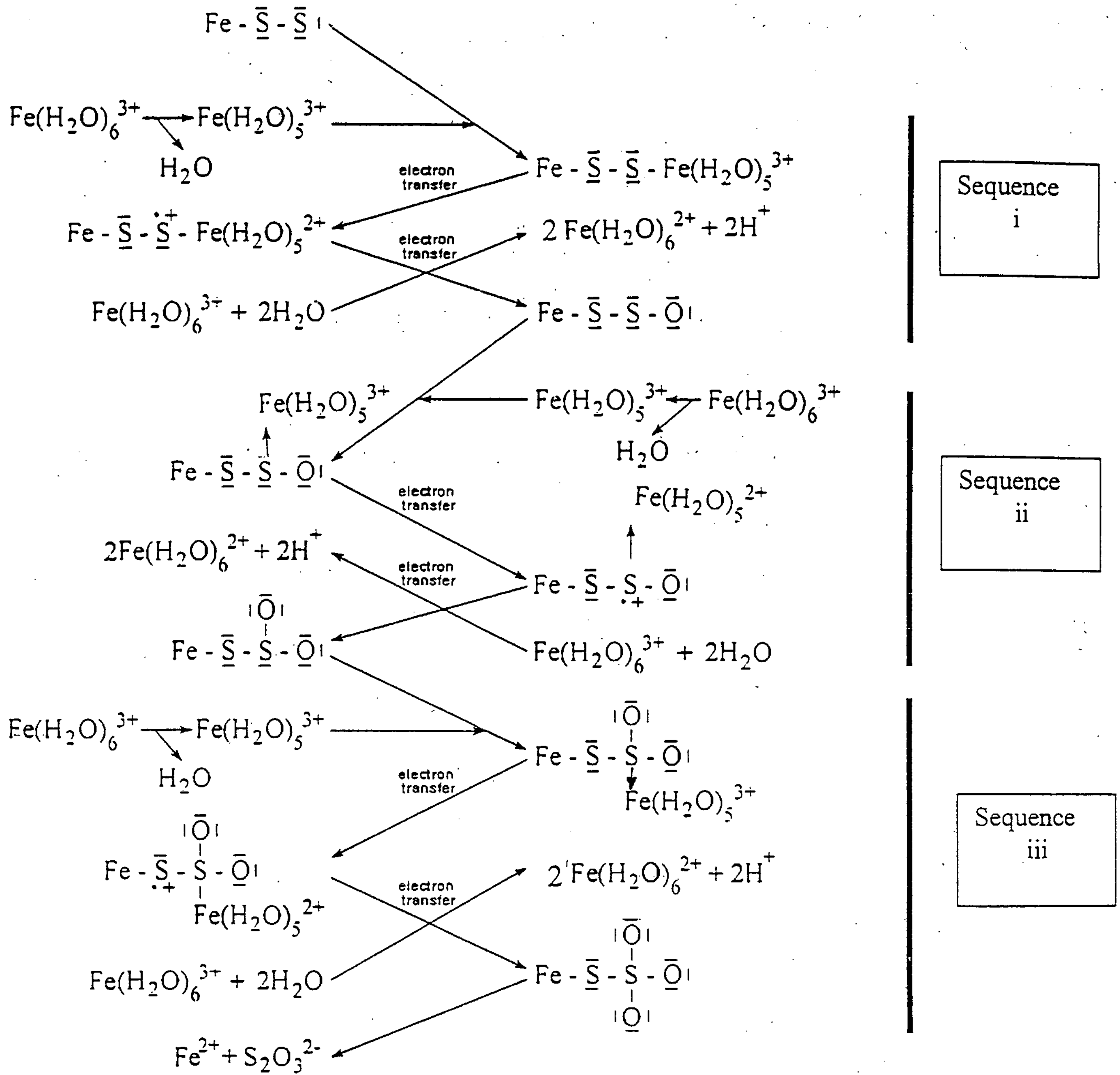

Fig. 1. Schematic diagram of the oxidation of pyrite by ferric hexahydrate, which by repetitive transfers of electrons to ferric iron from one of the pyrite sulphurs leads to the dissolution of pyrite as ferrous iron and thiosulphate. (Adapted from Evanglou and Zhang, 1995; Luther, 1987).

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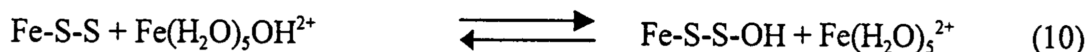
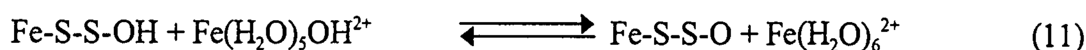
sphere electron transfer mechanism where OH^- and an electron are exchanged simultaneously between $Fe(H_2O)_5OH^{2+}$ and the disulphide.



$$Fe\text{-}S\text{-}S + Fe(H_2O)_5OH^{2+} \rightleftarrows Fe\text{-}S\text{-}S\text{-}OH + Fe(H_2O)_5{}^{2+} \quad (10)$$

This differs from the mechanisms of Moses et al. (1987) and Luther (1987), in that the ferric hexahydrate loses a hydrogen ion in solution rather than at the pyrite surface. Six electron transfers are needed before the sulphur can leave the pyrite structure as thiosulphate. Reaction 10, repeated three times, is sufficient to explain the transfer of three electrons by the addition of hydroxyl ions. Three more electrons could be transferred by the removal of hydrogen ions from the Fe-S-S-OH and subsequent hydroxylated intermediates, with the formation of water which may leave with the ferrous complex.



$$Fe\text{-}S\text{-}S\text{-}OH + Fe(H_2O)_5OH^{2+} \rightleftarrows Fe\text{-}S\text{-}S\text{-}O + Fe(H_2O)_6{}^{2+} \quad (11)$$

Repetition of reactions 10 and 11 leads to the formation of a thiosulphate leaving unit which is released into solution along with ferrous iron.

Moses and Herman (1991) proposed a mechanism for pyrite oxidation at circumneutral pH involving ferrous iron, adsorbed to the pyrite surface, giving up electrons to dissolved oxygen and the resulting ferric iron rapidly accepting electrons from the pyrite. The adsorbed iron is, therefore, cyclically oxidized and reduced while acting as a conduit for electrons travelling from pyrite to dissolved oxygen (Fig. 2).

Although the exact mechanism of pyrite oxidation may not be clear and may vary according to the pH at which the reaction proceeds, it is generally agreed that pyrite oxidation proceeds via hydro- or hydroxy-complexed iron electron carriers and that thiosulphate is the first intermediate formed (Brown and Jurinak, 1989; Moses and Herman, 1991; Moses *et al.*, 1987; Luther, 1987; Sand *et al.*,1995; Schippers *et al.*, 1996). Ferric iron is therefore the main oxidizing agent for pyrite, with oxygen playing a vital role by reoxidizing ferrous iron to ferric iron.

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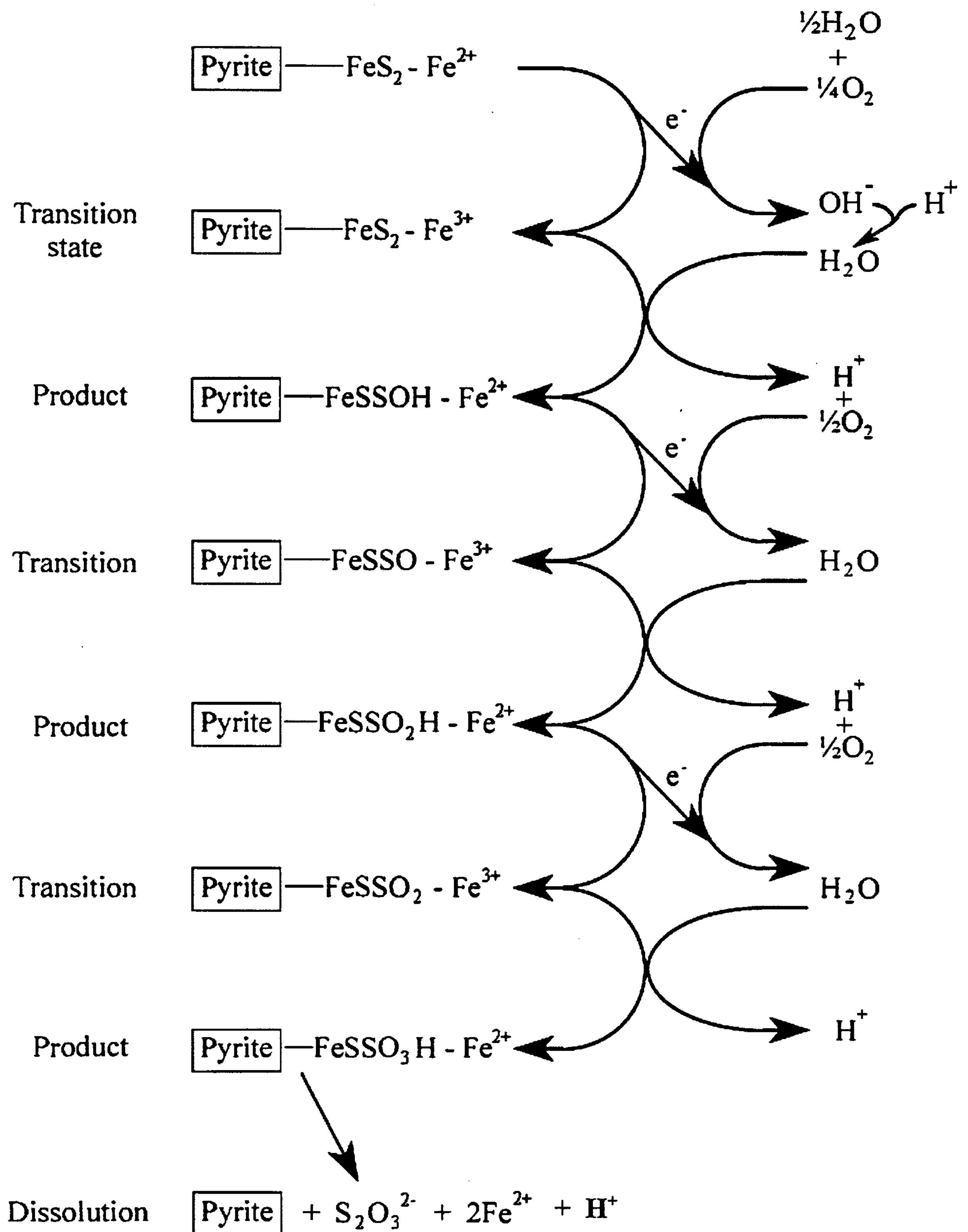

Fig. 2. Schematic model of pyrite oxidation at circumneutral pH, involving ferrous iron, adsorbed to the pyrite surface, giving up electrons to oxygen. Repeated electron transfers between oxygen and pyrite via the adsorbed iron conduit lead to the dissolution of pyrite. The adsorbed and liberated iron is hydrated. (Adapted from Moses and Herman, 1991).

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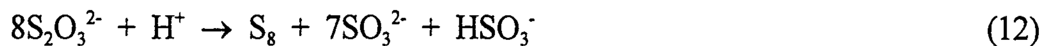
Thiosulphate formed during pyrite oxidation is unstable in acidic environments and various sulphoxy intermediates and sulphur form during its decay, with the reaction superficially being:



$$8S_2O_3^{2-} + H^+ \rightarrow S_8 + 7SO_3^{2-} + HSO_3^- \qquad (12)$$

However, reaction 12 is the sum of a sequence of reactions and there are numerous possibilities for the formation of side products (Moses *et al.,* 1987). Polythionates found during leaching operations may arise from chemical reactions starting from thiosulphate (Sand *et al.*, 1995; Schippers *et al.*, 1996).

New model of biotic pyrite oxidation

General. Sand *et al.* (1995) and Schippers *et al.* (1996) have considered the fate of the iron and sulphur of pyrite. Their reviews suggest a new model for the mechanisms of biotic pyrite oxidation by *T. ferrooxidans* and *L. ferrooxidans*. *Thiobacillus ferrooxidans* is the best characterized member of the lithotrophic organisms involved in pyrite oxidation through its metabolism (oxidation) of ferrous iron, sulphur and inorganic sulphur compounds (Blake *et al.,* 1994). *Leptospirillum ferrooxidans* is also capable of oxidizing pyrite through its metabolism (oxidation) of ferrous iron (Hallmann *et al.*, 1992). Although sulphate is formed during pyrite oxidation by *L. ferrooxidans*, this organism does not have the enzymes required for sulphur metabolism (Hallmann *et al.*, 1992), suggesting that the oxidation of sulphur or sulphoxy intermediates formed during the pyrite oxidation occurs abiotically if ferric iron is present as oxidant.

The following discussion of the bacterially mediated pyrite oxidation and dissolution process will deal with bacterial pyrite oxidation as an iron-dependant cyclical process where pyrite and the resulting intermediary sulphur compounds are oxidized with the concurrent reduction of ferric iron to ferrous iron, which then acts as electron donor for the lithotrophic bacteria. The oxidation of the intermediate sulphur compounds can proceed via purely abiotic processes (as with *L. ferrooxidans*) or abiotic as well as enzymatically catalysed processes (as with *T. ferrooxidans*). Pyrite oxidation in relation to the different growth phases observable during batch culture experiments with *T. ferrooxidans* and *L. ferrooxidans* (Mustin *et al.,* 1992; Fernandez *et al.,* 1995) will also be considered.

Chemical/biochemical processes (components) of the model. A comprehensive model can be developed for the chemical/biochemical processes by combining the following component models:

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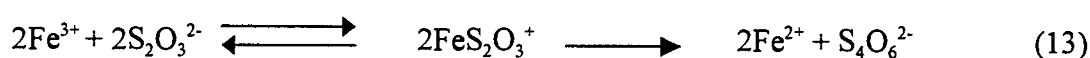
(ii) The first component model is the abiotic oxidation of pyrite to ferrous iron and thiosulphate (Luther, 1987; Moses et al., 1987) (Fig. 1). The bacteria may enhance this process by concentrating the ferric ions in their extracelluar matrix (Gehrke *et al.*, 1995). A cyclical iron oxidation-reduction process then occurs, in which the ferrous ions produced in the oxidation of pyrite are reoxidized by the bacteria to yield energy before again being reduced by the pyrite (Sand *et al.*, 1995).

(iii) The second component model describes the abiotic reactions of sulphur compounds originating from the thiosulphate produced by the oxidation of pyrite. Thiosulphate is unstable in acidic environments and decomposes to form various sulphur compounds, including all the sulphoxy intermediates detected in leaching operations, as well as sulphur (Sand *et al.* 1995; Schippers et al., 1996). Schippers *et al.* (1996) proposed a decomposition pathway for thiosulphate (Fig. 3), based on the detection of intermediary sulphur compounds in the oxidation of pyrite by sterile ferric iron or *T. ferrooxidans* or *L. ferrooxidans*. Only *T. ferrooxidans* produces enzymes that could assist with the oxidation of sulphur compounds. The pathway, which is similar to that proposed by Pronk *et al.* (1990), is cyclical and involves both ferric iron and oxygen as electron acceptors. Sulphate is an important product. The details of the pathway are as follows:

In the first step thiosulphate is oxidized to tetrathionate (reaction 13).



$$2Fe^{3+} + 2S_2O_3^{2-} \rightleftharpoons 2FeS_2O_3^{+} \longrightarrow 2Fe^{2+} + S_4O_6^{2-} \qquad (13)$$

As this reaction proceeds faster than the dissolution of pyrite (Fig. 1, 2 and reaction 9), thiosulphate is barely detectable. The hydrolysis of tetrathionate leads to the formation of highly reactive disulphane monosulphonic acid and sulphate (reaction 14)

$$S_4O_6^{2-} + H_2O \longrightarrow HSSSO_3^{-} + SO_4^{2-} + H^{+} \qquad (14)$$

Disulphane monosulphonic acid may react in several ways to form elemental sulphur, sulphite, thiosulphate, trithionate, pentathionate (via reactions 15 to 18) and various other polythionates (Pronk *et al.*, 1990; Schippers *et al.*, 1996).

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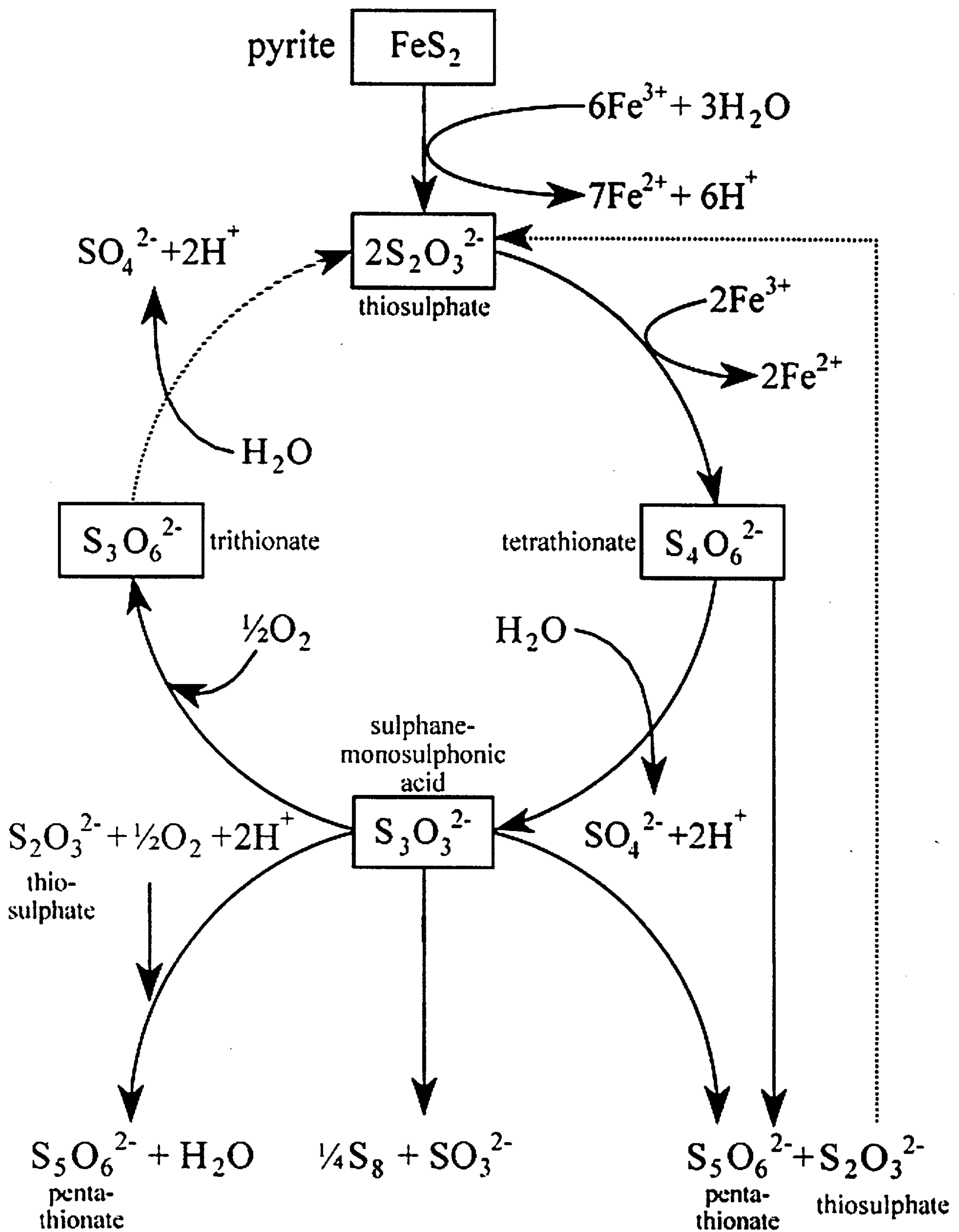

Fig. 3. Cycle of oxidative pyrite oxidation by chemical and/or bacterial leaching. Dotted lines indicate where thiosulphate may enter the cycle again. (Adapted from Pronk *et al.*, 1990; Schippers *et al.*, 1996).

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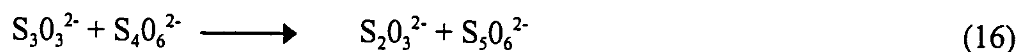
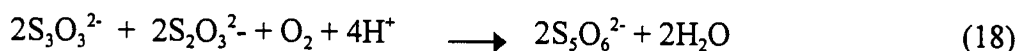
$$4S_30_3^{2-} \longrightarrow S_8 + 4S0_3^{2-} \qquad (15)$$



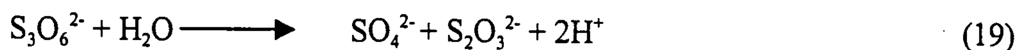
$$S_30_3^{2-} + S_40_6^{2-} \longrightarrow S_20_3^{2-} + S_50_6^{2-} \qquad (16)$$

$$2S_3O_3^{2-} + 30_2 \longrightarrow 2S_30_6^{2-} \qquad (17)$$



$$2S_3O_3^{2-} + 2S_2O_3^{2-} + O_2 + 4H^+ \longrightarrow 2S_5O_6^{2-} + 2H_2O \qquad (18)$$

The cycle is completed by hydrolysis of trithionate to yield sulphate and thiosulphate.



$$S_3O_6^{2-} + H_2O \longrightarrow SO_4^{2-} + S_2O_3^{2-} + 2H^+ \qquad (19)$$

This cycle of reactions is catalysed by the pyrite surface as vigorous shaking was required for the reactions to proceed in experiments where no bacterial catalyst was available (Schippers et al., 1996).

Reaction 15 seems to be the dominant reaction in which disulphane monosulphonic acid is involved, as S^0 was the dominant intermediary sulphur product formed in experiments using *T. ferrooxidans, L. ferrooxidans* or sterile ferric iron to oxidize pyrite (Schippers *et al.*, 1996). This observation provides an alternative explanation of how the sulphur 'intermediate' of reactions 4 and 5 in the early models of biotic pyrite oxidations may be produced.

(iv) The third component model is concerned with the bacterial oxidation of sulphur and sulphur compounds. *Thiobacillus ferrooxidans* is capable of metabolizing the sulphur and sulphur compounds formed during the oxidation of pyrite (Fig. 3), leading to less accumulation of these products than when the transformations are entirely abiotic (Schippers *et al.*, 1996). The S^o formed during the decomposition of thiosulphate (Pronk *et al.*, 1990; Schippers *et al.*, 1996) is deposited as storage globules extracellularly in a polymer matrix (Rojas *et al.*, 1995) or in the periplasmic space (Schippers *et al.*, 1996). Steudel *et al.* (1987) proposed a structure for these globules with deposits of polythionate ions on the surface (Fig. 4), which make the globules hydrophilic and should therefore assist enzymatic sulphur oxidation.

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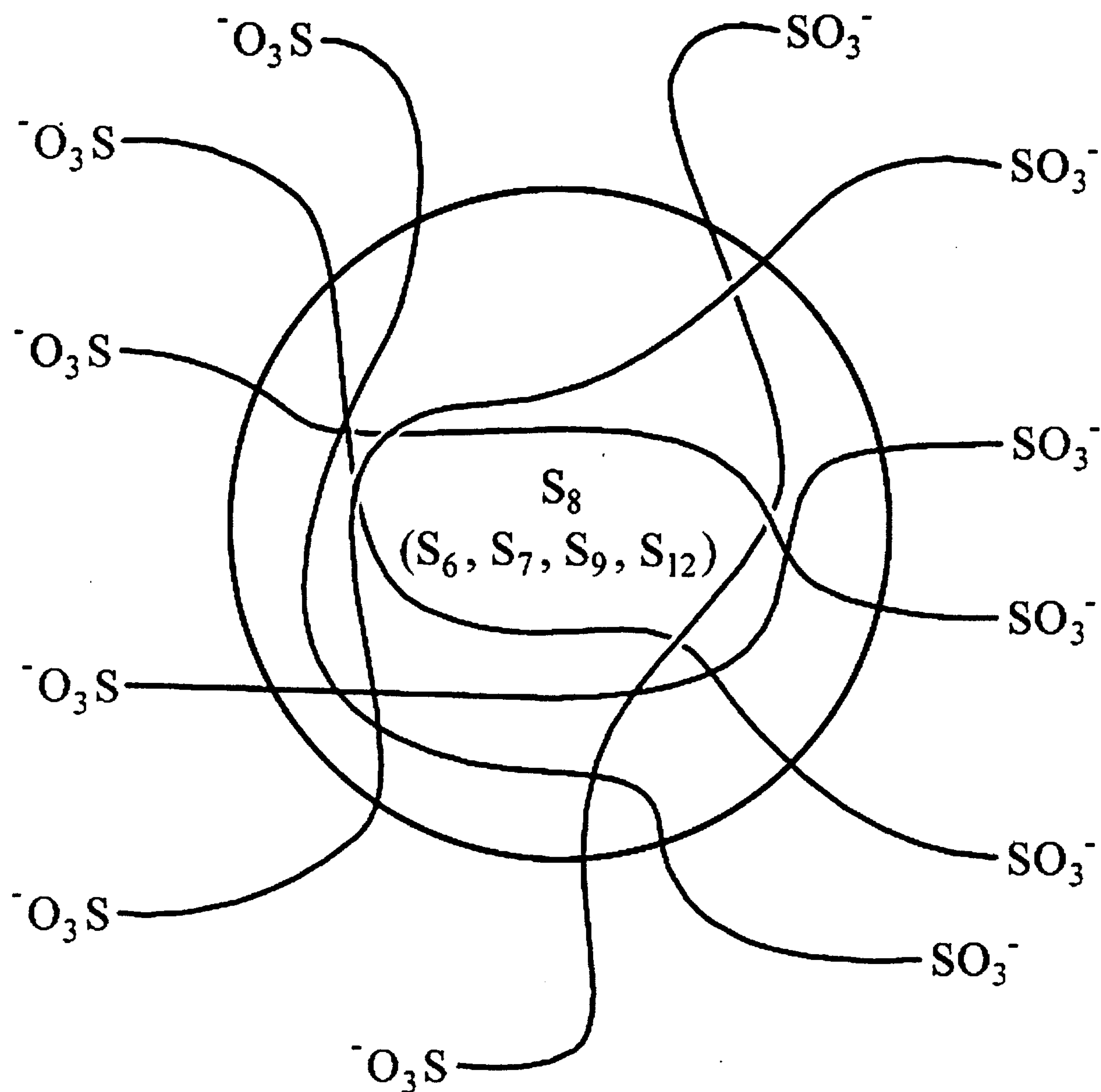

Fig. 4. Simplified model of a sulphur globule as it is formed extracellularly by *T. ferrooxidans*. It consists mainly of S_8 and small amounts of other molecules (S_6, S_7, S_9 and S_{12}) which impede crystallization, as in globules of supercooled liquid sulphur. Long chain polythionate ions ($^-O_3S.S_n.SO_3^-$) deposited on the surface make the globule hydrophilic. (Adapted from Steudel *et al.*, 1987).

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(v) A further component model linking sulphur and iron transformations comes from the studies of Sugio *et al.* (1985, 1987, 1988) who found in *T. ferrooxidans* an enzyme system catalysing sulphur oxidation to sulphite coupled to the reduction of ferric to ferrous iron (Fig. 5). The enzyme was first named sulphur:ferric iron oxidoreductase and subsequently named hydrogen sulphide:ferric iron oxidoreductase, following further studies of its catalytic activities (Pronk and Johnson, 1992; Sugio *et al.,* 1990). Sugio *et al.* (1988) reported that *T. ferrooxidans* also possessed a sulphite-oxidizing iron-reducing enzyme system, although the oxidation of sulphite to sulphate with the concomitant reduction of ferric to ferrous iron can also occur abiotically (Sugio *et al.*, 1985). The ferric iron reduction permits the reoxidation of ferrous iron for energy generation by the organism. The oxidation of sulphur is strongly inhibited by high concentrations of ferrous iron (Sugio *et al.*, 1990), indicating a preference by the bacteria for the metabolism of ferrous iron if it is abundant. The oxidation of thiosulphate to tetrathionate may also be coupled to ferric iron reduction (Schippers et al., 1996).

Comprehensive chemical/biochemical model. When these component models are brought together, a comprehensive model for microbially mediated pyrite oxidation can be constructed (Fig. 6). This model links the abiotic chemical oxidation of pyrite by ferric iron via thiosulphate (Luther, 1987; Moses *et al.*, 1987; Schippers *et al.*, 1996) to the important catalytic function of iron-oxidizing bacteria such as *T. ferrooxidans* and *L. ferrooxidans,* which oxidize ferrous iron to ferric iron for metabolic energy (Johnson, 1995a). The model also indicates the fate of sulphur compounds that form during the decomposition of thiosulphate.

Bacteria Involved in Pyrite Oxidation

Acidophilic iron-oxidizing bacteria

Various acidophilic bacterial groups possess the capacity to catalyse the formation of acid mine drainage by oxidizing ferrous iron to ferric iron, which is the most important oxidizing agent in the oxidation of pyrite. The most important groups are described in the following paragraphs.

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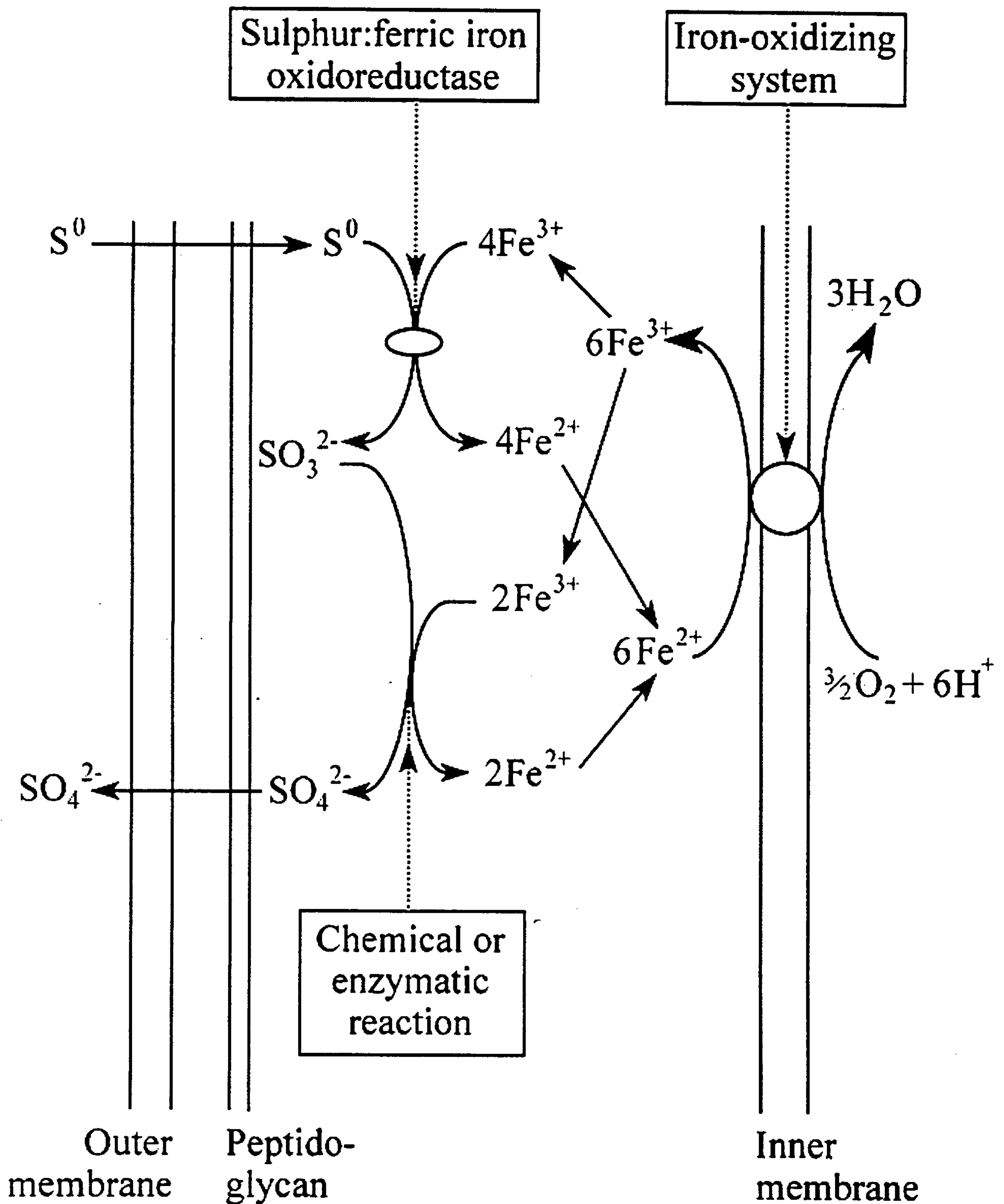

Fig. 5. Model linking the oxidation of sulphur and sulphite to the cyclic reduction of ferric iron to ferrous iron and reoxidation of ferrous by *T. ferrooxidans*. The oxidation of sulphur is mediated by sulphur:ferric iron oxidoreductase (subsequently renamed hydrogen sulphide:ferric iron oxidoreductase), while the oxidation of sulphite occurs spontaneously or enzymatically. (Adapted from Sugio *et al.*, 1985, 1987, 1988).

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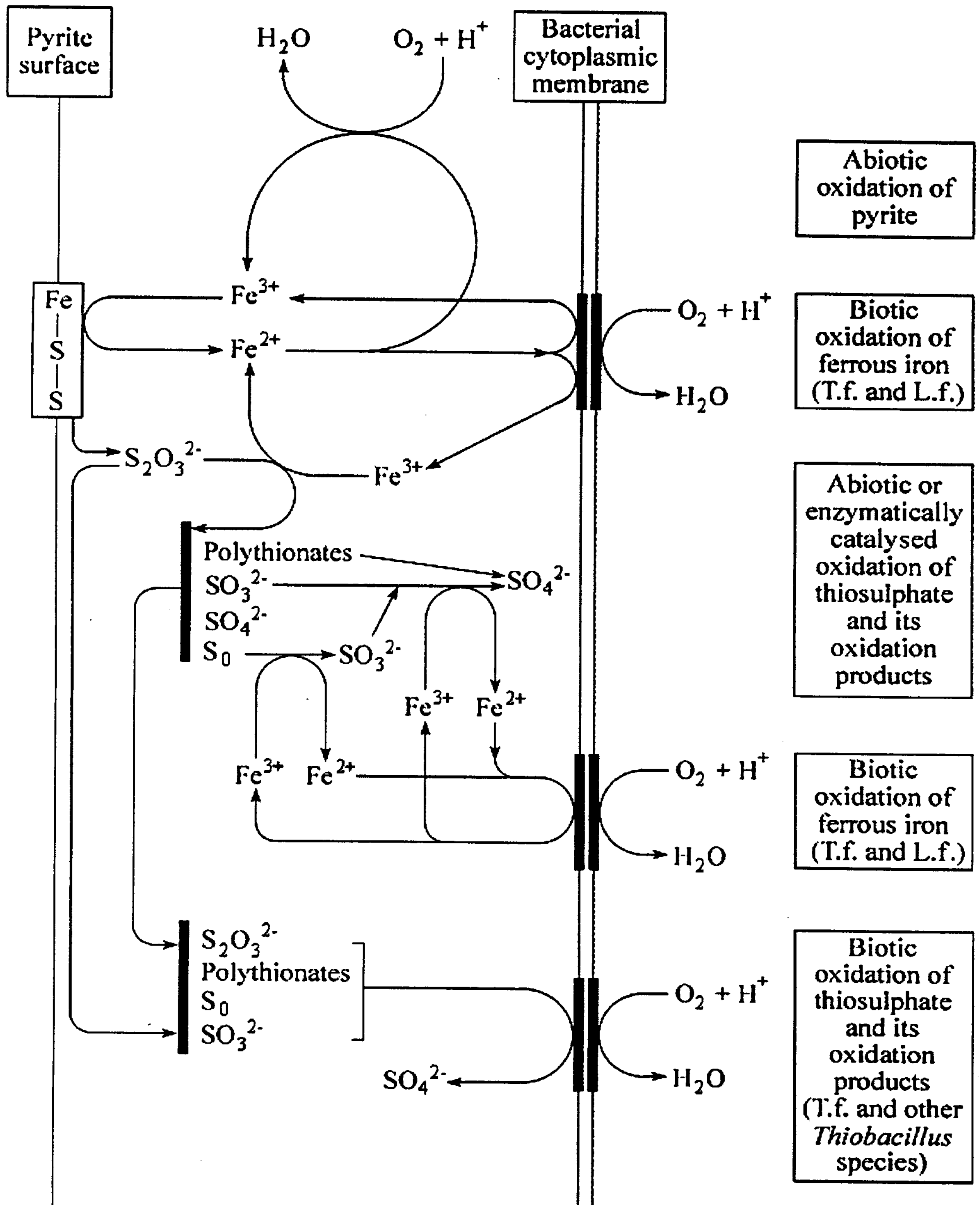

Fig. 6. Comprehensive model of abiotic and biotic oxidation processes involved in the oxidation of pyrite to sulphate, ferrous and ferric iron. The linked chemical transformstions in this diagram are not balanced. T.f. = *Thiobacillus ferrooxidans* and L.f. = *Leptospirillum ferrooxidans*.

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Thiobacillus ferrooxidans. *Thiobacillus ferrooxidans* is the best characterized member of the iron-oxidizing bacteria involved in the formation of acid mine drainage and bioleaching, and was for many years considered to be the only organism involved (Blake *et al.*, 1994; Hutchins *et al.*, 1986). Colmer and co-workers isolated *T. ferrooxidans* from bituminous coal mines in 1947-51 (Colmer and Hinkle, 1947; Colmer *et al.*, 1950; Temple and Colmer, 1951). Other iron-oxidizing bacteria isolated later were placed in the genus *Ferrobacillus* (Kinsel, 1960; Leathen *et al.*, 1956), but further investigation of these isolates indicated that they belonged to the same species as the organism isolated by Colmer *et al.* (1950) and that they should be included in the species *T. ferrooxidans* (Kelly and Tuovinen, 1972). This organism seems to be ubiquitous in acid mineral environments (Johnson, 1995a).

Morphologically *T. ferrooxidans* cells are short gram negative rods (0.5 x 1.0 μm), usually occurring singly or in pairs (Kelly and Harrison, 1989). Different strains possess flagella and/or pili (DiSpirito *et al.*, 1982).

The organism is an obligate chemolithotroph capable of growth on ferrous iron, sulphur and a range of sulphur compounds, including pyrite, thiosulphate, tetrathionate and sulphite (Kelly and Harrison, 1989). Shrader and Holmes (1988) observed phenotypic switching when *T. ferrooxidans* ATCC19859 and other strains of *T. ferrooxidans* were grown on media containing ferrous iron and thiosulphate. Under these conditions variants arose that formed large spreading colonies that utilized tetrathionate only. This switching was genetic and may be a way of adapting to changing environmental conditions. A detailed discussion of the mechanism of pyrite and sulphur oxidation by this bacterium is given in a previous section (see **New model of biotic pyrite oxidation**). *Thiobacillus ferrooxidans* is aerobic, but has been reported to grow and oxidize sulphur anaerobically using ferric iron as electron acceptor (Kelly and Harrison, 1989; Pronk *et al.*, 1991; Sugio *et al.*, 1985). Macintosh (1978) demonstrated nitrogen fixation by *T. ferrooxidans*; however, the organism prefers to grow on fixed nitrogen, with ammonium salts being the best source (Kelly and Harrison, 1989).

Thiobacillus ferrooxidans is mesophilic and grows between 2°C and about 40°C with an optimum in the vicinity of 30°C (Hallmann *et al.*, 1992; Kelly and Harrison, 1989; Leduc *et al.*, 1993). It is an

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obligate acidophile with a pH range for growth of approximately pH 1.3-4.8, but the range and/or optimum may vary according to the substrate on which it is growing under laboratory conditions. On thiosulphate, growth occurs between pH 1.5 and pH 4.3, with the optimal growth being at pH 3.6, while on tetrathionate growth also commences between pH 1.5 and pH 4.3, but with optimal growth at pH 2.5. The optimum pH for the oxidation of ferrous iron is approximately pH 2.0-2.5 (Ingledew, 1982; Kelly and Harrison, 1989).

Growth of *T. ferrooxidans* is highly susceptible to inhibition by organic compounds, as is clearly illustrated by the difficulty of growing this organism in media containing organic gelling agents such as agar or agarose (Johnson, 1995b; Mishra and Roy, 1979; Tuovinen and Kelly, 1973; Visca *et al.*, 1989). Colmer and Hinkle (1947) found that 1000 mg/l phenol and 100mg/l formaldehyde inhibited ferrous iron oxidation. Further studies indicated inhibition by ethylenediaminetetraacetic acid (EDTA), a complexing agent (Silver and Lundgren, 1968), anionic detergents, such as sodium lauryl sulphate (Dugan and Lundgren, 1964; Loos *et al.,* 1990a,b), as well as the antimicrobial benzoic and sorbic acids (Loos *et al.,* 1990a,b; Onysko *et al.*, 1984). Tuttle and Dugan (1976) found that ferrous iron and sulphur oxidation, as well as growth of *T. ferrooxidans*, were inhibited by a wide range of organic compounds (including citric acid cycle acids and amino acids). They found that inhibition by organic compounds was affected by the presence of inhibitory or stimulatory inorganic ions, the molecular structure of the organic compounds, pH, physical treatment of cells and temperature. Furthermore, the relative electronegativity of the organic inhibitor was found to be a major contributing factor in the inhibiton of ferrous iron oxidation. Their data led them to suggest that inhibitory organic compounds may directly affect the iron-oxidizing enzyme system, react abiotically with ferrous iron outside the cell, interfere with the roles of phosphate and sulphate during iron oxidation, and/or non-selectively disrupt the cell envelope or membrane.

Acid mine drainage is an environment with high concentrations of sulphate and metal ions (Silverman and Ehrlich, 1964), making metal ion tolerance a prerequisite for growth in these environments. *Thiobacillus ferrooxidans* is grown routinely in the 9K medium of Silverman and Lundgren (1959) containing 44.2 g/l $FeSO_4.7H_2O$ (= 8900 mg/l Fe). It is tolerant to zinc, nickel, cobalt, manganese and aluminium salts at metal concentrations exceeding 10 000 mg/l (Table 1). Heavy metal tolerance is strain dependant and varies according to the growth substrate, with cells growing on ferrous iron exhibiting the highest tolerance to the heavy metal ions (Tuovinen *et al.*, 1971). The inhibitory levels of other metal ions tested by Tuovinen *et al.* (1971) are summarized in Table 1.

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TABLE 1. Inhibitory levels of some metal salts for ferrous iron oxidation by *Thiobacillus ferrooxidans* (Tuovinen *et al.*, 1971)

Salt added	Inhibitory level of metal in mg/l		
$ZnSO_4.7H_{2}0$	Zn	>	10 000
$NiSO_4.6H_{2}0$	Ni	>	10 000
$CuSO_4.5H_2O$	Cu	>	1 000
$CoSO_4.7H_{2}0$	Co	>	10 000
$MnSO_4.4H_2O$	Mn	>	10 000
$Al_2(SO_4)_3.6H_{2}0$	Al	>	10 000
$UO_2SO_4.3,5H_2O$	U	<	700
Ag_2SO_4	Ag	<	50
$NaAsO_2$	As	<	200
SeO_2	Se	<	100
Na_2TeO_3	Te	<	100
$Na_2MoO_4.2H_2O$	Mo	<	5

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Thiobacillus ferrooxidans strains vary considerably in terms of temperature (optimum and range), colony and cell morphology, as well as heavy metal resistance (Kelly and Harrison, 1989; Leduc *et al.*, 1993; Roberto *et al.*, 1993; Tuovinen *et al.*, 1971). Harrison (1982) conducted a study on 23 strains from various geographical locations. He found that these strains belonged to seven different DNA homology groups that correlated with their physiological characteristics. Although two of these groups of organisms were unable to oxidize sulphur and were later found to be morphologically and phylogenetically far removed from the thiobacilli (Kelly and Harrison, 1989; Lane *et al.,* 1985), the DNA of the remaining five homology groups had base compositions ranging from 56 to 62 mol% G + C, suggesting that *T. ferrooxidans* is a phenospecies rather than a genospecies.

Leptospirillum ferrooxidans. Although *T. ferrooxidans* is the best characterized lithotrophic organism involved in pyrite oxidation and acid mine drainage formation, it is becoming increasingly clear that other organisms and especially *Leptospirillum ferrooxidans* may play as important a role in catalysing the process (Hallmann *et al.*, 1992; Johnson, 1995a; Pronk and Johnson, 1992; Sand *et al.,* 1992). *Leptospirillum ferrooxidans* was first isolated by Markosyan (1972) from a copper deposit in Armenia. Many similar organisms have been isolated from different parts of the globe, and as *L. ferrooxidans* has almost the same environmental parameters for growth as *T. ferrooxidans* (Hallmann *et al.*, 1992; Harrison and Norris, 1985; Sand *et al.*, 1992), it is conceivable that *L. ferroooxidans* is as widely distributed as *T. ferrooxidans*.

Morphologically *L. ferrooxidans* cells are characterized by a spiral or vibroid shape, but they may be morphologically variable. Filaments of up to 30 turns have been observed. The cells are motile by polar flagella and swim with a corkscrew motion. The cells are Gram-negative (Harrison and Norris, 1985).

The organism is an obligate chemolithotroph, deriving its energy from the oxidation of ferrous iron, but is incapable of oxidizing sulphur or any of the sulphur compounds oxidized by the thiobacilli (Hallmann *et al.*, 1992; Harrison and Norris, 1985; Sand *et al.*, 1992). When grown on ferrous iron-containing media, the optimum substrate cencentration for *L. ferrooxidans* lies between 6 and 8 g/l ferrous iron, hence below that of *T. ferrooxidans* which is generally about 9 g/l ferrous iron. The organism is aerobic.

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Leptospirillum ferrooxidans is a mesophilic organism which grows well between 20°C and 40°C and optimally between 28°C and 30°C. However, below 20°C its growth rate declines far more rapidly than that of *T. ferrooxidans* and it will therefore probably be outcompeted in leaching environments below 20°C (Hallmann *et al.*, 1992). The organism is obligately acidophilic, growing optimally at approximately pH 1.6, which is below the optimum pH for *T. ferrooxidans.*

Like *T. ferrooxidans, L. ferrooxidans* is inhibited by organic compounds such as glucose (Hallmann *et al.*, 1992; Tuttle and Dugan, 1976). *Leptospirillum ferrooxidans* is generally more sensitive to toxic metals than *T. ferrooxidans*, but has been shown to tolerate uranium, molybdate and silver better than certain strains of *T. ferrooxidans* (Harrison and Norris, 1985).

Leptospirillum ferrooxidans strains have lower G+C contents in their DNA than *T. ferrooxidans*, and phylogenetically do not resemble any known bacteria on the basis of 16S rRNA analysis (Lane *et al.*, 1992). The *L. ferrooxidans* strains analysed by Lane *et al.* (1992) were also not closely related to one another. Hallmann *et al.* (1992) also found large genetic variation among the strains tested by them and concluded that *L. ferrooxidans, like T. ferrooxidans,* is a phenospecies rather than a genospecies.

Moderately thermophilic, mixotrophic/facultatively lithotrophic iron-oxidizing bacteria. Le Roux *et al.* (1977) were the first to report the existence of moderately thermophilic facultatively lithotrophic acidophilic bacteria. These organisms could oxidize ferrous iron and catalyse the oxidation of pyrite (Hutchins *et al.*, 1986). Since then similar organisms have been isolated from various sources. These bacteria are short rods, with certain isolates exhibiting filamentous growth and/or endospores (Brierley and Lockwood, 1977; Ghauri and Johnson, 1991)

These organisms prefer to grow mixotrophically, on ferrous iron or pyrite and yeast extract, but certain isolates have appeared to grow chemo-autolithotrophically on iron or pyrite, as well as heterotrophically on yeast extract (Ghauri and Johnson, 1991; Norris and Barr, 1985). Although most of the moderately thermophilic facultatively lithotrophic iron-oxidizing bacteria seem to require reduced forms of sulphur for biosynthesis, strains have been isolated that could utilize sulphate as sulphur source (Hutchins *et al.*, 1986; Norris and Barr, 1985). The rate at which carbon dioxide was fixed by these organisms, was negatively influenced by the availability of yeast extract (a carbon

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source). The growth rate of the organisms decreased considerably when they were grown either heterotrophically or lithotrophically (Ghauri and Johnson, 1991; Norris and Barr, 1985).

Phylogenetically (based on 16S rRNA sequence analysis) the three strains of moderately thermophilic facultatively lithotrophic iron-oxidizing bacteria tested by Lane *et al.* (1992), grouped within the Gram-positive bacteria, despite being characterized as Gram-negative or Gram-indeterminate. The strains branched from very close to the origin of the phylogenetic tree of the Gram-positive bacteria, with representatives in both major sub-divisions of the Gram-positive bacteria (Lane *et al.*, 1992).

Mesophilic heterotrophic iron-oxidizing bacteria. Johnson *et al.* (1992) isolated from streamer growth in acid water a heterotrophic bacterium that was capable of oxidizing ferrous iron. In liquid medium the isolate (CCH7) appeared macroscopically as thread-like growths and was considered to be the main organism involved in acid streamer formation. A second heterotrophic iron-oxidizing bacterium (T-21) isolated by the same laboratory (Pronk and Johnson, 1992) grew as short rods and did not form any macroscopic growth in liquid media.

Neither of the two organisms fixed carbon dioxide and ferrous iron oxidation activity tended to be limited by the availability of a suitable organic substrate. Neither of the organisms possessed the capacity to oxidize sulphur compounds, but T-21 was shown to catalyse the oxidation of pyrite, via the production of ferric ions, if ferrous ions and yeast extract (a carbon source) were present. Pyrite dissolution by strain T-21 was only about 30% of that of *T. ferrooxidans* (Pronk and Johnson, 1992). Strain CCH7 was generally less tolerant to heavy metal inhibition than *T. ferrooxidans* or *L. ferrooxidans* (Johnson *et al.*, 1992).

***Metallogenium* spp.** In 1972 Walsh and Mitchell postulated a possible role for the moderately acidophilic bacteria of the genus *Metallogenium* in a succession of micro-organisms in mine waste dumps and the formation of acid mine drainage. At pH values above pH 4.5, abiotic oxidation of ferrous iron by dissolved oxygen proceeds rapidly, and at pH values below pH 3.5 ferrous iron oxidation by *T. ferrooxidans* becomes significant. They suggested that *Metallogenium* spp. catalyse the oxidation of ferrous iron in the range pH 3.5-4.5, which is too high for rapid iron oxidation by *T. ferrooxidans*.

Metallogenium spp. are polymorphic with cell shapes ranging from coccoid to threadlike. The cells are normally encrusted heavily with ferric precipitates. They are aerobic and multiply by means of

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budding (Dubinina,1970; Walsh and Mitchell, 1973). The bacterium isolated by Walsh and Michell (1972) was capable of oxidizing low concentrations of ferrous iron and grew optimally at pH 4.1.

However, subsequent research has questioned the role of *Metallogenium* in the bacterial succession involved in the formation of acid mine drainage as postulated by Walsh and Mitchell (1972). Kleinmann and Crerar (1979) found that *T. ferrooxidans* was able to adapt to a neutral macro-environment (pH 6.9), and to change it sufficiently to allow its own growth and survival. A previously reported study performed in our laboratory could detect only very low numbers of *Metallogenium*-like organisms in coal waste undergoing acidification (Cleghorn, 1997), while Harrison (1978) in his laboratory scale experiment on the microbial succession in coal waste dumps could not find *Metallogenium*-like organisms.

Acidophilic sulphur-oxidizing bacteria

This section will focus on the members of the genus *Thiobacillus* that are well known in acid drainage and other pyrite dissolution environments. Extremely thermophilic sulphur-oxidizing bacteria among the archaeobacteria (Staley *et al.*,1989) will not be discussed, even though Brierley (1978) discussed in considerable detail the potential of *Sulfolobus* spp. to assist the bacterial leaching of ores. *Sulfolobus* spp. occur typically in hot springs and have not been implicated yet as organisms of acid mine drainage generation.

During the biotic oxidation of pyrite by ferric iron, sulphur and a range of sulphur-containing compounds are formed (see **New model of biotic pyrite oxidation**). Although the capacity to oxidize inorganic sulphur compounds does not enable the responsible bacteria to catalyse directly the oxidation of pyrite and therefore the formation of acid mine drainage, they do catalyse acid mine drainage formation indirectly by oxidizing the sulphur compounds to sulphuric acid (Evangelou and Chang, 1995).

Certain iron-oxidizing bacteria, such as *T. ferrooxidans* and strains of moderately thermophilic facultatively lithotrophic iron-oxidizing bacteria, also possess the capacity to oxidize inorganic sulphur compounds. These orgainsms therefore catalyse both the primary oxidation of pyrite (via ferric iron formation) and the downstream oxidation of sulphur-containing intermediates. As these

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organisms have been described under the iron-oxidizing bacteria, descriptions of them will not be repeated.

Thiobacillus thiooxidans. This organism was first isolated by Waksman and Joffe (1922). It is a short Gram-negative rod (0.5 x 1.0-2.0 μm), occurring singly and in short chains (Kelly and Harrison, 1989).

Thiobacillus thiooxidans is an obligate chemolithotroph and autotroph. It is capable of oxidizing elemental sulphur and various inorganic sulphur compounds, including sulphide, thiosulphate, tetrathionate and sulphite, but not pyrite (Kelly and Harrison, 1989).

The organism is strictly aerobic. It grows between 10°C and 37°C, with optimal growth occurring at 29-30°C. *Thiobacillus thiooxidans* is an acidophilic organism, growing at pH 0.5-5.5, with optimal growth at pH 2.0-3.0 (Kelly and Harrison, 1989).

Phylogenetically, on the basis of 5S rRNA analysis, this organism is most closely related to the type strain of *T. ferrooxidans* (Lane *at al.,* 1985).

Facultatively lithotrophic *Thiobacillus* spp. Several acidophilic and moderately acidophilic *Thiobacillus* spp. are capable of heterotrophic growth on various organic substrates (Kelly and Harrison, 1989). The acidophilic *Thiobacillus acidophilus* was isolated from a culture of *T. ferrooxidans* (Guay and Silver, 1975). Moderately acidophilic thiobacilli, which may have been *Thiobacillus novellus, Thiobacillus thioparus* or *Thiobacillus intermedius,* were detected in large numbers in uranium mine waste dumps (Sand *et al*., 1995).

The organisms of this group are short Gram-negative rods. They are capable of growing lithotrophically on a range of inorganic sulphur compounds, but are incapable of oxidizing pyrite. They can also grow heterotrophically on various organic substrates, including glucose. *Thiobacillus intermedius* does not grow well in media containing only a single carbon source, but grows when yeast extract is added to the medium (Kelly and Harrison, 1989).

The facultatively lithotrophic *Thiobacillus* spp. may stimulate the oxidation of pyrite and acid mine drainage generation in two ways. Firstly, they oxidize sulphur-containg intermediates formed during pyrite oxidation in a similar way to the obligately lithotrophic *T. thiooxidans.* Secondly, the organisms might remove organic substances inhibitory to the obligately lithotrophic iron-oxidizing bacteria that

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are sensitive to inhibition by organic substances. This role has been indicated for the consortium of *T. acidophilus* in association with *T. ferrooxidans* (Harrison, 1984).

Acidophilic heterotrophic bacteria

Acidophilic heterotrophic bacteria occur in acid mine drainage water and have been found in cultures of *T. ferrooxidans* (Harrison, 1981; Wichlacz and Unz, 1981). The obligately chemolithotrophic iron-oxidizing bacteria which catalyse the oxidation of pyrite and the subsequent formation of acid mine drainage are very sensitive to inhibition by organic substances (Hallmann, *et al.*, 1992; Harrison, 1984; Tuttle and Dugan, 1976). It has been postulated that the acidophilic heterotrophic bacteria stimulate the growth of these bacteria by removing inhibitory organic molecules (Harrison, 1984). Harrison (1984), as well as Johnson and McGinness (1991a), used acidophilic heterotrophic bacteria to facilitate the growth of *T. ferrooxidans* and other iron-oxidizing bacteria on plates solidified with agar or agarose. Hallmann *et al.* (1992) found that heterotrophic bacteria could stimulate the oxidative dissolution of pyritic ores by mixed cultures of *T. ferrooxidans, L. ferrooxidans* and *T. thiooxidans*. Acidophilic heterotrophic bacteria isolated from acid mineral environments have been assigned mainly to the genus *Acidiphilium* (Hallmann *et al.*, 1992; Harrison, 1981, Harrison, 1984; Johnson and Kelso, 1983; Pronk and Harrison, 1995) and this genus is therefore considered to contain the most important heterotrophic bacteria stimulating the formation of acid mine drainage.

Harrison (1989), in Bergey's Manual of Systematic Bacteriology, described members of the genus *Acidiphilium* as straight Gram-negative rods (0.3-1.2 μm x 0.6-4.2 μm) with rounded ends. The organisms are aerobic and weakly catalase-positive. They are acidophilic, growing between pH 2.0 and pH 5.9. They are mesophilic, with optimum growth between 31°C and 41°C. They grow slowly below 20°C, do not grow at 47°C and die rapidly at 67°C. *Acidiphilium* spp. are organotrophic and do not grow on elemental sulphur, inorganic sulphur compounds or ferrous iron. However, these organisms are inhibited by the high concentrations of organic substances used in conventional organic media. Various members of the genus are capable of reducing ferric iron to ferrous iron, and may therefore play an important role in the biogeochemical cycling of iron in acid mine drainage environments (Johnson and McGinness, 1991b). Hallmann *et al.* (1992) found that *L. ferrooxidans* formed flocs in mixed cultures with *Acidiphilium* strains and suggested that the *Acidiphilium* might also stimulate pyrite oxidation by encouraging adhesion between iron-oxidizing lithotrophic bacteria and the pyrite surface.

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Based on 16S rRNA analyses, *Acidiphilium* spp. are closely related to *Thiobacillus acidophilus* (Lane *et al.*, 1992). Recently Kishimoto *et al.* (1995) proposed that the genus *Acidiphilium* be divided into two genera, *Acidiphilium* and *Acidocella*. The new genus *Acidocella was* proposed to accommodate the organisms previously known as *Acidiphilium facilis* and *Acidiphilium aminolytica*. The distinction between the two genera is based on 16S rRNA analysis, as well as differences in pigmentation, including the synthesis of photopigments (*Acidocella* spp. are not pigmented), and susceptibility to inhibition by organic acids.

Development and Interactions of Bacterial Populations Associated with Pyrite Degradation

Iron-oxidizing bacteria

Cyclical reactions involving iron oxidation and reduction lead to the oxidation of pyrite and the formation of acid mine drainage. A detailed description of the mechanisms of pyrite oxidation has been given under a detailed review of the organisms and reactions involved in iron oxidation and reduction in acid mineral environments presented by Pronk and Johnson (1992). The oxidative dissolution of pyrite to thiosulphate and ferrous iron requires ferric iron as oxidizing agent. During this process the ferric iron is reduced to ferrous iron (Moses *et al.*, 1987; Sand *et al.*, 1995; Schippers *et al.*, 1996). The ferrous iron produced in the oxidation of pyrite is oxidized by iron-oxidizing bacteria, such as *T. ferrooxidans* and *L. ferrooxidans,* to ferric iron. Catalysis of these reactions is assisted by the concentration of ferric iron in the extracellular matrices with which the bacteria adhere to the pyrite surface (Gehrke *et al.*, 1995; Sand *et al.*, 1995). The ferric iron can then once again act on the pyrite as oxidizing agent. A schematic summary of the transformations of pyrite and iron by attached and unattached bacteria in acid mine drainage-forming environments is given in Fig. 7. In view of the potential significance of the bacterial attachment to pyrite, the process and its role in iron oxidation and pyrite dissolution are described in detail below.

Phase 1: Bacterial adsorption to pyrite and the initiation of pyrite dissolution. In batch culture experiments using *T. ferrooxidans* to oxidize pyrite, an initial period where no observable dissolution of the pyrite takes place can be observed. During this period the bacteria attach themselves to the pyrite surface (Espejo and Ruiz, 1987; Fernandez *et al.*, 1995; Mustin *et al.*, 1992; Mustin *et al.*, 1993.). The adhesion of bacteria to pyrite is a complex and non-random process influenced by surface

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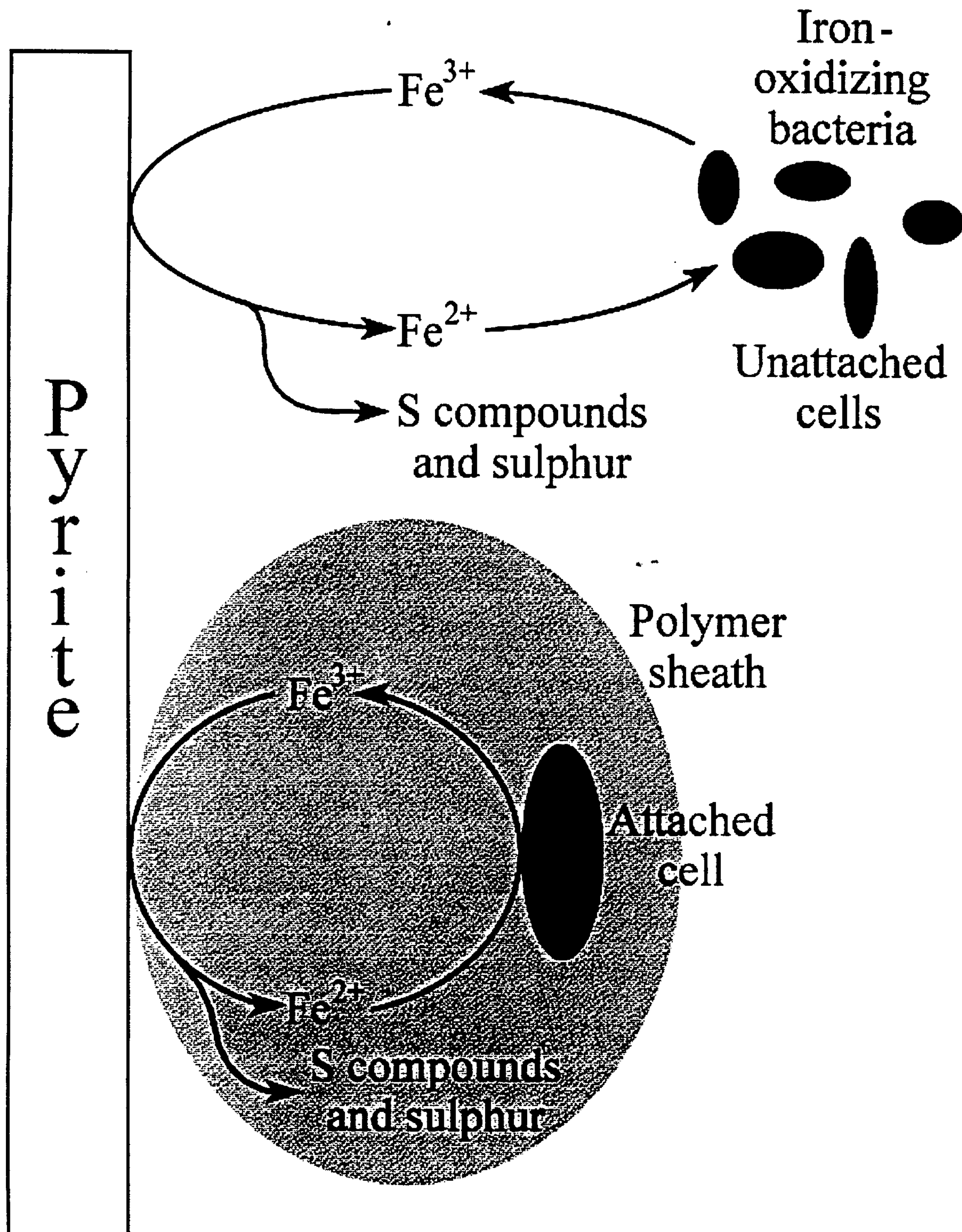

Fig. 7. Catalysis of the oxidation of pyrite by attached and unattached iron-oxidizing bacteria oxidizing ferrous iron to ferric iron. The ferric iron in turn drives the oxidation of pyrite, yielding sulphur and sulphur compounds and ferrous iron that can be reoxidized by the iron-oxidizing bacteria.

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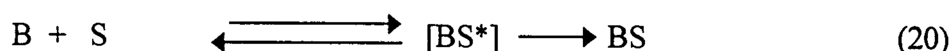
energy effects (Bagdigan and Myerson, 1986). Electrostatic and hydrophobic interactions between the bacteria and the pyrite (Blake *et al.*, 1994) and chemotaxis by the bacteria to sites where electrochemical dissolution due to semiconducting properties of pyrite takes place (Acuña *et al.*, 1992, 1992; Sand *et al.*, 1995), are also involved. Gehrke et al. (1995) noted that ferric iron in the exopolymer sheaths of *T. ferrooxidans* and *L. ferrooxidans* is necessary to mediate the electrostatic interaction between the negatively charged pyrite surface and the bacterium, thereby facilitating adhesion. Heterotrophic bacteria of the genus *Acidiphilium* may also aid the attachment of *L. ferrooxidans* to pyrite (Mustin *et al.*, 1993).

According to the model of Zobell (1943), bacterial adhesion is a two phase process, involving an initial reversible step, followed by a second irreversible step. This can be written as :



$$B + S \rightleftharpoons [BS^*] \longrightarrow BS \quad (20)$$

where the reactants B and S are the bacteria and the solid surface, respectively, and [BS*] a metastable state which results from the initial interaction between the bacteria and the substrate surface. In this state the bacterium is held at a finite distance from the surface, due to a balancing of attractive and repulsive forces between the two bodies. This complex may dissociate to leave a free, non-interacting cell and surface site. However, the bacterium may excrete the necessary exopolymers to produce a permanent association, BS, between the bacterium and the surface site.

Studies by Bagdigan and Myerson (1986) on the adhesion of *T. ferrooxidans* to pyrite in coal showed that a mathematical model based on the two phase adhesion model fitted the kinetics of the adhesion. They further showed a constant non-zero amount of reversibly adsorbed bacteria, which also suggested that the adsorption process takes place in two steps. Electron microscopic studies by Rojas *et al.* (1995) confirmed this model, for during the initial (reversible) interaction between *T. ferrooxidans* and pyrite, the bacteria retained their normal appearance. Subsequently, however, a dramatic change took place. The cells covered themselves with an organic substance that contained phosphorous (suggesting phospholipids). This layer formed a contact zone between the bacteria and the pyrite, by which the bacteria remained attached to the pyrite surface.

During the initial adhesion of the bacteria to the surface of the pyrite, very little or no observable pyrite dissolution takes place but ferric iron present in the liquid medium is reduced to ferrous iron, (Mustin *et al*, 1992). However, the electron microscopic observations of Rojas *et al.* (1995) clearly

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showed growth and pyrite oxidation activity after 12 hours, as the organic matrix surrounding the bacteria became dotted with sulphur colloids and cell division was observed. These observations can be explained by considering the chemistry of pyrite oxidation as well as the metabolic action of *T. ferrooxidans* in re-oxidizing (recycling) ferrous to ferric iron. Ferric iron acts as the electron acceptor in the oxidation of pyrite (Luther, 1987; Moses *et al.,* 1987) and is reduced to ferrous iron. This would explain the observed reduction of ferric iron early in phase 1. The sulphur atoms in pyrite are thermodynamically loose (Andrews, 1987) and iron is recycled and trapped in the reaction space, which could explain the apparent preference for sulphur solubilization in the non-stoichiometric solubilization of pyrite observed by Andrews (1987) and Mustin *et al.* (1992) during phases 1-3 of pyrite oxidation.

Phase 2: Early logarithmic growth phase of free bacteria. Phase 2 of bacterial pyrite oxidation in batch culture experiments is marked by sharp increases in the percentage of non-attached bacteria and ferric iron. The growth of free bacteria corresponds to the early logarithmic phase. The solubilization of iron and sulphur becomes significant, and pyrite or arsenopyrite crystals show surface cracks as the early corrosion pattern (Fernandez *et al.*, 1995; Mustin *et al.*, 1992).

The availability of ferrous iron in the surrounding medum (formed during phase 1), as well as competition for attachment sites on the pyrite, leads to the onset of the logarithmic growth of free bacteria. During this phase the biological oxidation of ferrous iron starts to exceed the rate at which ferric iron is reduced. This leads to the decline of observable ferrous iron in the medium while the increased availability of ferric ions increases the redox potential of the reaction medium and therefore the oxidative attack on the pyrite.

Phase 3: Late logarithmic growth phase of free bacteria. This phase corresponds to the later part of the logarithmic growth phase of free bacteria. It is characterized by high concentrations of ferric iron and very low concentrations of ferrous iron. The redox potential of the medum stabilizes at a high level and the solubilization of iron and sulphur increases strongly. Pits, which later become the dominant corrosion pattern, start to appear on pyrite or arsenopyrite crystals (Fernandez *et al.*, 1995; Mustin *et al.*, 1992).

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During this phase the increase in the rate of bacterial oxidation of ferrous iron is limited by the rate at which ferric iron is reduced during the oxidation of pyrite. This leads to the stabilization of the redox potential, and an increase in the oxidative attack on the pyrite surface.

Phase 4: Stationary growth phase of free bacteria. After the free bacteria enter the stationary growth phase, the ferrous iron concentration increases, but remains far below that of the ferric iron. Deep pores develop as the dominant corrosion pattern on the pyrite surface (Fernandez *et al.*, 1995; Mustin et al., 1992).

Bacterial metabolism of iron becomes low during this phase. The reduction of ferric to ferrous iron during the oxidation of pyrite exceeds the rate of reoxidation of the ferrous iron by the bacteria, so that ferrous iron increases to a higher concentration in the medium.

Sulphur-oxidizing bacteria

A schematic summary of the fate of the sulphur moiety of pyrite in reactions involving attached and non-attached sulphur-oxidizing bacteria during acid mine drainage formation is given in Fig. 8.

The oxidation of the sulphur moiety of pyrite by ferric iron (produced by iron-oxidizing bacteria such as *T. ferrooxidans* and *L. ferrooxidans*) leads first to the formation of thiosulphate (Moses *et al.*, 1987; Sand *et al.*, 1995; Schippers *et al.*, 1996). Thiosulphate is unstable in acidic environments and decomposes to form sulphur and a range of sulphur-containing compounds, including polythionates (Moses *et al.*, 1987; Schippers *et al.*, 1996). *Thiobacillus* spp. present in acid mine drainage-producing environments oxidize thiosulphate to sulphuric acid via similar intermediates and are therefore also capable of oxidizing the abiotically formed compounds (Evangelou and Chang, 1995; Pronk *et al.*, 1990; Schippers *et al.*, 1996).

During the initial stages of pyrite oxidation by *T. ferrooxidans*, the process proceeds non-stoichiometrically, with more sulphate being released than the stoichiometric ratio (Andrews, 1987). Rojas *et al.* (1995) found that attached cells of *T. ferrooxidans* deposited sulphur storage globules in their extracellular polymer matrix. These globules can subsequently be oxidized to yield energy for the organism and sulphate. The organism is also capable of oxidizing elemental sulphur under anaerobic conditons using ferric iron as electron acceptor (Suzuki *et al.*, 1990).

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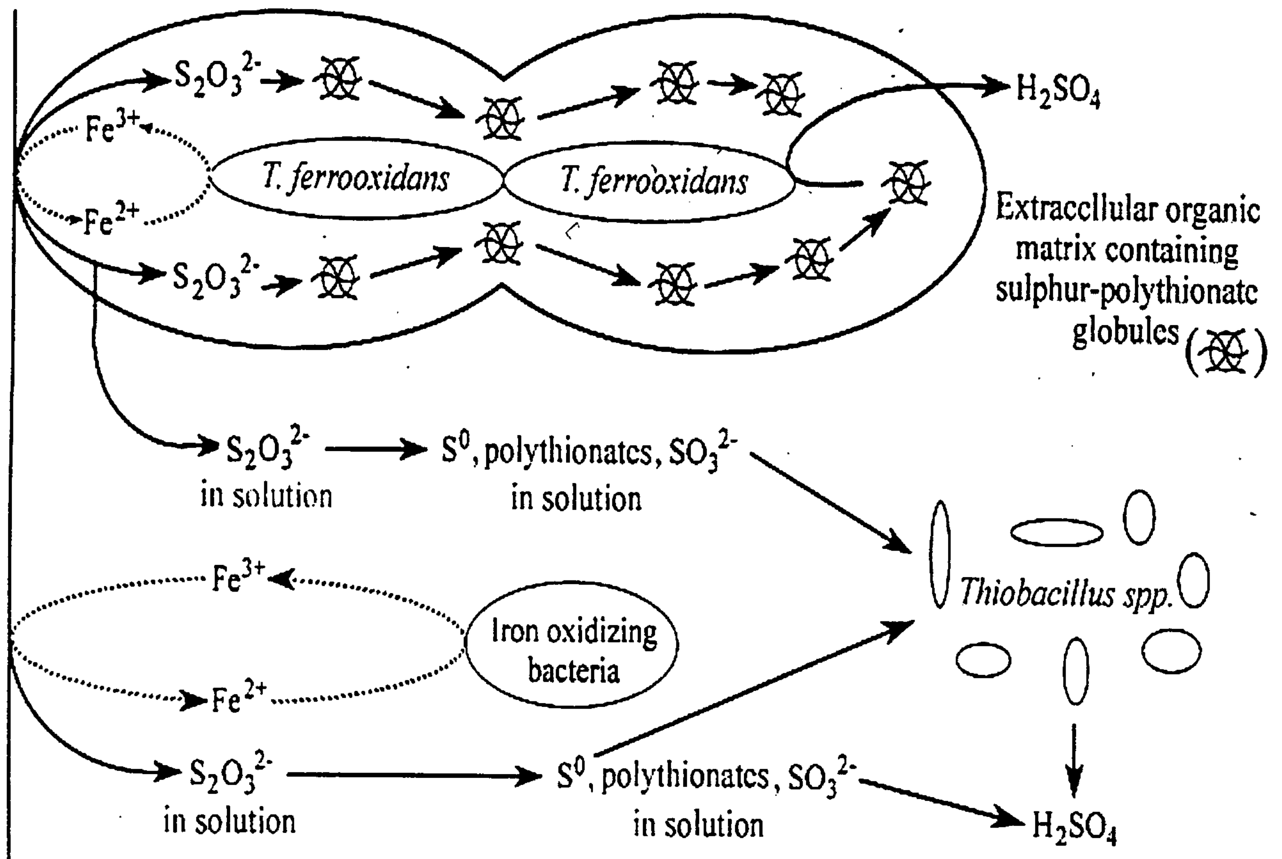

Fig. 8. Roles of attached and non-attached sulphur-oxidizing bacteria (*Thiobacillus* spp., including *T. ferrooxidans*) in the oxidation of thiosulphate, other sulphur compounds and sulphur formed during the oxidation of pyrite.

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Interactions between litho-autotrophs and heterotrophs in relation to organic nutrition and toxicity

Acid mine drainage-forming environments contain high numbers of litho-autotrophic or facultatively litho-autotrophic iron- and sulphur-oxidizing bacteria (Harrison, 1984; Johnson, 1995a; Sand *et al.*, 1992). These organisms are capable of fixing carbon dioxide and are the main source of recently fixed organic carbon in acid mine drainage-generating environments (Johnson, 1995a). Leakage of organic compounds or lysis of litho-autotrophic cells liberates organic substrates for use by heterotrophic organisms such as bacteria from the *Acidiphilium/Acidocella* group (Harrison, 1984, 1989; Johnson and McGinness, 1991a; Johnson, 1995a). As the litho-autotrophic iron-oxidizing bacteria *T. ferrooxidans* and *L. ferrooxidans* are strongly inhibited by organic compounds (Hallmann, *et al.*, 1992, Johnson, 1995a; Mishra and Roy, 1979; Tuovinen and Kelly, 1973; Tuttle and Dugan, 1976; Visca *et al.*, 1989), heterotrophic organisms may play an important role in the generation of acid mine drainage by consuming the organics and preventing inhibition of the litho-autotrophic bacteria that catalyse the oxidation of pyrite. Acidophilic protozoa and rotifers that graze on the bacteria present in acid mine drainage environments have been isolated (Johnson and Rang, 1993; McGinness and Johnson, 1992). A schematic summary of interactions among litho-autotrophic and heterotrophic microbial populations in respect of carbon nutrition, toxicity and detoxification of organic microbial products in acid mine drainage-producing environments is presented in Fig. 9.

Combined model of bacterial transformations and interactions involved in pyrite oxidation in acid mine drainage environments

The microbial catalysis of acid mine drainage formation in pyritic mineral environments is linked to transformations of iron, sulphur, carbon and oxygen. A combined model of these transformations, interactions among them and microbial populations that catalyse them is presented in Fig 10.

Environments where acid mine drainage is generated, are not closed systems. Biogeochemical cycles in these environments are therefore also not closed. Pyritic minerals are the primary energy source in these environments. The central reaction in the formation of acid mine drainage is the oxidation of pyrite by ferric ions (Luther, 1987; Moses *et al.*, 1987; Sand *et al.*, 1995, Schippers *et al.*, 1996). The pyrite is consumed with the production of ferrous iron and thiosulphate, which are subsequently

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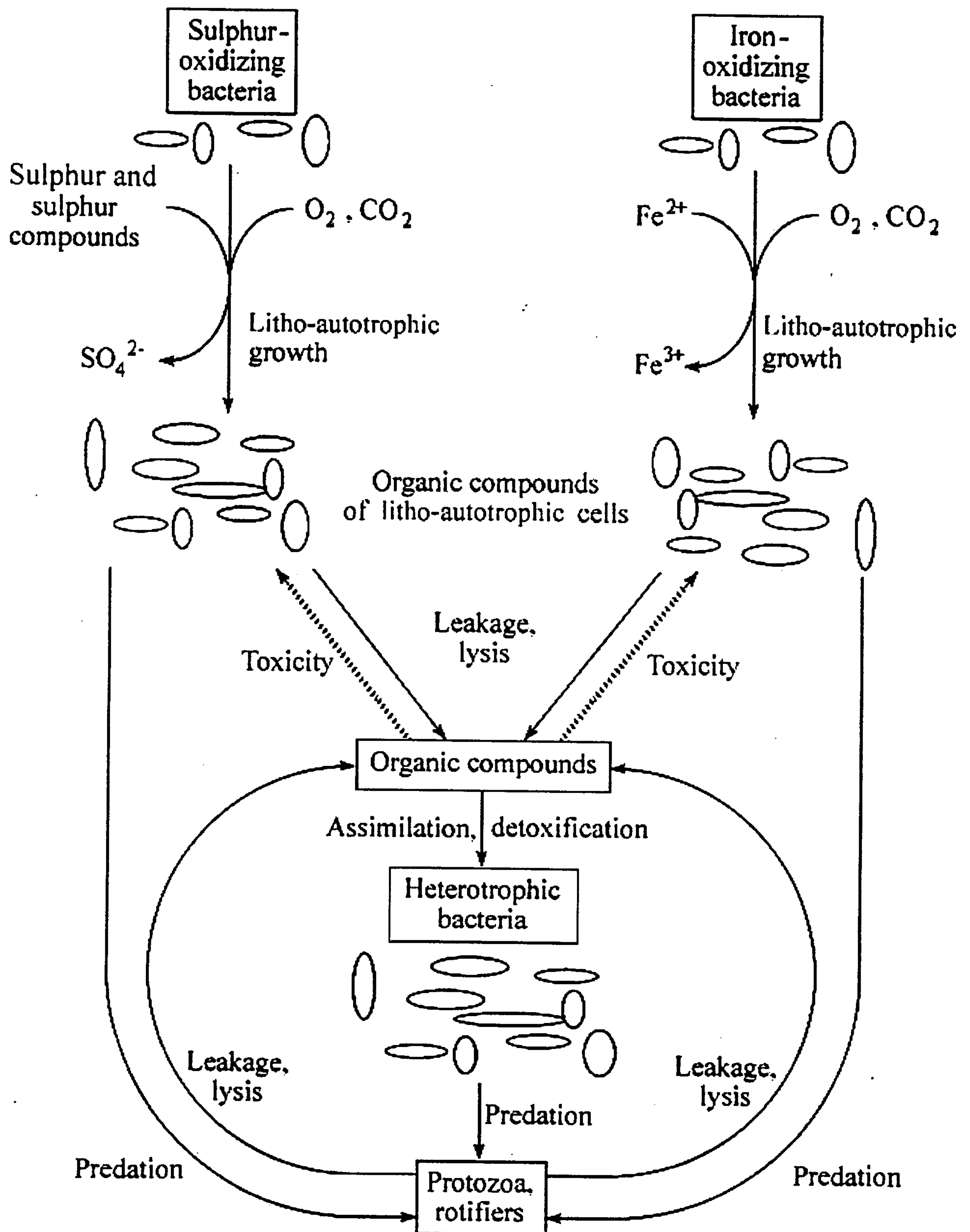

Fig. 9. Schematic summary of the carbon flow in acid mine drainage-generating environments, indicating the various groups of organisms and processes involved.

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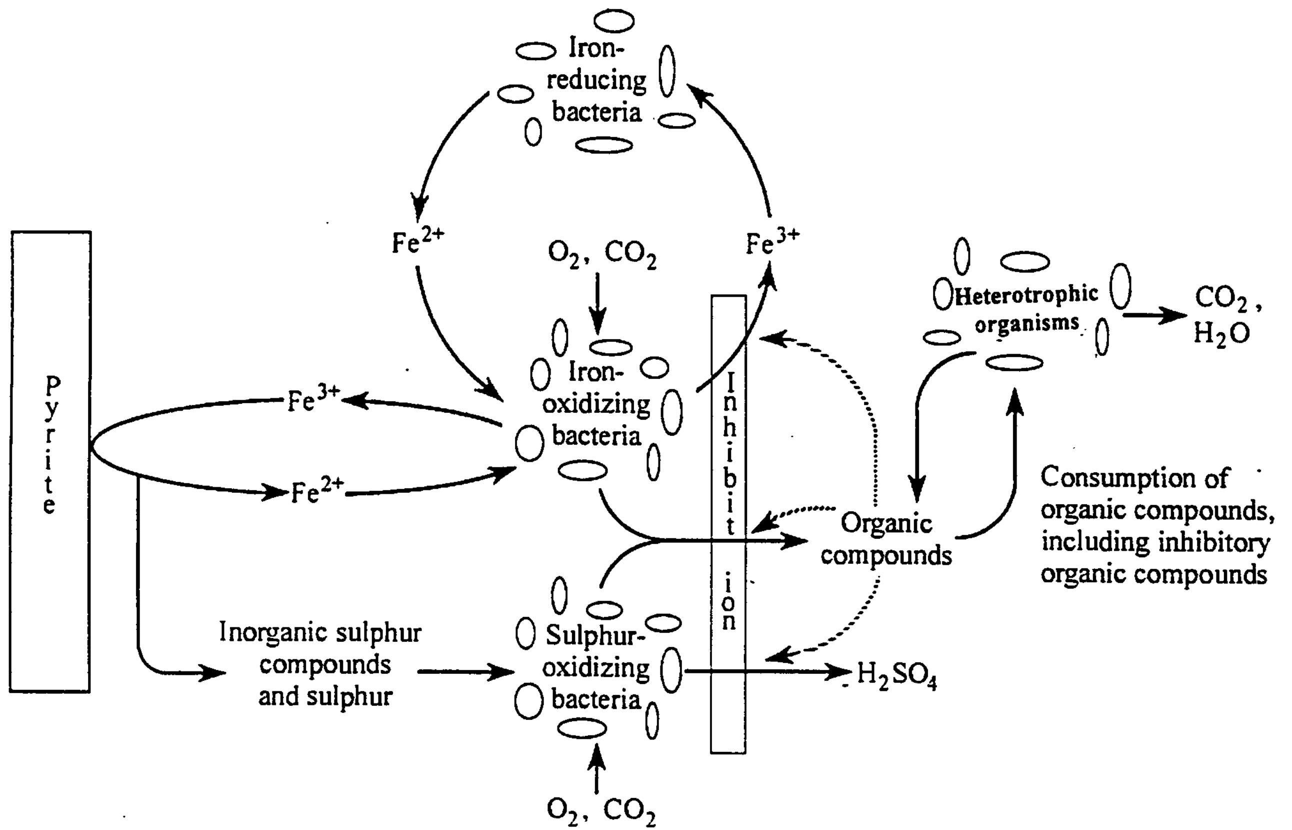

Fig. 10. Schematic summary of the ecology of acid mine drainage generation, in relation to the biogeochemical cycling of iron, sulphur, carbon and oxygen. Iron-oxidizing, sulphur-oxidizing and iron-reducing bacteria may be the same organism, for example *T. ferrooxidans*, with the particular activity or activities determined by the micro-environment.

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oxidized and released into the environment as ferric iron and sulphate (Atlas and Bartha, 1993). The oxidation of pyrite is therefore driven by ferric iron, which is also an end-product of the microbially catalysed oxidation of pyrite. Cyclic reactions of the ferrous iron-ferric iron redox couple and bacterial populations catalysing these reactions are thus central to acid mine drainage formation (Fig. 6, 7 and 10). Bacterial populations that catalyse the oxidation of thiosulphate, other inorganic sulphur compounds and sulphur also enhance the formation of acid mine drainage by oxidizing these components to sulphuric acid (Fig. 8 and 10). Furthermore, these litho-autotrophic iron- and sulphur-oxidizing bacteria are the primary poducers in acid mineral environments. However, they are inhibited by low concentrations of many organic compounds, including products of their metabolism (Harrison, 1984). Heterotrophic organisms that consume these organic compounds therefore form an essential component of the ecology of acid mine drainage formation (Fig. 9 and 10).

Conclusions

Pyrite oxidation is a complex phenomenon with many steps, which lead to the formation of various intermediates and side products, such as sulphur and sulphoxy anions. The process is further complicated by the interactions of abiotic and biotic transformations in the reaction sequences. The understanding of pyrite oxidation is by no means complete, but recent observations on the mechanism of pyrite oxidation suggest the following hypothesis:

i. Pyrite oxidation is primarily an abiotic process.

ii. Ferric hexahydrate is the main oxidizing agent in the primary oxidation of pyrite, as well as the subsequent oxidation of intermediary sulphur-containing compounds resulting therefrom.

iii. Chemolithotrophic iron-oxidizing bacteria, such *T. ferrooxidans* and *L. ferrooxidans*, catalyse the oxidation of pyrite by the production of ferric ions.

iv. Free and attached cells of the lithotrophic bacteria catalyse the oxidation of pyrite.

v. During the initial stages of attack, attached cells are the dominant catalytic agents, but non-attached cells play an increasingly important catalytic role as the leaching environment matures.

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vi. Various groups of acidophilic organisms interact synergistically to catalyse the oxidation of pyrite, which leads to the formation of acid mine drainage.

This discussion of pyrite oxidation supports the cyclic mechanism proposed by Sand *et al.* (1995), in which iron is oxidized by the bacteria and reduced on the pyrite surface. Using this model and taking into account the enzymatic systems of *T. ferrooxidans* as well as the chemistry of the intermediary sulphur compounds formed during the oxidation of pyrite, the model of biologically catalysed pyrite oxidation can be enlarged. Pyrite and intermediate sulphur-containing compounds formed by its dissolution in aqueous environments containing ferric iron act as indirect energy sources for chemolithotrophic iron-oxidizing bacteria in that they reduce ferric iron to ferrous iron which the bacteria can utilize as a source of electrons for their energy metabolism.

Although *T. ferrooxidans* is the best characterized member of the acidophilic iron oxidizing bacteria, it is becoming increasingly clear that other iron-oxidizing bacteria such as *L. ferrooxidans* and heterotrophic iron-oxidizing bacteria may also play a very important part in catalysing pyrite oxidation. Sulphur-oxidizing members of the genus *Thiobacillus* may also play an important part in the formation of acid mine drainage as they oxidize sulphur compounds formed as intermediates during pyrite oxidation to sulphuric acid. Heterotrophic organisms living in acid mine drainage-generating environments stimulate the growth of iron-oxidizing bacteria in two possible ways. Firstly, they remove inhibitory organic substances from the environment and secondly, they assist in the adhesion of iron-oxidizing bacteria to the pyrite surface.

The study of the ecology of acid mine drainage-generating environments necessitates an understanding of the transformations of iron, carbon, sulphur and oxygen, as well as the organisms involved. Litho-autotrophic iron-oxidizing bacteria, such as *T. ferrooxidans* and *L. ferrooxidans,* play a pivotal role in the ecology of acid mine drainage-generating environments, as they not only produce the ferric iron necessary for pyrite oxidation, but are also responsible for carbon fixation. Various organoheterotrophic and mixotrophic organisms then utilize the carbon fixed by the litho-autotrophic organisms.

The study of the ecology af acid mine drainage-generating environments has often been hampered by difficulties experienced in culturing organisms from these environments. The most probable number (MPN) technique is frequently used to enumerate iron-oxidizing bacteria, but this technique is

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inherently inaccurate. Organic inhibitors in gelling agents such as agar or agarose hamper the use of solid media for the culturing and enumeration of acid mine drainage-causing organisms. However, recent media that use the symbiotic relationship between heterotrophic and litho-autotrophic bacteria in double layered media can overcome this inhibition by organic compounds (Johnson, 1995b) and may permit more acurate determinations of litho-autotrophic bacteria by plate counts.

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EXPERIMENTAL PART 1. ABIOTIC ECOLOGICAL DETERMINANTS (TEMPERATURE, MOISTURE, OXYGEN, CARBON DIOXIDE AND pH) AND IRON-OXIDIZING BACTERIA IN PILOT SCALE COAL WASTE DUMPS IN RELATION TO COVERS USED FOR DUMP REHABILITATION

INTRODUCTION

In 1993, as part of the Water Research Commission Project K5/454, the Department of Microbiology at the University of Stellenbosch, in conjunction with the Department of Water Affairs and Forestry and consulting engineers Wates, Meiring and Barnard, started a pilot scale experiment to study the effects of various dump cover treatments on the acidification of coal waste dumps and the microbial populations that could be involved in accelerating the process. It was hoped that results from this study would provide guidelines for the successful rehabilitation of coal waste dumps and the prevention of acid mine drainage.

Initial studies of the acidification of the pilot scale dumps (mini-dumps or cells) and abiotic factors and organisms associated with it, until August 1995, were conducted by Cleghorn (1997). Experimental Part 1 of this thesis is a continuation of the studies of Cleghorn (1997). The construction of the mini-dumps and the experimental methodology are described in detail by Cleghorn. (1997) and Loos *et al.* (1997). Where experimental procedures in the present thesis are those of Cleghorn (1997), they are indicated by reference to his thesis and Loos *et al.* (1997). Where necessary to provide a complete picture of the acidification process, Cleghorn's data pertaining to pH, other abiotic factors affecting the mini-dumps and the populations of bacteria capable of oxidizing high ferrous iron concentrations before August 1995, have been included in the present thesis with acknowledgement of their source.

The research of the present thesis was not only a continuation of the studies started by Cleghorn (1997) but was expanded to include:

(i) The determiniation of populations of bacteria oxidizing a high concentration of ferrous iron and capable of growth at the elevated temperature of 37°C and the lowered pH of 1.0-1.3.

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(ii) The determination of populations of bacteria oxidizing a moderate concentration of ferrous iron and including a wider range of bacteria than the high iron oxidizers.

(iii) The testing of cultures of moderate ferrous iron-oxidizing bacteria for utilization of sulphur and thiosulphate, to ascertain the occurrence of *T. ferrooxidans* among them.

(iv) The testing of a plate count method involving the FeSo-medium of Johnson (1995b), as a possible method to determine various populations of acidophilic bacteria involved in acid mine drainage generation.

It was hoped that these studies and those of Cleghorn (1997) would indicate the main groups of bacteria involved in the generation of acidity in the control and unsuitably rehabilitated mini-dumps.

MATERIALS AND METHODS

Construction and Materials of Pilot Scale Dumps

A diagram summarizing the construction of the variously treated mini-dumps (cells) in the pilot scale experiment at the Kilbarchan mine is presented in Fig. 11. Detailed descriptions of the construction of the cells are given by Cleghorn (1997) and Loos *et al.* (1997).

The coal waste used in the experiment was obtained from the Kilbarchan Mine. The waste consisted of fine particles of which at least 95% were < 3 mm in cross-section. Two soil types, namely, an Avalon soil and an Estcourt soil, were used to cover the cells. Compacted Escourt soil, which had the highest bulk density and the lowest water permeability, was used as the clay barrier between the coal waste and the Avalon topsoil in covers consisting of both soil types and for the vertical barrier walls separating and surrounding the cells. Analyses of the coal waste, Avalon and Estcourt soils are presented by Cleghorn (1997) and Loos *et al.* (1997).

As vegetation may play an important role in the stabilization and prevention of erosion of the cover material, cells 3-10 were seeded as descibed by Cleghorn (1997) and Loos *et al.* (1997). By November 1995 the vegetation was not well established and the Chamber of Mines Vegetation Unit again seeded these cells. The cells were fertilized with 500 kg/ha 2:3:2 N-P-K

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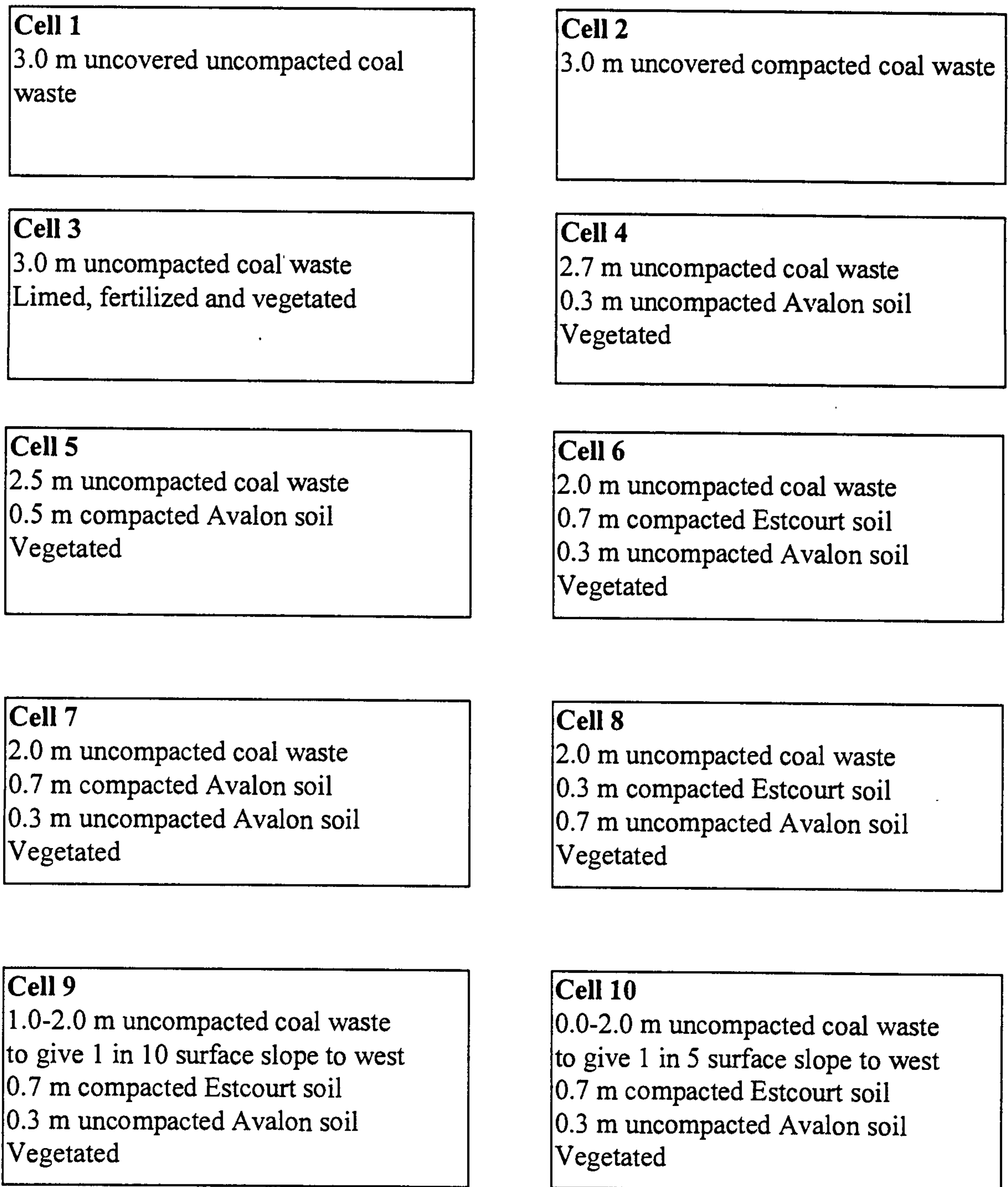

Fig. 11. Diagram summarizing the construction, treatment and relative positions of the cells of the pilot scale coal waste dump rehabilitation experiment at the Kilbarchan mine. The horizontal dimensions of all cells were 10 x 10 m. The cells were constructed on a plastic liner and were seperated by 1-m-wide barrier walls of compacted Estcourt soil, which was also used in a sloped containment barrier around the block of cells.

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fertilizer and bare areas lightly scarified, seeded with a grass cocktail and lightly cultivated to cover the seed and fertilizer. In addition to this treatment, cell 3 was limed (30 tons/ha dolomitic lime) and planted with stargrass (*Cynodon ethiopicus*). The stargrass plants were planted in shallow holes prepared on a grid of approximately 0.5 m by 0.5 m and watered (data supplied by B. L. Dawson, Chamber of Mines Vegetation Unit, Regents Park). The 1995/1996 rainy season was particularly wet and the grass covers on the cells became well established.

Studies of Abiotic Ecological Determinants

Rainfall

Rainfall was measured weekly in two rain-gauges on the pilot scale dumps as detailed by Cleghorn (1997) and Loos *et al.* (1997).

Oxygen and carbon dioxide in the coal waste

The oxygen and carbon dioxide concentrations of the atmosphere within the upper 15 cm of coal waste in the cells were measured weekly using buried sintered stainless steel probes and a portable carbon dioxide/oxygen monitor as described by Cleghorn (1997) and Loos *et al.* (1997a). However, no readings could be taken from February to May 1996 due to malfunctions in the carbon dioxide/oxygen monitor.

Sampling and analysis of coal waste for moisture and pH

Sampling. Sampling of the upper 15 cm of coal waste in the covered dumps or at 15-30 cm depth in uncovered dumps was performed using a clay auger as described by Cleghorn (1997) and Loos *et al.* (1997). Until September 1996, samples were transported to Stellenbosch the same day by car and plane. Thereafter samples for pH determination only were taken in the same way by the Department of Water Affairs and Forestry, but sent to Stellenbosch by fast mail to arrive usually 2 days after sampling.

Moisture and pH analysis of coal waste. The coal waste samples were analysed for moisture and pH as described by Cleghorn (1997) and Loos *et al.* (1997).

Microbiological Studies

Experimental approach

As *T. ferrooxidans* was generally regarded as the main acid-generating chemolithotrophic bacterial species in coal waste dumps (Blake *et al.,* 1994; Harrison, 1978; Kleinmann *et al.*, 1981), populations of this organism were monitored by most probable number (MPN) counts in

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samples from near the top of the coal waste in the mini-dumps throughout the period of the study. The selective medium for the MPN counts was the modified 9K medium used by Harrison *et al.* (1980), designated HJJ medium.

Populations of various other groups of iron-oxidizing bacteria were counted alongside the routine counts of high ferrous iron-oxidizing organisms believed to be *T. ferrooxidans*. These included bacteria able to grow at higher temperature than the usual *T. ferrooxidans* in an acidified HJJ medium (designated L medium) and bacteria requiring the lower iron concentration of the JLFe medium developed by D.B. Johnson (University of Wales, Bangor, personal communication). From these studies it might be possible to obtain indications of possible roles of iron-oxidizing bacteria able to function under relatively high temperature, high acid and high ferrous iron conditions (unusual strains of *T. ferrooxidans*; see Norris, 1990), or high acid and moderate but not high ferrous iron concentrations (*L. ferrooxidans* and others; see Sand *et al.,* 1992).

Subsequently, bacteria growing in the JLFe medium were tested for sulphur metabolism (which identifies *Thiobacillus*) in the S^0 medium of D.B. Johnson (personal communication). Those that metabolized sulphur were tested further for their ability to metabolize thiosulphate in Starkey's medium (Allen, 1957).

Plates of the FeSo medium developed by Johnson and his colleagues (Johnson, 1995b; Johnson *et al.,* 1987; Johnson and McGinness 1991a) were inoculated from the same dilution series used for the MPN counts of the abovementioned groups of iron-oxidizing bacteria to test the possiblity of using a more accurate plate count method to determine the numbers of acidophilic bacteria involved in acid mine drainage generation.

Media

HJJ medium. This medium, which was named after Harrison, Jarvis and Johnson (1980), was a modification (reduced ammonium sulphate concentration) of the widely used 9K liquid medium of Silverman and Lundgren (1959) for *T. ferrooxidans.* It was used for the MPN determinations as well as enrichment culturing of these and possibly other similar acidophilic high ferrous iron-oxidizing bacteria in the coal waste. The basal medium consisted of 2.00 g $(NH_4)_2SO_4$, 0.10 g KCl, 0.50 g K_2HPO_4, 0.50 g $MgSO_4.7H_2O$, 0.01 g $Ca(NO_3)_2$ and 1.0 ml 10 N H_2SO_4 in 700 ml distilled H_2O. To the sterilized (121°C for 15 min) basal medium were added 44.2 g $FeSO_4.7H_2O$ in 300 ml distilled water, which was sterilized by passage through a 0.2 µm nitrocellulose membrane filter (Millipore SA, Bellville). The pH of the basal medium and of the

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$FeSO_4.7H_2O$ solution was adjusted before sterilization to pH 2.0 by the addition of a 15 % (v/v) H_3PO_4-15 % (v/v) H_2SO_4 solution or H_2SO_4.

L medium. This medium was used for MPN determinations of high ferrous iron-oxidizing bacteria with relatively high temperature (37°C) and low pH growth parameters (extreme conditions) and differed from HJJ medium only in that the pH was lowered to pH 1.0-1.3.

JLFe medium. This moderate ferrous iron medium developed by D.B. Johnson (personal communication), contained about one third of the ferrous iron contained in HJJ medium, as some iron-oxidizing bacteria (including *L. ferrooxidans*) may be inhibited by the ferrous iron concentration employed in HJJ medium. The JLFe medium was used for more inclusive MPN determinations of acidophilic iron-oxidizing bacteria than those achieved with HJJ medium.

For the preparation of this medium a basal salts stock solutions (BSS) and a trace element stock solution (TES) were first prepared. The BSS contained 1.50 g $(NH_4)_2SO_4$, 0.50 g KCl, 5.0 g $MgSO_4.7H_2O$, 0.50 g KH_2PO_4 and 0.10 g $Ca(NO_3)_2$ in 1 000 ml distilled water and was acidified to pH 2.0-2.5 with H_2SO_4. The TES (modified slightly) contained 10 g $ZnSO_4.7H_2O$, 1.0 g $CuSO_4.5H_2O$, 1.0 g $MnSO_4.4H_2O$, 1.0 g $CoSO_4.7H_2O$, 0.5 g $Cr_2(SO_4)_3.K_2SO_4.24H_2O$ (modified component), 0.5 g $Na_2B_4O_7.10H_2O$ and 0.5 g $NaMoO_4.2H_2O$ in 1 000 ml distilled water and was acidified to pH 2.0 with H_2SO_4. A salts solution was then prepared from 100 ml BSS, 0.5 ml TES and 800 ml distilled water. To this was added (after sterilization) a solution containing 14 g $FeSO_4.7H_2O$ in 100 ml distilled water. Both the salts and ferrous sulphate solutions were adjusted to pH 2.0 with H_2SO_4 before sterilization. The salts solution was sterilized by autoclaving (121°C for 15 min), while the ferrous sulphate solution was sterilized by passage through a 0.2 µm nitrocellulose membrane filter (Millipore SA, Bellville).

S^0 **medium.** This medium of D. B Johnson (personal communication) was used to test positive MPN cultures in JLFe medium for the utilization of sulphur (S^0), thereby confirming the presence of *T. ferrooxidans* (Harrison, 1978; Kelly and Harrison, 1989). It had the same basal ingredients as the JLFe medium, containing 100 ml BSS, 0.5 ml TES and 900 ml distilled H_2O plus 10 g ground sulphur. The pH of the solution was adjusted to pH 2.7 using H_2SO_4. The medium was sterilized by steaming at 100 °C for 1 hour.

Starkey's medium. The medium as specified by Allen (1957) was used to test MPN cultures that grew in the JLFe medium and subsequently in the S^0-medium for thiosulphate utilization. Basal medium consisted of 0.30 g $(NH_4)_2SO_4$, 3.00 g KH_2PO_4, 0.50 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2$ and 0.01 g $Fe_2(SO_4)_3.9H_2O$ in 980 ml of distilled water. Thiosulphate solution was prepared as 5.00 g $Na_2S_2O_3.5H_2O$ in 20 ml distilled water. The solutions were sterilized separately at 121°C for 15 min then mixed in the ratio 49:1.

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FeSo medium. This solid medium developed by Johnson and his colleagues (Johnson, 1995b; Johnson *et al.,* 1987; Johnson and McGinness 1991a) was tested for the enumeration of acidophilic bacteria in coal waste by plate count. It consisted of four solutions that were sterilized separately and mixed after cooling to 45°C in a water bath. The basal salt stock solution (BSS) and trace element stock solution (TES) were as described under **JLFe medium**.

Solution A consisted of 575 ml distilled water, 100 ml BSS, 0.5 ml TES, 0.25 g Tryptone Soy Broth (Biolab Diagnostics, Midrand) and 1.1 g $(NH_4)_2SO_4$. The pH was adjusted to pH 2.5 using H_2SO_4 and the solution was heat-sterilized (121 °C for 15 min). Solution B consisted of 5 g agarose (FMC Bioproducts, Rockland, USA) in 250 ml distilled water and was heat-sterilised (121 °C for 15 min). Solution C consisted of 7 g $FeSO_4.7H_2O$ dissolved in 50 ml distilled water. This solution was adjusted to pH 2.0 using H_2SO_4 and sterilized by passage through a 0.2 µm nitrocellulose membrane filter (Millipore SA, Bellville). Solution D consisted of 1.51 g $K_2S_4O_6$ dissolved in 25 ml distilled water and sterilized by passage through a 0.2 µm nitrocellulose membrane filter (Millipore SA, Bellville). After sterilization, the heat-sterilized solutions (A and B) were allowed to cool to 45 °C in a water bath . The other solutions were warmed to 45 °C and mixed into A in the order C, D and then B. This molten medium was then split in two and one of the halves returned to the water bath to prevent gelling. To the other half were added 10 ml of an active culture of *Acidiphilium* strain E1A (isolated during this study from enrichment culture K/E1 from a fine soft waste from a duff-washing process at the Kilbarchan Mine). After thorough mixing, this inoculated medium was used to pour a thin underlayer in a standard Petri dish and allowed to gel. After gelling of the underlayer, a sterile overlayer of the same thickness as the underlayer was poured using the rest of the medium.

Coal waste samples from pilot scale dumps for microbiological analyses

Samples for MPN counts of bacteria in the coal waste in the pilot scale dumps were portions of those described previously under the heading **Sampling and analysis of coal waste for moisture and pH**.

MPN counts: General procedures

For MPN determinations of specific chemolithotrophic bacterial groups, flasks of the appropriate medium (see next section) were inoculated in triplicate with coal waste or dilutions thereof using the basal medium as diluent. The first two inoculations were 10 g coal waste in 50 ml medium and 1 g coal waste in 20 ml medium. The first dilution was 5 g coal waste in 50 ml basal medium (10^{-1} dilution), which was shaken for 5 min at maximum speed (approximately 600 oscillations/min) on a Griffin wrist action flask shaker (Griffin and George, London). Subsequent tenfold dilutions were prepared by suspending 10 ml of the 10^{-1} and subsequent

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dilutions in 90 ml of the appropriate basal medium. Flasks containing 20 or 50 ml of complete medium were inoculated with 1 ml of the appropriate dilution and incubated at 26 ^{0}C (most studies) or 37°C as specified in the next section. After appropriate incubation times, flasks were recorded as positive or negative for bacterial growth as specified in the following section.

From the patterns of cultures showing growth of the specific organisms in the MPN series, the populations of the organisms were determined using the appropriate MPN table of De Man (1983) or the American Public Health Association *et al.* (1955). The indicated populations were corrected to populations per g dry coal waste using the moisture content values for the samples. The reliability of each MPN test result, as the likelihood of obtaining the specific test result, was noted from Table 1 of De Man (1983), which was also used to determine the 95 % confidence limits of the MPN values. Confidence limits (95%) that could not be obtained from the De Man tables were computed according to Dr. J. H. Randall (Biometrician, Faculty of Agricultural Sciences, University of Stellenbosch).

MPN counts: Specific procedures for different bacterial groups

Acidophilic high ferrous iron-oxidizing bacteria. These iron-oxidizing bacteria were believed to be *T. ferrooxidans.* They were investigated in all the coal waste samples from the pilot scale dumps throughout the study period using HJJ medium. The diluent for preparing the dilutions was basal HJJ medium without the ferrous sulphate. The inoculated MPN flasks were incubated at 26 °C in the dark for 4 weeks and tubes which had changed colour from light green to a reddish brown colour (caused by the presence of ferric ions) were scored as positive.

Acidophilic relatively high temperature high ferrous iron-oxidizing bacteria. The L medium and incubation at 37 °C used in this study would select iron-oxidizing bacteria able to grow under extreme conditions of high iron concentration, low pH (1.0-1.3) and relatively high temperature. High temperature strains of *T. ferrooxidans* (Norris, 1990) tolerant of low pH (Razzell and Trussell, 1963) and possibly strains of *L. ferrooxidans* would be counted by this procedure. The basal salts solution of the HJJ medium was used as diluent and the MPN flasks were 250-ml Erlenmeyer flasks containing 100 ml L medium. The inoculated flasks were incubated at 37°C in the dark for 4 weeks and scored for growth in the same way as the cultures growing in HJJ medium.

Selected active cultures of these bacteria from MPN flasks inoculated with 10 g coal waste and the highest dilution giving growth, were subcultured into L medium and incubated at 40°C in the dark to investigate their ability to grow at this elevated temperature.

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Acidophilic moderate ferrous iron-oxidizing bacteria (count in JLFe medium). This medium was the least selective of all the media used for iron-oxidizing bacteria, and should have allowed growth of *T. ferrooxidans, L. ferrooxidans* and possibly other species that might be inhibited by the high iron concentration of HJJ medium. The basal HJJ medium without ferrous sulphate was used to prepare the dilutions, which were inoculated into 250 ml Erlenmeyer flasks containing 50 ml JLFe medium. Incubation was for 4 weeks at 26°C in the dark, after which the flasks were scored in the same way as for the MPN counts in HJJ and L medium.

Iron- and sulphur-oxidizing bacteria able to grow in both JLFe and S^0 medium. The positive MPN cultures in JLFe medium were inoculated (1 ml in 10 ml) into S^0 medium in 15 x 150-mm test tubes which were then incubated for 8 weeks at 26 °C in the dark. Positive tubes containing S^0-metabolizing bacteria were identified by measuring the pH of each culture. A decline in the pH of more than 0.3 pH units relative to the pH of a non-sulphur-oxidizing control inoculated with *L. ferrooxidans* CF12 (supplied by Dr. D. B. Johnson, University of Wales, Bangor) was taken as positive. *Thiobacillus ferrooxidans* ATCC 23270 (American Type Culture Collection, Rockville, Maryland, USA) was used as a positive control.

Iron-, sulphur- and thiosulphate-oxidizing bacteria able to grow in JLFe, S^0 and Starkey's medium. Cultures that had grown in JLFe medium and subsequently in S^0 medium were subcultured from the latter after 8 weeks of incubation (1 ml inoculum into 50 ml of Starkey's media in 250-ml Erlenmeyer flasks) and incubated at 26°C in the dark for 4 weeks. After incubation, thiosulphate metabolism was detected by titration of 10 ml samples of the cultures with a 0,02 N I_2-0,4% (m/v) KI solution using starch as indicator (Vogel, 1951).

Plate counts of acidophilic bacteria in coal waste

For the determination of acidophilic bacteria in coal waste samples by plate count, 1 ml quantities of inoculum from the dilutions prepared for MPN determinations of acidophilic high ferrous iron-oxidizing bacteria were spread evenly over FeSo plates. The inoculated plates were inverted after 1 day, then incubated in the dark until countable colonies formed (usually 4-6 weeks).

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RESULTS

Abiotic Ecological Determinants in Pilot Scale Coal Waste Dumps

Moisture conditions

Rainfall. Rainfall on the pilot scale dumps during the study period January 1994 to July 1997 is shown in Fig. 12. The 1994/1995 rainy season was rather dry. This, combined with the dry winters in 1994 and 1995, caused the soil covers to dry out by September 1995. Because of the clayey nature of the cover materials, large cracks appeared in the covers as well as in the barrier walls separating the individual cells.

Although the 1995/1996 rainy season started late (October), it was particularly wet (Fig. 12). The cracks in both the covers and the walls separating the cells closed by the middle of the rainy season. Good rainfall continued throughout the rainy season which lasted until the end of May, then sporadically during June to August 1996. The cracks did not reappear during the dry winter months in 1996.

Good rains fell during the 1996/1997 rainy season, particularly during the last part of the season which extended to July 1997. As there was enough moisture in the cover material at the onset of the 1996 dry period, the cracks did not reappear.

Moisture content of coal waste. The mean moisture contents of the duplicate coal waste samples from each cell over the entire period of the experiment to September 1996 are shown in Fig. 13. Details of the moisture in individual samples from February 1995 to September 1996 are provided in Appendix Tables 1-9a.

The moisture content of the uncovered coal waste in cells 1, 2 and 3 was affected by rainfall or desiccation conditions prior to sampling as the coal waste in these cells was directly exposed to the elements, resulting in considerable fluctuation of the moisture content through the samplings. The moisture levels were generally higher during the very wet 1995/1996 summer, after they had decreased to their lowest levels in September 1995.

The moisture content of the coal waste in the cells covered with Avalon soil only (cells 4, 5 and 7) remained close to the orginal level of approximately 10%, although with a slight decline as dry conditions persisted, until the very wet 1995/1996 rainy season when it increased sharply before returning to the original levels during the winter of 1996.

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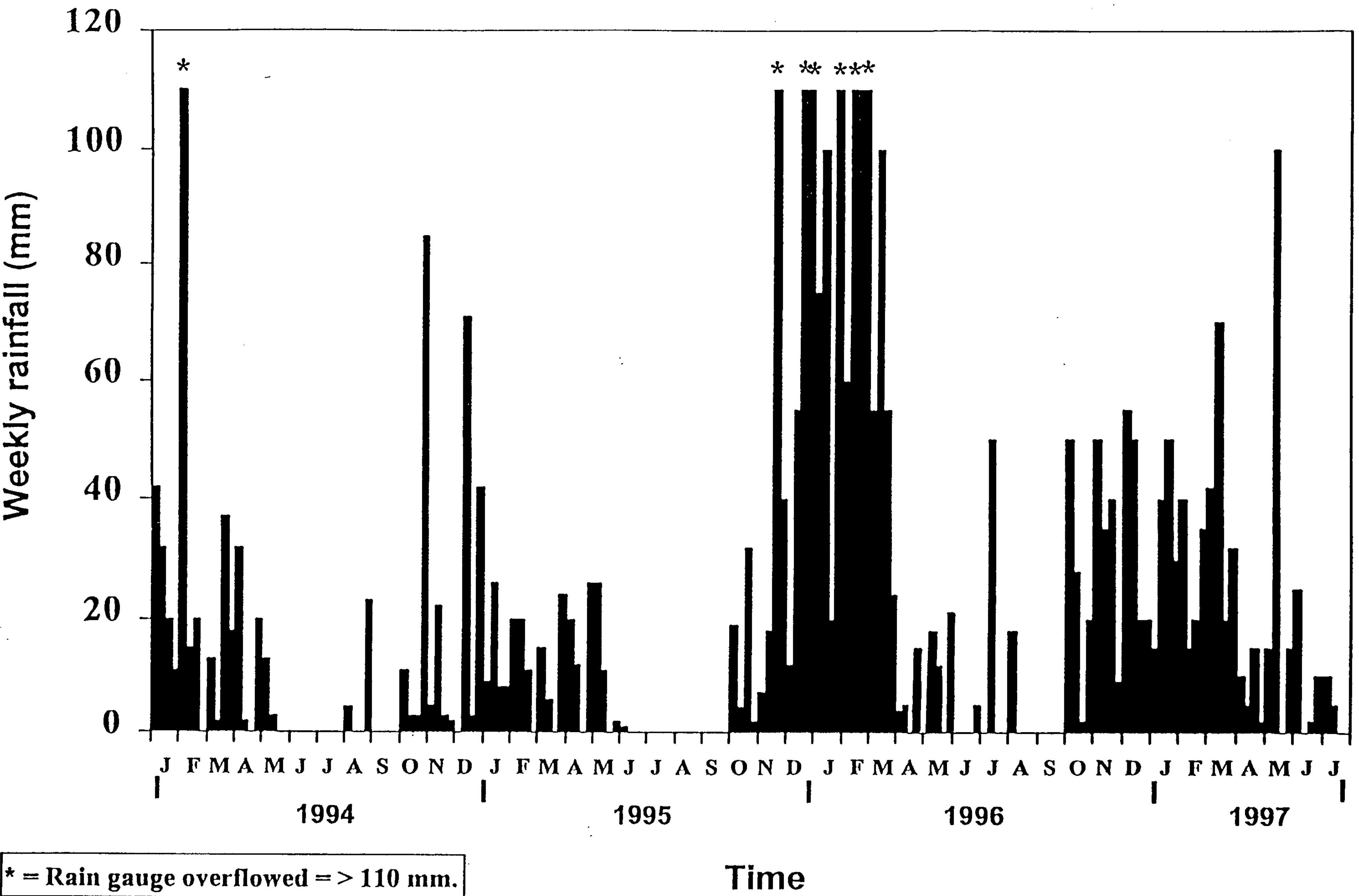

Fig. 12. Weekly rainfall on the pilot scale dumps from January 1994 to July 1997. (Data prior to August 1995 were obtained from Cleghorn, 1997.)

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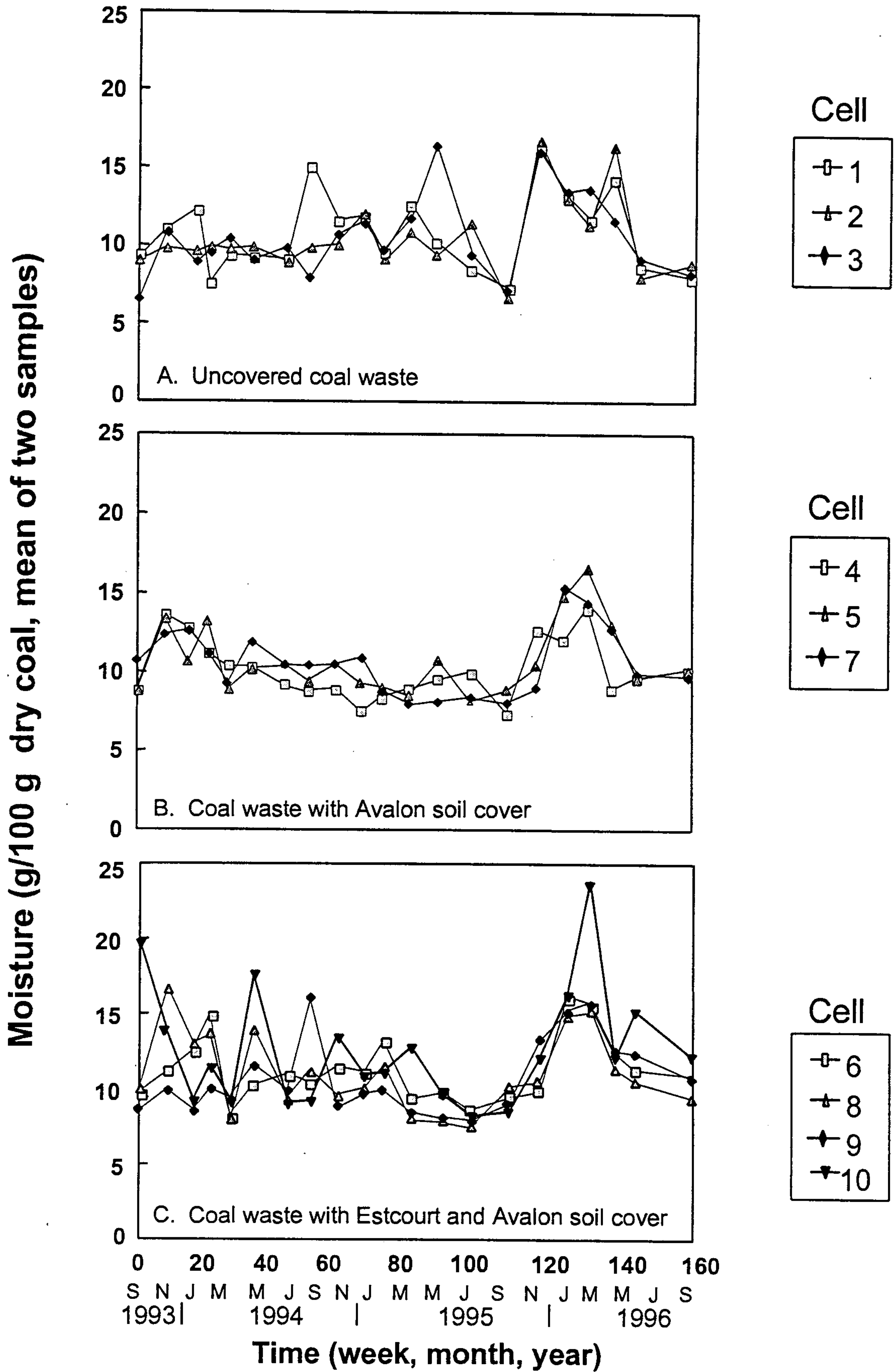

Fig. 13. Mean moisture content of duplicate samples from the upper 30 cm of coal waste in the cells of the pilot scale dump rehabilitation experiment in relation to the time of sampling. (Data prior to August 1995 were obtained from Cleghorn, 1997.)

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During the period September 1993 to September 1995, the cells covered with both an Estcourt and an Avalon soil layer (cells 6, 8, 9 and 10), seemed stabilised at a moisture content of about 10 %. During the 1995/1996 rainy season, the moisture content rose sharply before returning to the original levels during the winter of 1996.

Oxygen and carbon dioxide concentrations in coal waste

Oxygen and carbon dioxide concentrations in the atmosphere in the upper 15 cm of coal waste in the various cells are shown in Fig. 14 and 15. The concentrations are monthly mean values determined from weekly readings.

The oxygen and carbon dioxide concentrations generally had an inverse relationship; the higher the oxygen concentration (more aerobic) in the coal waste, the lower was the carbon dioxide concentration and vice versa. As the monitoring apparatus could measure carbon dioxide levels only up to 5%, concentrations of 5% shown in Fig. 15 could have been higher.

Uncovered cells (1, 2 and 3). All three uncovered cells remained aerobic during the course of the experiment, with the mean oxygen concentrations remaining mostly above 15% and carbon dioxide concentrations below 2%. The vegetated cell 3 containing uncompacted waste was slightly more aerobic than cells 1 and 2 containing uncompacted and compacted waste, respectively, hence no effect could be ascribed to compaction. During the extended very wet summer of 1995/1996, all three cells showed temporary declines in oxygen concentration, but not always a corresponding rise in carbon dioxide concentration.

Avalon soil-covered cells (4, 5 and 7). These cells were covered with different depths of the cover material, resulting in their oxygen and carbon dioxide profiles differing substantially.

Cell 4, covered with 30 cm of uncompacted Avalon soil remained aerobic; however, it was usually slightly less aerobic than the uncovered cells, and often contained a higher carbon dioxide concentration.

Cell 5, covered with 50 cm of compacted Avalon soil, became anaerobic soon after construction. However, the cover was permeable to oxygen during dry periods, particularly after July 1995 when it developed cracks. Although the cracks closed during the subsequent wet season, cell 5 retained a concentration of oxygen higher than 10% until July 1996, after which it slowly declined to become anaerobic in September 1996. Thereafter it remained anaerobic until the end of the experimental period.

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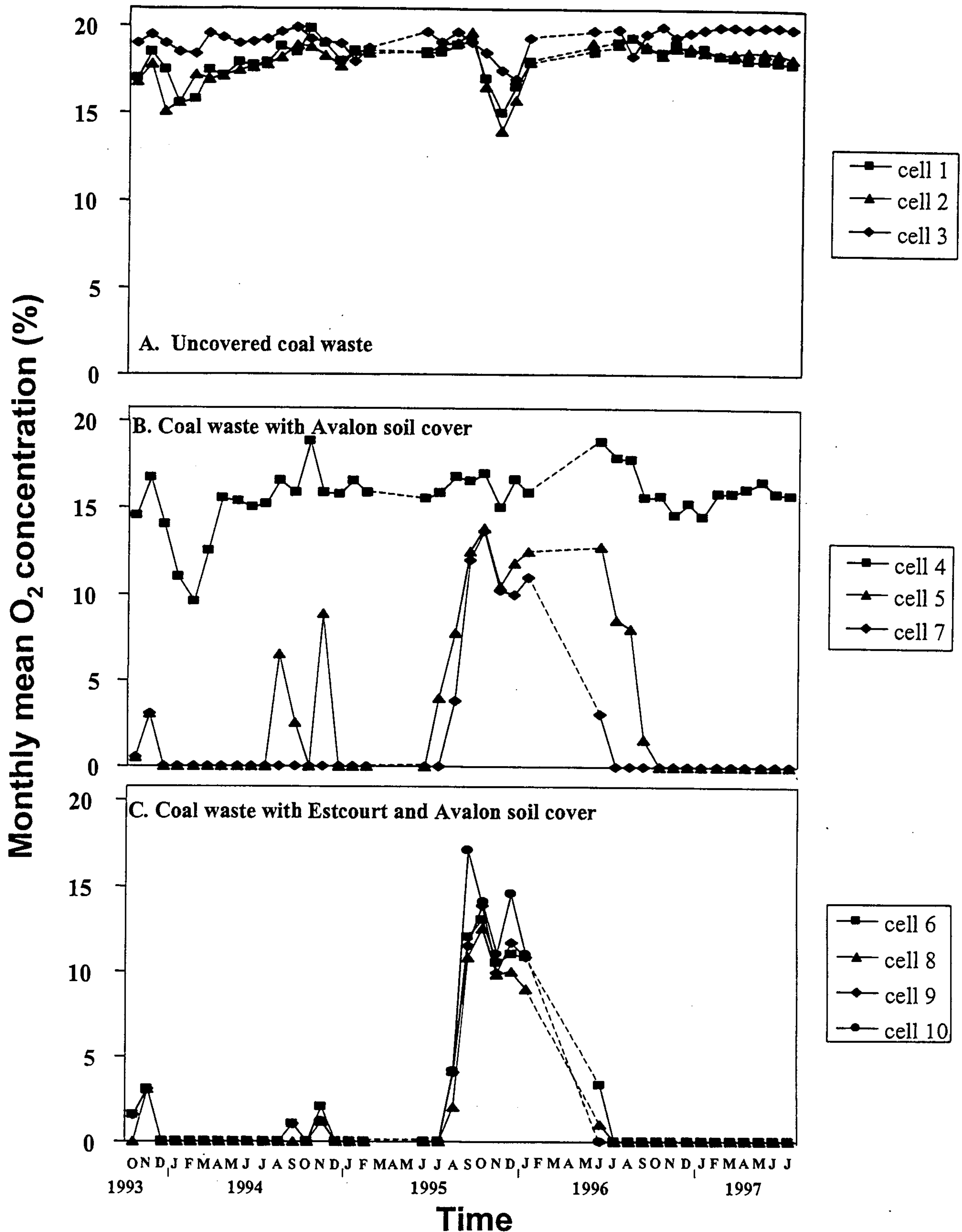

Fig 14. Monthly mean oxygen concentrations (from weekly measurements) at depths of 5-15 cm below the upper surface of the coal waste in cells of the pilot scale dump rehabilitation experiment. Dotted lines indicate periods when no determinations could be made. (Data prior to August 1995 were obtained from Cleghorn, 1997.)

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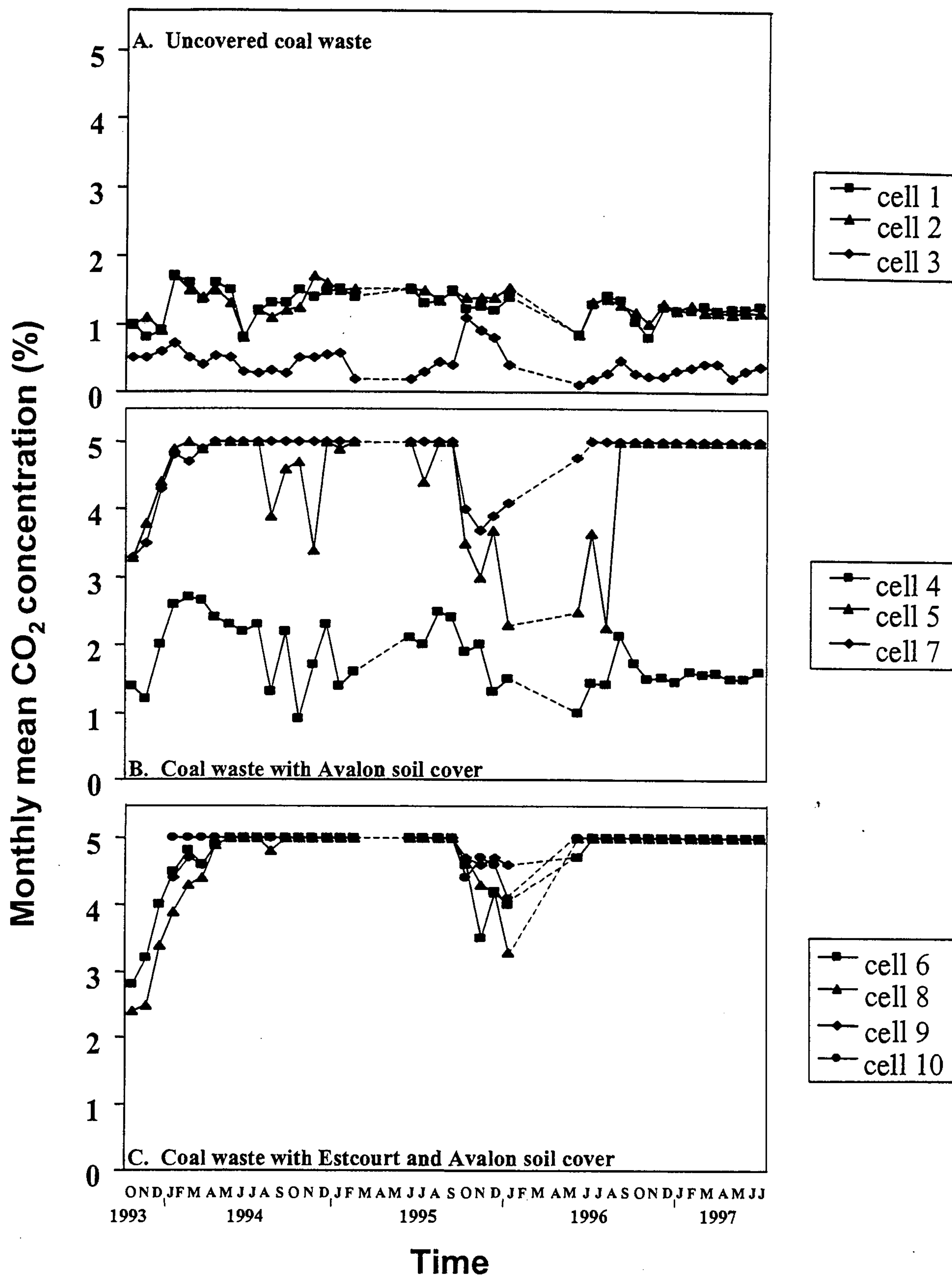

Fig. 15. Monthly mean carbon dioxide concentrations (from weekly measurements) at depths of 5-15 cm below the upper surface of the coal waste in cells of the pilot scale dump rehabilitation experiment. Dotted lines indicate periods when no determinations could be made. (Data prior to August 1995 were obtained from Cleghorn, 1997.)

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Cell 7, covered with 70 cm compacted and 30 cm uncompacted Avalon soil, became anaerobic soon after construction. The cover remained an effective barrier to oxygen penetrating into the cell (which remained anaerobic) until it cracked during the latter part of the 1995 dry season. However, cell 7 again become anaerobic by July 1996, about 2 months earlier than cell 5. It then remained anaerobic until the end of the experimental period.

Estcourt and Avalon soil-covered cells (6, 8, 9 and 10). The very dry conditions during the latter half of the 1995 dry season caused the covers to crack, allowing considerable gas exchange with the atmosphere. Aerobic conditions developed in the coal waste and persisted despite the good rains in the 1995/1996 rainy season, until June 1996. Thereafter, the cells returned to being anaerobic and remained so until the end of the experimental period.

pH of coal waste

The mean pH for the two samples of coal waste from each cell at each sampling was determined from the values in Appendix Tables 1-10. These mean pH values and those of Cleghorn (1997) are plotted in Fig. 16.

Uncovered cells (1, 2 and 3). These cells acidified at approximately similar rates, acidifying from an initial pH of just below 6 to approximately pH 3 by April 1995 (Cleghrorn, 1997), after which the pH in cells 1 and 2 remained more or less constant.

The pH of cell 3 rose sharply to pH 4.5 after the lime treatment of November 1995, after which it returned to its previous levels. Subsequently, however, pockets of relative alkalinity probably due to the lime treatment were sampled frequently, leading to fluctuation of the mean pH values in the range approximately pH 3-6.

Avalon soil-covered cells (4, 5 and 7). As these cells differed considerably in construction and aerobicity, their acidification trends also differed substantially.

Cell 4, covered with 30 cm of Avalon soil, remained aerobic during the experimental period. Its acidification proceeded similarly to that of the uncovered cells. A sharp rise in the mean pH (from a high pH of one sample) was observed during March 1996 in the very wet rainy season, but it returned thereafter to approximately pH 3.

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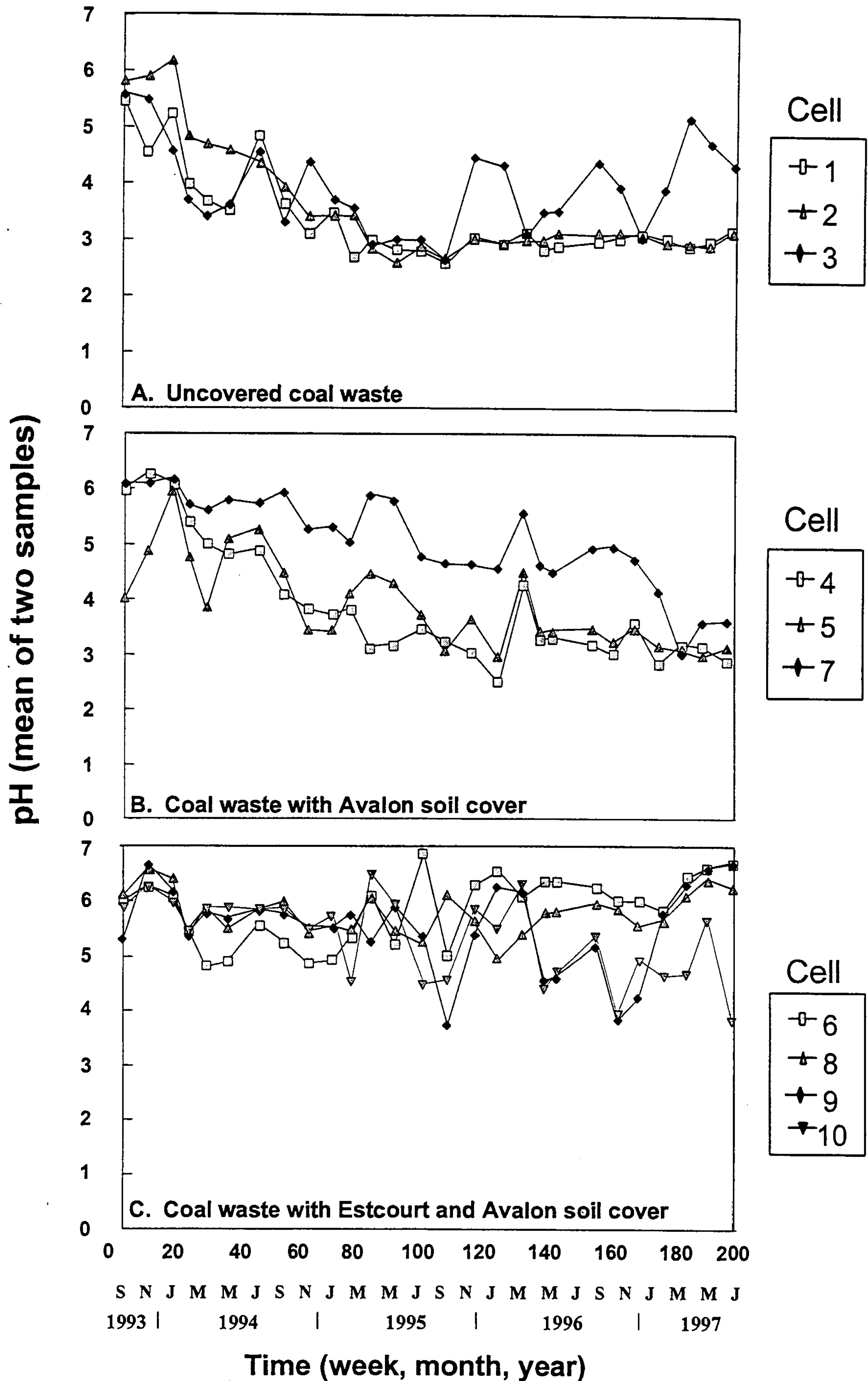

Fig. 16. Mean pH of duplicate samples from the upper 30 cm of coal waste in the cells of the pilot scale dump rehabilitation experiment in relation to the time of sampling.(Data prior to August 1995 were obtained from Cleghorn, 1997.)

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Cell 5, covered with 50 cm of compacted Avalon soil, fluctuated between periods of being aerobic in the dry seasons and anaerobic during the wet seasons. It already contained pockets of acidification at the onset of the experiment causing an extreme scatter of pH values around the general pH trend (Cleghorn, 1997). However, from Fig. 16 it appears that the pH declined to about pH 3 by January 1996, then showed a sharp rise similar to that in cell 4 before returning to about pH 3 for the remainder of the experimental period.

Cell 7, covered with a total thickness of 1 m of Avalon soil, of which the lower 70 cm was compacted, remained anaerobic until the cover cracked during the extremely dry winter of 1995. Slight and slow acidification occurred over most of the experimental period, but seemed to increase from December 1996 to March 1997 after the aerobic period. A pH of about 3-4, comparable to the pH at which the more aerobic cells stabilized, was measured in the samples taken from March to June 1997.

The sharp, short-lived pH increases measured in cells 4, 5 and 7 during March 1996 seemed to result from random sampling of pockets of coal waste that had not yet acidified substantially, giving mean pH values above the general pH trends for the respective cells (see Appendix Table 6).

Estcourt and Avalon soil-covered cells (6, 8, 9 and 10). These cells remained mainly anaerobic throughout the experiment, except when the covers cracked after the dry winter of 1995, allowing gaseous exchange which resulted in aerobic conditions in the waste from August 1995 to June 1996. From September 1995, pockets of acidification were detected during sampling, suggesting that the acidification process may have started in these cells, although the overall pH of the four cells did not drop appreciably during the almost 4-year duration of the experiment. Cells 9 and 10, sloped 1 in 10 and 1 in 5, respectively, seemed to yield acidic samples more frequently than the flat-topped cells 6 and 8.

Microbial Populations in Coal Waste of the Pilot Scale Dumps

Acidophilic high ferrous iron-oxidizing bacteria

Population sizes of acidophilic iron-oxidizing bacteria capable of growth at 26 ^{0}C in HJJ-medium from various cells of the pilot scale experiment from September 1995 to September 1996 are shown in Table 2 and Appendix Tables 3-9 (with 95% confidence limits). Fig. 17 shows the mean log populations of duplicate samples from each cell at the various samplings, including the period September 1993-June 1995 from Cleghorn (1997).

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Table 2. Populations (MPN) of acidophilic high ferrous iron-oxidizing organisms capable of growth at 26°C in HJJ medium in coal waste samples from the pilot scale dump rehabilitation experiment from September 1995 to September 1996

Cell and (sample)	MPN/g dry coal waste in samples of						
	24/09/95	20/11/95	21/01/96	18/03/96	06/05/96	03/06/96	02/09/96
1 (1)	21 673	27	49	105	1 056	10 096	47
1 (2)	478 260	496	39	1 663	50	250	259
2 (1)	308 799	107	17	2 557	5 025	377	47
2 (2)	1 172	110	17	1 037	50	2 275	47
3 (1)	2 454	127	170	239	2 382	316	224
3 (2)	24 712	1 755	182	2 608	319	25 244	131
4 (1)	99 666	3 347	169	26	1 632	10 200	164
4 (2)	10 003	473	1 034	8 475	1 634	395	266
5 (1)	101 536	429	106	4 976	2 437	47	165
5 (2)	467	25	107	1 764	231	82	25
6 (1)	4 702 716	25	27	50	3	10	509
6 (2)	3	82	87	26	167	2 535	26
7 (1)	463 231	261	231	4 834	2 291	164	25
7 (2)	21 636	47	323	1 748	103	103	25
8 (1)	1 648	168	330	50	26	103	168
8 (2)	254 166	47	32	17 221	2 551	10 267	25
9 (1)	163 785	104	27	10	486	485	263
9 (2)	468	26	262	111	1 686	3 132	168
10 (1)	45 406	47	109	55	26	4	263
10 (2)	13 052	3	86	519	43	2 974	26

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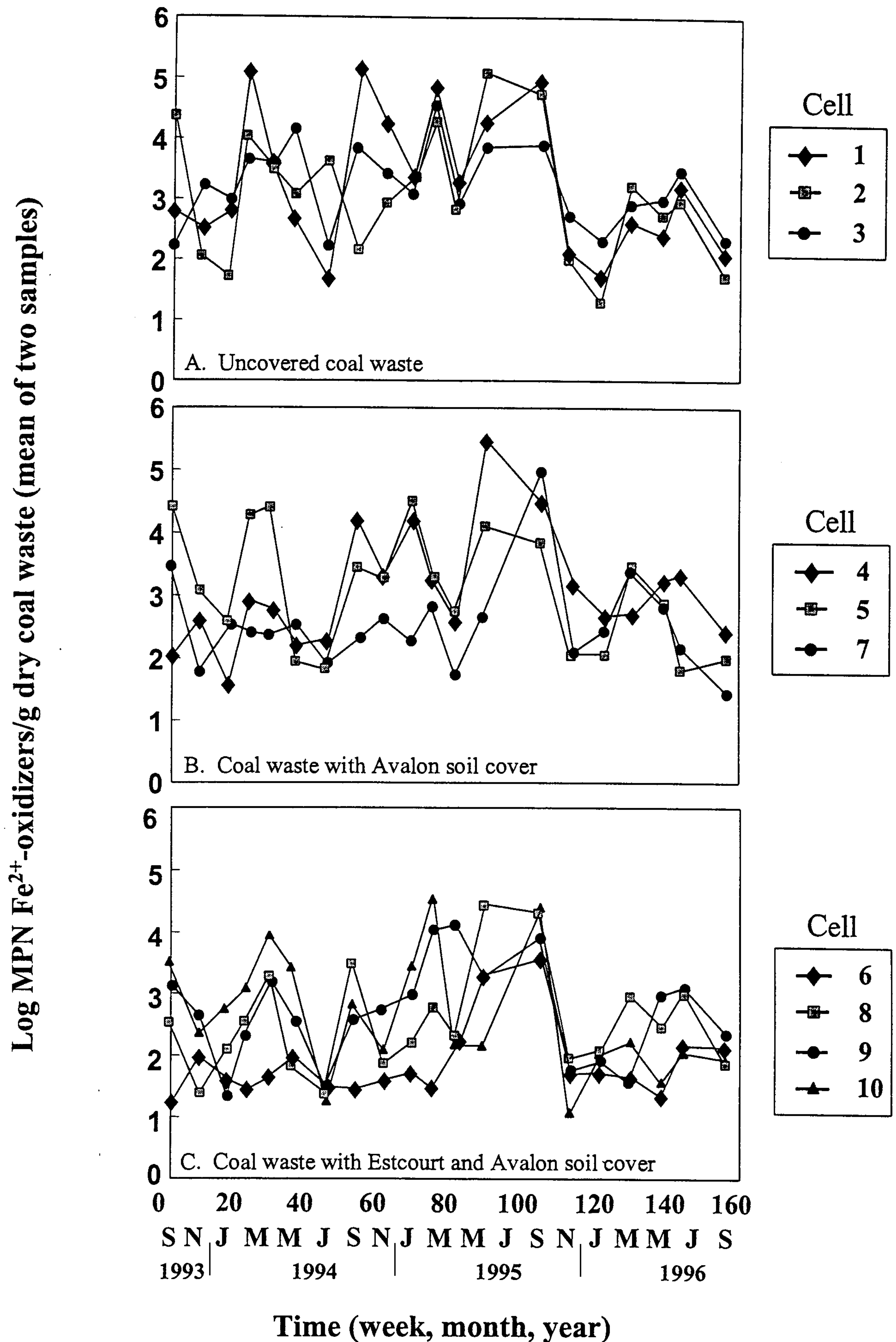

Fig. 17. Mean log MPN counts of acidophilic ferrous iron-oxidizing bacteria in duplicate samples from the upper 30 cm of coal waste in the cells of the pilot scale dump rehablitatiom experiment in relation to the time of sampling.(Data prior to August 1995 were obtained from Cleghorn, 1997).

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Large fluctuations of the population sizes (in some cases reflecting growth not occurring homogeneously throughout the coal waste) complicate analysis of the results; however, the following observations can be made:

(i) The uncovered consistently aerobic cells 1-3 generally had the highest populations, ranging mostly between 100 and 100 000 per g dry coal waste.

(ii) The Avalon soil-covered cells 4 and 5, which were aerobic for all or part of the time, respectively, showed similar populations to those of the uncovered cells. However, samples from cell 7, which was covered by 1 m of Avalon soil and was anaerobic for most of the time, had populations mainly between 100 and 10000 per g dry coal waste, hence comparable with the lowest counts for cells 4 and 5.

(iii) The cells covered with Estcourt and Avalon soil (cells 6, 8, 9 and 10) generally had the lowest populations, mostly ranging between 10 and 10 000 per g dry coal waste.

The fluctuations suggest the possibility of cyclic seasonal fluctuations, with peak populations in spring and late summer to autumn, when temperature and moisture conditions would be favourable for microbial development. Declining temperatures and drying may be the cause of the autumn declines. Wet conditions with poor gas diffusion to growth sites in the coal waste, could be the cause of the summer declines of the populations.

Acidophilic relatively high temperature, high ferrous iron-oxidizing bacteria

The populations of these organisms which grew at 37°C in L medium are given in Table 3 and Appendix Tables 1-3 and 5. The counts were often lower but sometimes higher than the corresponding counts at 26 °C in HJJ medium (also see Appendix Tables 11 and 12 of Cleghorn, 1997), although the differences were usually slight (less than one order of magnitude) and often not statistically significant. This suggested that MPN determinations at 37°C in L medium might have counted very similar if not the same organisms as the 26°C counts in HJJ medium. However, some strains which grew at 26°C in HJJ medium of pH 2.0 could apparently not grow under the more extreme conditions of incubation at 37°C in L medium of pH 1.0-1.3.

Table 3 suggests further that a large proportion of the cultures growing at 37°C in L medium could also grow in this medium at 40°C.

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Table 3. Populations (MPN) of acidophilic high temperature (37°C) high ferrous iron-oxidizing bacteria able to grow in L medium in coal waste samples from the pilot scale dump rehabilitation experiment and growth of selected subcultures at 40°C

Cell and (sample)	MPN/g dry coal waste and growth of selected subcultures at 40°C					
	27/02/95		10/04/95		24/09/95	21/01/96
	MPN (37°C)	subcultures growing at 40°C[a]	MPN (37°C)	subcultures growing at 40°C[a]	MPN (37°C)	MPN (37°C)
1 (1)	13 158	10^1 (2)	855	10^1 (2)	47	307
1 (2)	16 434	10^1 (2)	5118	10^1 (2), 10^{-3} (1)	46	1 352
2 (1)	82	10^1 (2)	51313	10^1 (2)	99	5
2 (2)	1016	10^1 (2), 10^{-3} (1)	25	10^1 (2), 10^{-1} (2)	46	1 056
3 (1)	360	10^1 (2)	4258	10^1 (2)	10	32
3 (2)	262	10^1 (2)	266	10^1 (2), 10^{-2} (2)	31	318
4 (1)	3 767	10^1 (2)	25	10^1 (2), 10^{-1} (1)	15	846
4 (2)	38	10^1 (2)	25	10^1 (2), 10^{-1} (2)	31	1 667
5 (1)	47	10^1 (2)	10	10^1 (2), 10^{-1} (1)	4	1 062
5 (2)	50117	10^1 (2)	24891	10^1 (2)	1 629	70
6 (1)	3	10^1 (2)	0.3	10^1 (2)	25	5
6 (2)	5	10^1 (2), 10^0 (1)	0.4	10^1 (1)	10	27
7 (1)	47	10^1 (2)	2	10^1 (2)), 10^{-1} (1)	3	173
7 (2)	25	10^1 (2), 10^{-1} (2)	2	10^1 (2), 10^0 (2)	<0.3	23
8 (1)	402	10^1 (2)	16	10^1 (2), 10^{-1} (1). 10^{-2} (1)	22	106
8 (2)	26	10^1 (2), 10^{-1} (2)	2	10^1 (2), 10^0 (2)	< 0.3	108
9 (1)	2 462	10^1 (2)	1498	10^1 (2), 10^{-1} (1)	4 695	27
9 (2)	16902	10^1 (2)	1	10^1 (2)	41	106
10 (1)	>110000	10^1 (2)	100	10^1 (2)	3	11
10 (2)	1 042	10^1 (2)	1 752	10^1 (2)	5	173

[a] Value in brackets shows number of subcultures that grew at 40°C, out of two inoculated from 37°C 10^1 cultures and two inoculated from 37°C cultures from the indicated end-point dilution or dilutions (not tested for the MPN counts of 24/09/95 and 21/01/96).

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Acidophilic moderate ferrous iron-oxidizing bacteria

The populations of acidophilic moderate ferrous iron-oxidizing bacteria capable of growth in JLFe medium are shown in Table 4 and Appendix Tables 7-9. Counts of these organisms were usually higher than the corresponding counts in HJJ medium. The difference was generally at least one order of magnitude, suggesting that more groups of organisms were counted using JLFe medium. As the JLFe medium is suitable for *T. ferrooxidans*, the difference indicates those organisms that can grow in this moderate ferrous iron medium, but not in the high iron HJJ medium. These organisms might include *L. ferrooxidans* as a major group. There is no clear indication that the populations may be generally larger in the aerobic cells 1-4 than in the usually anaerobic cells 6 and 8-10 which showed no or little overall acidification.

Acidophilic moderate ferrous iron- and sulphur (S^0)-oxidizing bacteria

Cultures showing iron oxidation in MPN determinations in JLFe medium were tested for sulphur utilization in S^0-medium. *Thiobacillus ferrooxidans* utilizes ferrous iron and sulphur as energy sources (Kelly and Harrison, 1989), but neither *L. ferrooxidans* nor expected heterotrophic associates of iron-oxidizing bacteria in enrichment cultures (see **EXPERIMENTAL PART 2**) utilize sulphur. This test could therefore be used to indicate JLFe cultures in which *T. ferrooxidans* was the organism responsible for iron oxidation (Harrison, 1978), and to obtain MPN counts of presumptive *T. ferrooxidans* in the coal waste samples. A serious problem was that sometimes the patterns of sulphur-oxidizing cultures were confusing in that only the highest dilutions were positive, suggesting repression of sulphur-oxidizing by non-sulphur-oxidizing iron-oxidizing bacteria in the low dilution MPN cultures in JLFe medium. However, this phenomenon was observed only with samples from the aerobic uncovered cells. Where the pattern of positive results at higher dilutions was suitable, an MPN was determined making the assumption that negative tubes in the lower dilutions would have been positive in the absence of this repression.

The populations of the sulphur-oxidizing organisms are shown in Table 4, alongside the populations of moderate ferrous iron-oxidizing bacteria, as well as in Appendix Tables 7a-9a. Counts for these organisms were generally much lower than those for the moderate ferrous iron-oxidizing bacteria, with some MPN series yielding no sulphur-oxidizing tubes. These results suggest that *T. ferrooxidans* may have formed only a small portion of the total iron-oxidizing populations in the coal waste samples. If the results of Tables 4 and 2 are compared, it appears that the counts of acidophilic high ferrous iron-oxidizing bacteria in HJJ medium estimate not only *T. ferrooxidans* populations, but populations of other species as well. Possibly these could

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Table 4. Populations (MPN) of acidophilic moderate ferrous iron-oxidizing bacteria capable of growth in JLFe medium, as well as the populations (MPN) of these organisms also capable of growth on sulphur (S^0-medium) and thiosulphate (Starkey's medium), in coal waste samples from the pilot scale dump rehabilitation experiment

Cell and (sample)	**MPN/g dry coal waste**						
	06/05/96		**03/06/96**		**02/09/96**		
	JLFe	S^0	JLFe	S^0	JLFe	S^0	Starkey
1 (1)	2 612	34	2 497	< 0.3	11 905	17	10
1 (2)	265	< 0.3	21 776	806	49 584	4 958	4
2 (1)	18	7	22 649	669	50 104	32	25
2 (2)	337	< 0.3	47 675	8	119 582	1 196	8
3 (1)	2 609	4	16 320	3	213 416	800	117
3 (2)	4 730	< 0.3	47 195	670	78 908	2 301	25
4 (1)	2 503	< 0.3	102 000	22	50 404	1 205	47
4 (2)	10 130	12	10 212	33	166 161	2 326	510
5 (1)	406	50	47	< 0.3	121 011	15	15
5 (2)	23 139	4	17 452	< 0.3	121 448	8	4
6 (1)	< 0.3	< 0.3	105	48	1 660	830	7
6 (2)	480	< 0.3	474	< 0.3	511	48	10
7 (1)	106 512	< 0.3	4 715	22	102	103	17
7 (2)	16	10	474	< 0.3	251 275	120170	<0.3
8 (1)	168	< 0.3	476	< 0.3	2 529	506	8
8 (2)	1 664	10	15 455	< 0.3	5 015	1 635	<0.3
9 (1)	226	4	846	4	25 248	23	<0.3
9 (2)	483	33	10 402	10	2 572	1 230	4
10 (1)	157	4	18	< 0.3	252	25	<0.3
10 (2)	49	17	16 520	10	2 649	1 267	<0.3

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include high ferrous iron-oxidizing strains of *L. ferrooxidans*. The September 1996 sampling yielded far greater numbers of iron- and sulphur-oxidizing bacteria than the May and June 1996 samplings. This observation can possibly be explained by seasonal variation in the population of iron-oxidizing bacteria, as *T. ferrooxidans* tends to outcompete *L. ferroxidans* at lower temperatures (Hallmann *et al.*, 1992).

Acidophilic moderate ferrous iron-, sulphur- and thiosulphate-oxidizing bacteria

As the type strain, *T. ferrooxidans* ATCC 23270, and many other strains of *T. ferrooxidans* can utilize thiosulphate, the presumptive *T. ferrooxidans* cultures from the 3 June 1996 and the 2 September 1996 samplings growing in the S^0 medium were subcultured into flasks containing Starkey's medium to test their ability to utilize thiosulphate. However, of the 27 sulphur-utilizing cultures, only two tested positive for thiosulphate utilization. When this procedure was repeated with the cultures from the sampling of 2 September, a greater percentage yielded positive results and therefore confirmation of the presence of *T. ferrooxidans*. The MPN counts of these confirmed *T. ferrooxidans* are also given in Table 4.

The positive *T. ferrooxidans* ATCC 23270 control utilized thiosulphate in this test, as well as the iron in the JLFe medium and sulphur in the S^0 medium.

Acidophilic bacteria by plate count

The plate counts of acidophilic bacteria capable of growth on FeSo plates are given in Table 5 and Appendix Table 6 and 8. The counts are mean counts of bacterial colonies on three replicate plates. As the FeSo plates permit the growth of most types of organisms known to be directly or indirectly involved in catalysing acid mine drainage formation (Johnson, 1995b), the counts were generally higher than the corresponding MPN determinations of iron-oxidizing bacteria using HJJ or JLFe medium. However, fungal growth often interfered with bacterial growth on the plates, causing many plates of the reported studies and all plates of two entire additional samplings to be of no use for counting acidophilic bacteria. Only plates with low numbers of bacteria could be counted, as precipitation of ferric salts by iron-oxidizing bacteria hampered the viewing of single colonies. The low numbers of bacteria counted per plate negatively affected the statistical value of the plate counts.

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Table 5. Plate counts of acidophilic bacteria capable of growth on FeSo plates in coal waste samples from the pilot scale dump rehabilitation experiment

Cell and (sample)	Plate count/g dry coal waste	
	18/03/96	3/06/96
1 (1)	236460	ND[a]
1 (2)	ND[a]	166949
2 (1)	311220	352310
2 (2)	1036764	657323
3 (1)	102501	232107
3 (2)	ND[a]	274400
4 (1)	9205	5209800
4 (2)	644100	874740
5 (1)	266166	ND[a]
5 (2)	70566	ND[a]
6 (1)	ND[a]	ND[a]
6 (2)	ND[a]	ND[a]
7 (1)	314776	219300
7 (2)	ND[a]	ND[a]
8 (1)	6565	73767
8 (2)	68886	ND[a]
9 (1)	16243	ND[a]
9 (2)	ND[a]	ND[a]
10 (1)	38073	ND[a]
10 (2)	54333	ND[a]

[a]ND = not determined.

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DISCUSSION

Characteristics of Coal Waste and Cover Materials used in Construction of Pilot Scale Dumps

General Characteristics

The coal waste used in the construction of the mini-dumps for this experiment had a high acid potential, equivalent to 0.77% unoxidized sulphur according to an analysis at the Institute for Ground Water Studies at the University of the Orange Free State, Bloemfoentein (Cleghorn, 1997; Loos *et al.*, 1997). This sulphur content was conducive for oxidative reactions which would lead to the production of acidity and to the growth of organisms catalysing the formation of acid mine drainage. The fine texture of the coal waste used in this experiment facilitated handling and sampling. The texture of the coal waste was comparable to that of a sandy soil (Soil Classification Working Group, 1991), allowing good water drainage and diffusion of gases (Gray and Williams,1971).

The clayey nature of the cover materials used in the construction of the mini-dumps should limit the diffusion of gases and the infiltration of water into the cells (Cleghorn, 1997; Loos *et al.*, 1997). This was particularly evident in cells covered with 1 m of cover material (Avalon soil only or the various combinations of Estcourt and Avalon soil). Under conditions of extreme desiccation clays have a tendency to crack (Daniel and Wu, 1993), as happened at the end of the extremely dry 1995 winter season. Large cracks appeared in the Estcourt soil side walls separating the cells from each other and smaller cracks were noted on the surface of the Avalon soil cover on most of the cells. This was not suprising, as both the Avalon and Estcourt soil had a clay content of 30-34% and a silt content of 20-29% (Cleghorn, 1997; Loos *et al.*, 1997). The cracks allowed much oxygen, which is the electron acceptor for the oxidation of ferrous to ferric iron, to penetrate the cells. The ferric iron in turn is the main electron acceptor in the oxidation of pyrite which leads to acid mine drainage formation (Luther, 1987; Moses *et al.*, 1987). The infiltration of oxygen (also the terminal electron acceptor for the reactions arising from the oxidation of pyrite) due to the cracking of the cover material could therefore have far-reaching consequences for acidification of the coal waste underlying the covers. Oxygen infiltrating the underlying material could create conditions favourable for a bacterial bloom as soon as sufficient moisture became available, causing the biotic oxidation of ferrous iron to become increasingly rapid as pyrite oxidation proceeded, liberating substrate for further bacterial activity and lowering the pH (Atlas and Bartha, 1993; Kleinmann *et al.* 1981). After the good rains of the 1995/1996 rainy season the cracks closed, once again limiting the infiltration of water and the diffusion of gases into the mini-dumps.

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Because of the relatively wet conditions during the remainder of the experiment, the cells maintained their integrity after the cracks had closed at the end of the 1995/1996 rainy season.

Abiotic Conditions Affecting Bacterial Growth in the Pilot Scale Dumps

Moisture conditions

Rainfall. The rainfall occurred mainly during the summer and other warm months (October to May), with drought occurring during the winter months (June to September). In the area in which the experimental site was located, the rainfall occurs mainly as events lasting for 1 to 2 hours. Continuous rain over long periods (> 1 day) rarely occurs (Wates, Meiring and Barnard,1995a, b).

Although the rainfall in this area is seasonal, the duration and total rainfall of both the wet and the dry seasons varied considerably during the almost 4-year experimental period (Fig. 12). The most extreme wet and dry seasons were the summer of 1995/1996 and the winter of 1995, respectively. Both of these extreme seasons had profound effects on the soil covers and hence on the coal waste. The dry conditions during the winter of 1995 desiccated the cover materials and caused the covers to crack, whereas the extremely wet rainy season that followed, saturated the soil cover material and restored the integrity of the covers. The effect on the coal waste, apart from changes in the moisture content, was a marked effect on the penetration of oxygen through soil covers into the underlying waste (see later).

Moisture content of coal waste. The moisture content of the coal waste samples taken over a 3-year period of the experiment ranged mainly between 8 and 16 % (Fig. 13). If the moisture characteristic curve for a sandy or sandy loam soil (Gray and Williams, 1971) is applicable to the coal waste, the moisture would at all times have exceeded that at the permanent wilting point for plant growth, where suppression of microbial growth may also occur. Moisture did not therefore appear to be a limiting factor for bacteria in the coal waste of the pilot scale dumps.

The lack of a soil cover on cells 1, 2 and 3 made their moisture contents most responsive to rainfall or desiccation. The coal waste in these cells tended to be dryer during the dry seasons and responded more rapidly to the early rains of the very wet 1995/1996 rainy season than that in the other cells. The moisture contents of cells 4, 5 and 7 showed the least fluctuation and hence the clearest trends, notably the general drying trend through the winters of 1994 and 1995, as well as the intervening rather dry 1994/1995 summer, until it increased sharply during the 1995/1996 rainy season. Cells 6, 8, 9, and 10 covered with both an Estcourt and an Avalon soil layer, showed a

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rather similar trend but with more fluctuation, particularly when the collected drainage water that accumulated at the bottom of cell 10 was sometimes included in a sample.

The moisture content patterns suggest that the soil covers protected the coal waste from rapid and extensive drying, but did not prevent water penetration to the waste in the extremely wet 1995/1996 rainy season. The main effect of the seasonal moisture changes relevant to the activity of acid-generating bacteria in the coal waste, is likely to have been its effect on the aeration of the coal waste, discussed in detail in the next section. Heavy rains, which saturated the cover materials, would have been the main factor causing anaerobic conditions to develop in covered cells.

Oxygen and carbon dioxide concentrations in coal waste

The three uncovered cells 1, 2, and 3 remained highly aerobic during most of the experiment, with oxygen concentrations mainly above 15% and carbon dioxide concentrations below 2% (Fig. 14 and 15). This is understandable as coal waste is porous (with particle size distribution resembling that of a sandy soil) which would allow gases to diffuse in and out of the coal waste. The effect of high rainfall, filling pores with water, displacing oxygen and hindering its diffusion was evident during wet periods such as the summers of 1993/1994, 1995/1996 and 1996/1997, when the cells became slightly less aerobic. Chemical reactions of the coal and microbial metabolism under warm, wet conditions might also help to lower the oxygen concentration in the atmosphere of the coal waste. This could be the explanation for increases in the carbon dioxide concentration coinciding with reduced oxygen levels. As the cells remained aerobic, proliferation of iron-oxidizing bacteria and pyrite oxidation could ensue, leading to the acidification of the cells.

The Avalon soil-covered cells 4, 5 and 7 formed an interesting series. The coal waste was covered with different depths of soil cover material, and therefore their oxygen and carbon dioxide profiles differed substantially. The 30 cm of uncompacted Avalon soil on cell 4 was insufficient to create the anaerobic conditions which would inhibit bacterial iron oxidation and the acidification of the coal waste. However cell 4 consistently showed a higher carbon dioxide concentration than the uncovered cells, because the covering partially inhibited diffusion of gases in and out of the coal waste layer. Cell 5, covered with 50 cm of compacted Avalon soil, became anaerobic soon after construction, but the cover became permeable to oxygen during extended dry periods, such as the winters of 1994 and 1995. Aerobic conditions became well established following cracking of the covers during the 1995 winter, and despite the good rains of the 1995/1996 rainy season, persisted until September 1996. The periodic exposure of the coal waste in this cell to oxygen, allowed acidification to take place (see next section on **pH of coal waste**). Cell 7 covered with 70 cm compacted and 30 cm uncompacted Avalon soil, also

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became anaerobic soon after construction. It remained anaerobic throughout the dry 1994 winter, hence the 1-m cover was a more effective barrier to oxygen penetration than the 50-cm cover of cell 5. Nonetheless, during the latter part of the 1995 dry season, the cover showed cracks and became permeable to oxygen. As cell 7 remained anaerobic through most of the experiment, the chemical and bacterial oxidation of pyrite, as well as acidification of the cell, were inhibited to a considerable extent until very late in the experiment. During the last six months of the experimental period, pockets of acidification were sampled regularly, indicating that acidification may have become established during the aerobic period and subsequently progressed steadily despite the anaerobic conditions prevalent in the cell at this stage (see next section).

Cells 6, 8, 9 and 10 with 1-m-thick covers of 30 or 70 cm uncompacted Avalon soil on 70 or 30 cm compacted Estcourt soil, showed the same trends in oxygen and carbon dioxide concentrations as cell 7. Cracking of the covers at the end of the dry 1995 winter resulted in aerobic conditions which persisted until June 1996, despite the good rains in the 1995/1996 rainy season. As these cells remained anaerobic through most of the experiment, the chemical and bacterial oxidation of pyrite, as well as the acidification of the cells, were almost completely inhibited (see next section).

This study has clearly shown that all cells without a soil cover or covered with less than 0.5 m soil (cells 1-4), remained essentially aerobic during the course of the experiment, allowing the aerobic iron-oxidizing bacteria to grow and acidification to take place. From the results with cells 5-10, a minimum cover depth of between 0.5 and 1.0 m seems necessary to create the required anaerobic conditions needed to inhibit iron oxidation in coal waste dumps, but desiccation and the resulting increased permeability to atmospheric gases or, in extreme cases, cracking of the cover may permit the development of temporary aerobic conditions. Where desiccation is not so pronounced, a 1-m cover but not a 0.5-m cover, may maintain anaerobic conditions (as during the 1994 winter). Further studies are necessary to establish the effect of limited periods of aerobiasis during and following drought conditions on the acidification of the coal waste

pH of coal waste

The pH of the coal waste was of major interest as an indicator of acidification of the various cells, in addition to its possible effects on the microbial populations as an ecological determinant. The uncovered cells 1-3 showed steadily progressing acidification (Fig. 16), stabilizing at a mean pH of approximately 3 from April 1995, but with cell 3 showing pockets of higher pH following liming. A similar decline in pH was observed in cell 4 where the 0.3-m cover of uncompacted Avalon soil caused little reduction of the oxygen concentration in the

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underlying coal waste. The acidification of these aerobic cells would have created conditions favourable for high populations of iron-oxidizing bacteria and continued acidification at an accelerated rate (Harrison, 1984; Kleinmann *et al.*, 1981). However, acidification of the cells over an 80-week period was slow in comparison with that in a pilot scale experiment conducted near Witbank (Mpumalanga) by Loos *et al.* (1990b). All the uncovered dumps in that experiment showed acidification within 81 days of dump construction, while the pH of the effluent from all the dumps was below pH 3 within 120 days. The coal waste in the Witbank mini-dumps was not a small particle rather homogenous material as in the Kilbarchan mini-dumps, but contained material of all sizes to the size of small boulders.

The outer layers of the aerobic cells 1-4 seemed to have acidified at an almost uniform rate. This could be expected as these outer layers seemed to be wholly aerobic, and therefore ideal for the growth of iron-oxidizing bacteria and acidification. Localized differences in the rate of acidification would therefore not be as pronounced as in the more effectively covered cells where oxygen penetrated only occasionally and less uniformly. The form of the acidification curves of the aerobic cells (cells 1-4), resembled decay curves (Fig. 16). This is understandable, as provided bacterial activity is substantial and the supply of ferric ions is not limiting, the generation of acidity is linked to the oxidative decay of the pyrite in the coal waste.

Cell 5 covered with 50 cm of compacted Avalon soil fluctuated between periods of being aerobic in the dry seasons and anaerobic during the wet seasons, except during the 1995/1996 wet season following cracking of the cover. It already contained pockets of acidification at the onset of the experiment, causing extreme fluctuations of the mean pH values from that time. However, the mean pH values declined to just above pH 3 in 102 weeks (by September 1995) before rising slightly during the 1995/1996 rainy season. The cover was therefore not effective in preventing acidification of the coal waste.

Cell 7 with the 1-m of Avalon soil cover, of which the lower 70 cm was compacted, remained anaerobic until cracking occurred during the extremely dry winter of 1995. Slow acidification seemed to occur over the experimental period. Considerable fluctuation of the pH was observed among samples, supporting the concept of acidification starting in pockets. It is not clear to what extent acidification might have progressed if the cell had not become aerobic as a result of the cover cracking, but it seems that the aerobic period stimulated adification as evidenced by the pH of samples taken over the last six months of the experimental period. However, it appears that the 1-m Avalon soil cover of cell 7 was not as efficient a barrier to oxygen penetration as the covers of cells 6, 8, 9 and 10 consisting of layers of both Estcourt and Avalon soil. The cells covered with Estcourt and Avalon soil also remained mostly anaerobic throughout the experiment, except when cracking of the cover during the dry winter of 1995 allowed oxygen entry from the atmosphere. For about the first 18 months very little change in

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the mean pH of these cells occurred, with the mean pH values remaining mainly between 5 and 6. But thereafter, pockets of acidification were detected more frequently, suggesting that the acidification had started in these cells, although overall the pH of the four cells did not seem to drop appreciably over the 4-year duration of the experiment. The aerobic period did not seem to have the same adverse effect on the cells covered with both Estcourt and Avalon soil as it had on the other cells; this was especially true for the flat-topped cell 6 covered with 0.7 m compacted Estcourt soil below 0.3 m uncompacted Avalon soil. Although the oxygen profiles for cell 8 (covered with 0.3 m compacted Estcourt and 0.7 m uncompacted Avalon soil) and the sloped cells 9 and 10 (with the same cover as cell 6) were similar to that of cell 6, pockets of acidity were more frequently detected in these cells.

As samples of coal waste were taken only from the upper layers of the coal waste, the observed acidification may yield a distorted picture of the acidification of the total coal waste in a cell. The elevated sulphate concentrations measured in leachate from the experimental cells, suggest that bacteria caused the oxidation of pyrite, although a leachate pH of 6.7-7.4 was noted (Wates, Meiring and Barnard, 1995a,b). Neutralization of acid by carbonate present in the coal waste may occur at sites separated from those where pyrite oxidation becomes established in localized acidic regions ('pockets'). The overall high pH in the cells, reflected in the leachate pH, may have a relatively small effect on such localized acid production.

Microbial Populations in Coal Waste of Pilot Scale Dumps

Acidophilic high ferrous iron-oxidizing bacteria

The uncovered cells (1-3) tended to show higher counts of acidophilic high ferrous iron-oxidizing bacteria than the cells covered with 1 m of Avalon soil (cell 7) or Estcourt plus Avalon soil (cells 6, 8, 9 and 10). This tendency can be explained by the aerobic nature of these bacteria which were expected to be *T. ferrooxidans* (Belly and Brock, 1974; Kleinmann *et al.*, 1981), but could include *L. ferrooxidans* or even heterotrophic iron-oxidizing bacteria (Johnson, 1995a), in relation to the oxygen concentrations in the various cells. The aerobic conditions and the moisture in the upper 30 cm of the coal waste in the uncovered cells and cell 4 with the 0.3 m cover of uncompacted Avalon soil would have been favourable for their development most of the time. Moderately large populations (often 10^3-10^5/g waste) of acidophilic high ferrous iron-oxidizing bacteria have persisted in these cells from September 1993 to September 1996, coinciding with the decline in pH and the subsequent persistence of low pH conditions. Table 6 clearly shows the overall favourable effect of low pH on the size of the populations of these acidophilic bacteria.

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Table 6. Distribution of acidophilic high ferrous iron-oxidizing microbial populations of different sizes in coal waste samples of different pH, sampled every 6-11 weeks from 27 September 1993 to 2 September 1996 from the mini-dumps of the pilot scale dump rehabilitation experiment. (Data prior to August 1995 were obtained from Cleghorn, 1997.)

Sample pH	**Number (and percentage) of samples with iron-oxidizing microbial populations/g**					**Total**
	> 100 000	**10 000 - 100 000**	**1 000 - 10 000**	**100 - 1 000**	**< 100**	
2-3	6 (1.5)	14 (3.5)	9 (2.3)	9 (2.3)	8 (2.0)	**6 (11.6)**
3-4	9 (2.3)	17 (4.3)	26 (6.5)	23 (5.8)	9 (2.3)	**84 (21.1)**
4-5	2 (0.5)	12 (3.0)	15 (3.8)	23 (5.8)	8 (2.0)	**60 (15.1)**
5-6	4 (1.0)	11 (2.8)	27 (6.8)	47 (11.8)	42 (10.6)	**131 (32.8)**
6-7	1 (0.3)	2 (0.5)	6 (1.5)	27 (6.8)	41 (10.3)	**77 (19.3)**
Total	**22 (5.5)**	**56 (14.1)**	**83 (20.9)**	**129 (32.4)**	**108 (27.1)**	**398 (100.0)**

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The 0.5 m of compacted Avalon soil, covering cell 5, was unable to sustain anaerobic conditions and populations of iron-oxidizing organisms between 10^4 and 10^5/g waste were often observed, particularly in acid samples. The distribution pattern of samples with low pH and high iron-oxidizing bacterial populations suggests that the coal waste contained pockets of acidified materials at the time of construction of the cell. The acidophilic iron-oxidizing bacteria in the aerobic cells 1-4 and the partially aerobic cell 5 seemed to respond to reduced oxygen diffusion into the coal waste during the wet season with declines in their populations. The 1-m thick covers (Avalon soil or Estcourt and Avalon soil) on cells 6-10 caused the atmospheric oxygen to diffuse very slowly or not at all to the underlying coal waste, resulting in anaerobic conditions and relatively low counts (mostly less than 10^4/ g dry coal waste) of acidophilic iron-oxidizing bacteria most of the time. The pH also remained high except in isolated pockets and therefore unfavourable for the presumed *T. ferrooxidans*. According to Kelly and Harrison (1989), *T. ferrooxidans* actively oxidizes ferrous iron in the pH range 1.3-4.5, with the optimum pH between 2 and 4.

An important question arises concerning the specificity of these MPN counts in the HJJ medium at 26°C. The medium and incubation conditions were certainly favourable for *T. ferrooxidans*, but to what extent would they have favoured growth of other chemolithotrophic or heterotrophic iron-oxidizing bacteria? An acidophilic iron-oxidizing bacterium that appears to play an important role alongside *T. ferrooxidans* in metal bioleaching, is *L. ferrooxidans* (Johnson, 1995a; Norris, 1990; Norris and Kelly, 1982). The only possible limiting factor for this organism in the MPN determinations would be the high ferrous iron concentration of the HJJ medium, but *L. ferrooxidans* can grow in medium with 30 g/l $FeSO_4 .7H_2O$ (Hallmann *et al.*, 1992). This specificity issue will be considered further in later sections.

Acidophilic relatively high temperature high ferrous iron-oxidizing bacteria

Counts of these organisms using L medium of pH 1.0-1.3 at 37°C were often lower but sometimes higher than those of the acidophilic iron-oxidizers in HJJ medium of pH 2.0 at 26°C, but the differences were usually slight (less than one order of magnitude) and often not statistically significant (Appendix Tables 1-3 and 5). The two different MPN determinations may have counted the same or mainly the same organisms, with a few being eliminated by the more extreme conditions (higher temperature and lower pH) of the former counting procedure. This explanation seems likely, as many strains of *T. ferrooxidans* can grow at 40 °C (Norris, 1990), as could most of the cultures incubated at 40 °C in our study. A pH of 1.0 can also be suitable for growth of strains of *T. ferrooxidans* (Razzell and Trussell, 1963).

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Acidophilic moderate ferrous iron-oxidizing bacteria

The populations of these iron-oxidizing bacteria capable of growth in JLFe medium containing only 14 g/l $FeSO_4.7H_2O$ were usually higher than those in HJJ medium containing 44 g/l $FeSO_4.7H_2O$ (Appendix Tables 7-9). The difference was usually one order of magnitude or larger, suggesting that more groups of organisms were counted using JLFe medium. The count should include *T. ferrooxidans* and *L. ferrooxidans*, of which strains may have been counted in HJJ medium, and possibly heterotrophic iron-oxidizing bacteria (Johnson, 1995a). The *T. ferrooxidans* ATCC 23270 and *L. ferrooxidans* CF12 used as control cultures both grew in the JLFe medium, but only the former grew in HJJ medium. However, one culture of the latter containing a filamentous fungus contaminant did grow in HJJ medium. Some of the moderately thermophilic iron- and sulphur-oxidizing bacteria described by Norris (1990) might also have been counted using the JLFe medium, but whether they could have grown at the high iron concentrations of HJJ or L medium was not indicated in the descriptions of these organisms. The higher populations of iron-oxidizing bacteria detected using this medium, indicate that the JLFe medium may be better than HJJ medium for determining the total population of iron-oxidizing bacteria responsible for catalysing the oxidation of pyrite and the subsequent formation of acid mine drainage in coal waste dumps.

Acidophilic moderate ferrous iron- and sulphur-oxidizing bacteria

Tubes testing positive for iron oxidation in JLFe medium in MPN determinations were tested for sulphur utilization in S^0-medium, as *T. ferrooxidans* oxidizes ferrous iron and sulphur as energy sources (Kelly and Harrison, 1989). This test was therefore used to indicate in which tubes *T. ferrooxidans* was the organism responsible for iron oxidation (Harrison, 1978). The pattern of tubes positive for sulphur utilization was used to determine the MPN of presumptive *T. ferrooxidans* in a given sample. This cascade of MPN determinations was analogous to that employed for *Escherichia coli* (American Public Health Association *et al*, 1955). The counts of presumptive *T. ferrooxidans* determined in this way were generally much lower than those for both the moderate and high ferrous iron-oxidizing bacteria and some coal waste samples yielded no sulphur-oxidizing tubes. Harrison (1978) in his laboratory scale experiment, found that *T. ferrooxidans*, which could oxidize both iron and sulphur after transfer from one medium to the other, was the most important acid mine drainage-causing organism. However, our results suggest that *T. ferrooxidans* may form a far smaller percentage of the total iron-oxidizing population than originally suspected.

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Acidophilic moderate ferrous iron-, sulphur- and thiosulphate-oxidizing bacteria

This study failed to confirm the identity of most of the 27 presumptive *T. ferrooxidans* cultures from the 3 June 1996 sampling that metabolized sulphur in the S^0 medium, as only two showed thiosulphate utilization. However, the identity of the other 25 could still be *T. ferrooxidans* (non-thiosulphate-utilizing strains) as they agree with the description "acidophilic iron- and sulphur-oxidizing chemolithotrophic bacteria", which is a significant part of the definition of the species (Kelly and Harrison, 1989). Among the iron-oxidizing MPN cultures from the 2 September 1996 sampling, a greater number tested positive for sulphur and thiosulphate utilization. However, the number of confirmed *T. ferrooxidans* was similar to or lower than the number of high ferrous iron-oxidizing organisms from the same sampling. This suggests that MPN determinations in HJJ medium counted not only *T. ferrooxidans,* but also a small number of other organisms, notably *L. ferrooxidans* which does not utilize sulphur (Norris, 1990).

Acidophilic bacteria by plate count

As could be expected, plate counts on FeSo medium that allows the growth of most acid mine drainage-causing organisms (Johnson, 1995b), were generally higher than any of the MPN determinations that counted only iron-oxidizing organisms. The plate counts would also have been more accurate than the MPN determinations. Despite these two advantages over the MPN determinations, it was found that FeSo-plates were not suitable for routine determinations of acid mine drainage-causing populations for the following reasons:

(i) Coal waste particles obscured growth on plates inoculated with dilutions lower than 10^{-3}.

(ii) Ferric iron precipitation often made the counting of individual colonies impossible where plates had moderate colony density. The consequence was that only plates with a very low number of colonies could be counted, which compromised the statistical validity of the counts.

(iii) Fungal growth very often interfered with or obscured bacterial growth leading to many individual plates and even the plates of two entire samplings having to be discarded.

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EXPERIMENTAL PART 2. MICROORGANISMS OF IRON-OXIDIZING CONSORTIA INVOLVED IN THE GENERATION OF ACID MINE DRAINAGE IN NORTHERN KWAZULU-NATAL

INTRODUCTION

Acid mine drainage is caused by the biologically catalysed oxidation of pyrite. It has been suggested (for example, by Norris and Kelly, 1982) that various groups of organisms may interact as consortia to cause or enhance the oxidation of pyrite which leads to the production of ferric iron and sulphuric acid in coal waste dumps. The production and regeneration of ferric iron by iron-oxidizing bacteria, such as *T. ferrooxidans* and *L. ferrooxidans*, normally in association with heterotrophs, is the rate-limiting step in the oxidation of pyrite in low pH environments. The investigation of Experimental Part 1 has thrown some light on groups of iron-oxidizing bacteria involved in ferrous iron oxidation in the coal waste of the pilot scale dump rehabilitation experiment, but left various problems of bacterial identity unanswered. In this study, an attempt was made at a better understanding of the ecology of iron oxidation in coal mine dumps in the Klip River Coal Field, particularly the responsible microorganisms. Iron-oxidizing bacteria in samples of coal waste and acid mine drainage water were enriched in selective culture media, isolated (from water samples only) and characterized. Heterotrophic organisms that were closely associated with the iron-oxidizing bacteria during enrichment, were also isolated and characterized. Several fungi were found in close association with the iron-oxidizing bacteria in the enrichment cultures from the coal waste, and their interactions with *T. ferrooxidans*, the iron-oxidizing species identified in the cultures, were studied to investigate whether they might have a positive influence on the rate of iron oxidation in the presence of organic compounds that could inhibit *T. ferrooxidans*.

MATERIALS AND METHODS

Microbiological Media

HJJ, 9K, L, S^0 and FeSo media

The composition and use of the HJJ, L, S^0 and FeSo media have been described in Experimental Part 1. The 9K medium (Silverman and Lundgren, 1959) was the same as HJJ medium except that it contained a higher ammonium sulphate concentration (3.00 g/l).

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H medium

This medium was devised for the enrichment of iron-oxidizing bacteria, such as those described by Ghauri and Johnson (1991) and Johnson *et al.* (1992). These bacteria oxidize ferrous iron, but have a lower iron tolerance than *T. ferrooxidans* and require yeast extract for growth. The basal medium consisted of 1.3 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g $MgSO_4.7H2O$, 0.5 g K_2HPO_4, 0.01 g $Ca(NO_3)_2{\cdot}4H_2O$ and 0.5 g of yeast extract (Biolab Diagnostics, Midrand) dissolved in 700 ml distilled water. The pH was adjusted to pH 2.0 with H_2SO_4 and the medium was sterilized at 121°C for 15 minutes. To this basal medium, a filter-sterilized solution of 14 g $FeSO_4.7H_2O$ in 300 ml distilled water was added aseptically. The pH of the $FeSO_4.7H_2O$ solution was adjusted to pH 2.0 before sterilization, using H_2SO_4.

***Thiobacillus* solid medium (TSM)**

This medium was modified from the TSM media developed by Visca *et al.* (1989) for the isolation of *T. ferrooxidans.* It comprised a basal salts solution, a substrate solution and a gelling agent solution. The basal salts solution consisted of 3.0 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.05 g $K_2HPO_{4,}$, 0.5 g $MgSO_4.7H_2O$ and 0.015 g $Ca(NO_3)_2{\cdot}4H_2O$ dissolved in 600 ml distilled water, acidified to pH 2.0 using H_2SO_4, sterilized at 121°C for 15 minutes and cooled to between 45 and 50°C. The substrate solution comprised 22.0 g $FeSO_4{\cdot}7H_2O$ in 150 ml distilled water, which was acidified to pH 2.5, filter-sterilized and warmed to between 45 and 50°C. The gelling agent solution for TSM was 5 g SEAKEM GTG agarose (FMC Bioproducts, Rockland, Maine, cat. no. 50071) as the modified component in 250 ml distilled water, which was autoclaved at 121°C for 15 minutes and cooled to 55°C. The solutions were mixed gently prior to pouring the plates.

***Acidiphilium* solid medium (ASM)**

This medium, which was similar to that of Harrison (1989), was used to detect, culture and isolate acidophilic heterotrophic organisms associated with *T. ferrooxidans.* The ASM consisted of a basal salts solution comprising 2.0 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4 and 0.5 g $MgSO_4{\cdot}7H_2O$ in 500 ml distilled water, which was acidified to pH 2.0 with H_2SO_4, and an organic solution comprising 1.0 g glucose, 0.1 g yeast extract and 12.0 g Oxoid Ionagar no. 2 (Oxoid Ltd, Basingstoke, Hampshire, England) in 500 ml non-acidified distilled water. Both solutions were autoclaved at 121°C for 15 minutes and cooled to 45-50°C. After cooling, the solutions were mixed gently and poured aseptically into sterile Petri dishes.

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Enrichment Cultures

Cultures from coal waste

Mines at which coal waste was sampled are shown in Fig. 18. Three enrichment cultures of iron-oxidizing bacteria in HJJ medium were supplied in October 1994 by Mr. C. Cleghorn and have been described in detail in his M.Sc. thesis (Cleghorn, 1997). They were CD1 from Corby Rock duff, K/E1 from a fine soft waste from a duff-washing process at the Kilbarchan Mine and K/G1 from coal waste from the Kilbarchan Mine. Culture WC1 was an enrichment culture line from an iron-oxidizing MPN culture from the Kilbarchan pilot scale dump rehabilitation experiment. Further enrichment cultures were started from coal waste from the other listed mines in the northern KwaZulu-Natal area in June 1995. This was done by inoculating 1 g of coal waste sample into 100 ml of HJJ, H and L medium for enrichment of the groups of iron-oxidizing bacteria indicated under **Microbiological Media**. The source, moisture content and pH of these samples are indicated later under **RESULTS**. Cultures in HJJ and H medium were incubated stationary at 26°C and other cultures in L and H medium at 40°C. After 1 month, iron oxidation was determined by titration with acidic dichromate (Loos *et al.*, 1990a). The cultures were transplanted (10% v/v inoculum) into fresh medium when most of the ferrous iron had been metabolized. Subsequently these enrichment cultures were transplanted into fresh medium every 4-6 weeks.

Cultures from mine dump drainage water

Enrichment cultures of iron-oxidizing bacteria were started in May 1994 by inoculating 10 ml mine drainage water collected at the listed mines into 100 ml HJJ medium. The pH of the samples from which active enrichment cultures were derived, is reported under **RESULTS**. The cultures were incubated at 26 °C with shaking at 80 r.p.m. on a Gerhardt RO 20 rotary shaker (Laboratory and Scientific Equipment Co., Cape Town). Active cultures were transplanted (10% v/v) into fresh medium every 2-6 weeks, when most of the ferrous iron had been oxidized as determined by the acidic dichromate titration.

The isolations of organisms from the cultures (as described in the following section) were attempted after at least eight transfers of the cultures to fresh media to allow the selected populations to be enriched and to establish stable consortia.

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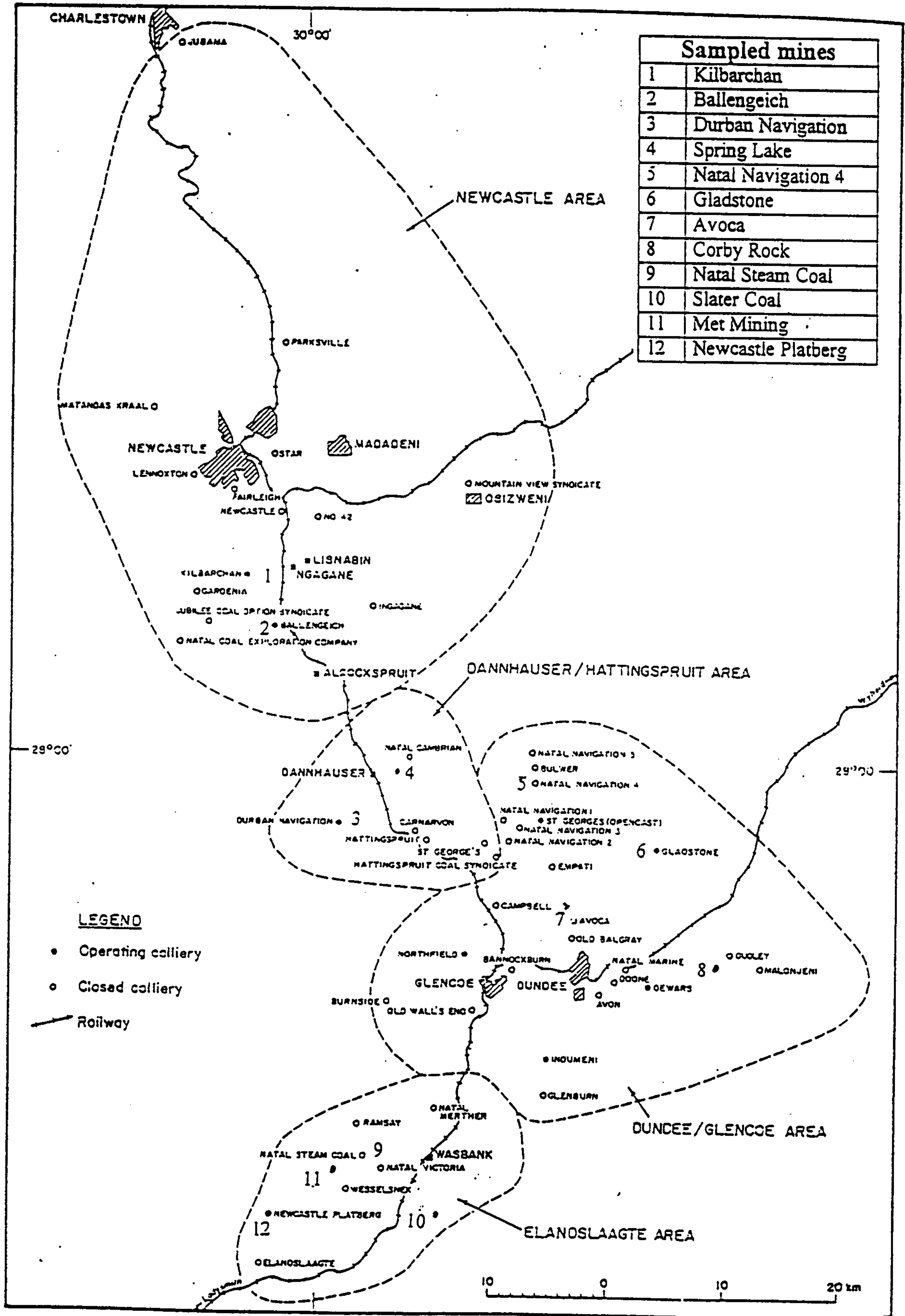

Sampled mines	
1	Kilbarchan
2	Ballengeich
3	Durban Navigation
4	Spring Lake
5	Natal Navigation 4
6	Gladstone
7	Avoca
8	Corby Rock
9	Natal Steam Coal
10	Slater Coal
11	Met Mining
12	Newcastle Platberg

Fig. 18. Mines of the Klip River Coalfield in northern KwaZulu-Natal (Bell and Spurr, 1986), with sampled mines indicated by numbers on the map.

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Isolation of Iron-oxidizing Bacteria

Plating procedures

Drops of inoculum removed with the aid of a loop from active iron-oxidizing enrichment cultures were streaked on TSM plates and incubated for 2-4 weeks at 26°C to allow single colony development. Colonies from these plates were each streaked over two plates and incubated at 26°C for 2 weeks to yield single colonies. Representative single colonies were streaked repeatedly in this manner for the isolation of iron-oxidizing bacteria in pure culture. After a minimum of four repetitions of the single colony streaking procedure, isolated strains were inoculated into HJJ liquid medium and incubated at 26°C for 2 weeks to test for iron-oxidizing activity.

Test for heterotrophic associates

Heterotrophic organisms, especially bacteria of the genus *Acidiphilium*, commonly occur as associates in *T. ferrooxidans* cultures. The combination of these two organisms forms colonies resembling single organism colonies (Harrison, 1984, 1989). The testing of iron-oxidizing cultures derived from single colonies for the presence of acidophilic heterotrophs was therefore necessary. This was done by inoculating a single colony from a TSM plate into 100 ml HJJ medium. After 2 weeks of growth, inoculum from this culture was streaked in duplicate on ASM plates, which were incubated at 26°C for 2 weeks. Growth on these plates would indicate the presence of heterotrophic organisms.

Culture maintenance

Isolated strains of iron-oxidizing bacteria and 'single colony' mixed cultures, were maintained by transplanting them every 2 weeks into fresh HJJ medium, using a 10% (v/v) inoculum and incubating them with shaking (80 r.p.m.) at 26°C.

Isolation of Heterotrophic Organisms

In view of the close association often found between *T. ferrooxidans* and acidophilic heterotrophic bacteria of the genus *Acidiphilium* (Harrison, 1981, 1984, 1989; Harrison *et al.*, 1980) attempts were made to isolate these bacteria and other heterotrophic microorganisms from enrichment cultures of iron-oxidizing bacteria in HJJ medium for investigation of their possible role in iron-oxidizing consortia.

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Plating procedures

Active iron-oxidizing enrichment cultures from coal waste and mine drainage, which had been subjected to at least eight successive transfers, were used to inoculate ASM plates by streaking for single colonies. The plates were incubated at 26°C for 2 weeks. Different colonies from these plates were each streaked over two plates to yield single colonies. Single colonies thus obtained, were streaked in a similar way. After at least three restreakings and picking of single colonies, the isolates were considered to be pure strains. Bacteria, yeasts and filamentous fungi, which grew on the ASM plates streaked with some of the enrichment cultures, were isolated in this fashion.

Culture maintenance

Pure cultures of acidophilic heterotrophs from single colonies were streaked on ASM plates, which were incubated at 26°C. These streaked cultures were transplanted every 3-6 weeks onto fresh ASM plates.

Characterization of Iron-oxidizing Bacteria

For microscopic observation of morphology and Gram-reaction, cells were collected by centrifuging early stationary phase cultures in HJJ medium at 6000 r.p.m. for 30 min, then washed repeatedly with 0.01N H_2SO_4 (pH 2) and collected by centrifuging at 13 000 r.p.m. Gram-stained smears were viewed microscopically at 1250 x magnification.

Sulphur utilization was tested by inoculating 2-week-old cultures into S^0-medium (1 ml in 10 ml) and incubating at 26°C for 5 weeks. *Thiobacillus ferrooxidans* ATCC 23270 and *L. ferrooxidans* CF12 were included as positive and negative controls, respectively. A drop in medium pH of 0.3 relative to that of the negative control was taken as positive for sulphur utilization.

Characterization of Heterotrophic Organisms

Bacteria

Cell morphology and motility were determined by bright field microscopic observation at 1250 x magnification of wet preparations of cells from 7-day-old cultures on ASM plates.

Catalase and oxidase tests were performed on streak cultures on ASM plates, using, respectively, 30 % (m/v) H_2O_2 solution and Oxidase Identification Sticks from Oxoid Ltd, as directed by the manufacturer.

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Pigmentation was observed in 2-week-old streak cultures on ASM plates.

Sulphur utilization tests were performed in 15- x 150-mm test tubes, using the S^0 medium. Inoculated tubes were incubated for 4 weeks at 26°C. *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus* were included as positive controls, while *Acidiphilium organovorum* and *Acidiphilium rubrum* served as negative controls for sulphur utilization.

All isolates were tested for growth at pH 2.0, 2.5 and 7.0 on ASM plates modified to the appropriate pH.

Yeasts

Cell morphology was determined by bright field microscopic observation at 1250 x magnification of wet preparations of cells from 10-day-old cultures grown on ASM plates at 26°C.

Physiological tests used for yeast identification were similar to those described by Barnett *et al.* (1990). Inoculum of 0.1 ml of a 4-day-old culture in *Acidiphilium* liquid medium (ASM without agar) in 10 ml test medium was used.

Carbon source utilization was tested in 15- x 150-mm test tubes, covered with metal caps and containing 10 ml of Yeast Nitrogen Base (Difco Laboratories) supplemented with 50 mM test carbon source. The basal medium was sterilized at 121°C for 15 minutes and the carbon sources filter sterilized through a 0.2 μm nitrocellulose membrane filter (Millipore SA, Bellville). Inoculated tubes containing only the Yeast Nitrogen Base served as negative controls. The tubes were incubated at 26°C with shaking at 100 r.p.m. After 10 days tubes showing turbidity greater than that of uninoculated controls containing the same carbon source, were scored as positive.

Fermentation ability was tested using 10 ml of yeast extract-glucose medium in which Durham tubes were submerged. The medium consisted of 0.5% (m/v) yeast extract (Difco Laboratories) containing 50 mM glucose. Inoculated tubes containing no glucose served as negative controls. Tubes were incubated stationary at 26°C for 10 days. Gas accumulation in the Durham tubes would indicate a positive result (Barnett *et al.*, 1990).

Tests for the production of extracellular starch-like compounds were performed by adding six drops of a solution containing 4 g/l KI and 2,54 g/l I_2 to 10 ml of a 1-week-old culture grown in *Acidiphilium* liquid medium.

Tests for nitrogen source utilization were similar to those for carbon source utilization, except that the test medium was Yeast Nitrogen Base without Amino Acids and Ammonium Sulphate

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(Difco Laboratories) to which 5 mM test nitrogen source and 1 g/litre glucose were added (Barnett *et al.*, 1990).

Filamentous fungi

The morphology of filamentous fungi isolated from the enrichment cultures derived from coal waste was determined by bright field microscopic observation at 400 x magnification. Colony and cultural characteristics were observed in 1-week-old cultures on ASM-plates.

Studies of Interactions Between *T. ferrooxidans* and Fungi from Enrichment Cultures

Five sets of media were prepared, consisting of JLFe medium with no organic supplementation and four sets where the JLFe medium was supplemented with yeast extract added to the basal salts solution to yield final concentrations (after addition of the ferrous sulphate solution) of 0.25, 0.50, 1.0 and 5.0 g/l yeast extract, respectively. Duplicate flasks (100 ml medium in 250 ml Erlenmeyer flasks) of each set of media were inoculated with the following combinations of organisms:

(i) *T. ferrooxidans* ATCC 23270 alone,

(ii) *T. ferrooxidans* ATCC 23270 and *Acidiphilium organovorum*, a known bacterial associate of *T. ferrooxidans*,

(iii) *T. ferrooxidans* ATCC 23270 and a *Penicillium* sp.,

(iv) *T. ferrooxidans* ATCC 23270 and a fungus of the unidentified Type 2, and

(v) *T. ferrooxidans* ATCC 23270 and a fungus of the unidentified Type 3.

Thiobacillus ferrooxidans ATCC 23270 was inoculated as 0.5 ml of a 4-week-old stationary phase culture in JLFe medium, while the heterotrophic organisms were inoculated by means of an inoculation loop from 1-week-old cultures on ASM-plates. The cultures were incubated stationary at 26°C for 15 days. At approximately 24-hour intervals, the percentage ferrous iron oxidized in each flask relative to the mean concentration of unoxidized ferrous iron in duplicate uninoculated control flasks, was determined by dichromate titration as described by Loos *et al.* (1990a). The mean percentage of ferrous iron that had been metabolized was calculated for the duplicate flasks of each medium set as an indicator of growth of the *T. ferrooxidans* in the different media with the different associates.

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RESULTS

Enrichment Cultures

Cultures from coal waste

Enrichment cultures CD1, K/E1, K/G1 and WC1 in HJJ medium started from 1992 to 1994 by Cleghorn (1997) from samples became stable and remained active over a long period of subculturing. Attempts to isolate iron-oxidizing bacteria from the cultures were not successful on account of overgrowth of the TSM isolation plates by fungi, but these enrichment cultures were used for the isolation of acidophilic heterotrophic associates of the iron-oxidizing bacteria. Other iron-oxidizing enrichment cultures from Corby Rock duff and Kilbarchan coal waste samples in 9K or HJJ medium died out, sometimes after surviving many successive subcultures.

The ferrous iron in all of the enrichment cultures started from coal waste in June 1995 was completely oxidized after 1 month of incubation. The subcultures in HJJ medium incubated at 26°C, remained active and became stable by February 1996. They were used for the isolation of acidophilic heterotrophic associates of the acidophilic high ferrous iron-oxidizing bacteria, as TSM and FeSo plates on which isolation of the iron oxidizers was attempted, became overgrown with fungi. The cultures in L medium incubated at 40°C (selection for acidophilic high temperature high ferrous iron-oxidizing bacteria), and cultures in H-medium, which were incubated at 26 and 40°C (selection for acidophilic iron-oxidizing bacteria with low iron tolerance and a need for yeast extract), initially grew very well, but soon lost viability and were terminated.

Cultures from mine dump drainage water

All of the enrichment cultures from drainage water in HJJ medium oxidized the ferrous iron fully within 2 weeks. Usually the characteristic brown-red colour indicative of growth and iron oxidation by *T. ferrooxidans* appeared 3-5 days after transfer. Stabilized cultures were used for the isolation of acidophilic high ferrous iron-oxidizing bacteria and their heterotrophic associates.

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Isolation and Identification of Iron-oxidizing Bacteria from Enrichment Cultures

Isolation

Iron-oxidizing bacteria could be successfully isolated and maintained only from cultures derived from mine drainage.

In most cases, well defined single colonies of iron-oxidizing bacteria developed on TSM plates. Various colony morphologies were observed, ranging from small black-brown colonies which were less than 1 mm in diameter to large spreading colonies with orange ferric iron precipitation. Generally only one or two of these morphologies could be observed on plates inoculated from a specific enrichment culture. However, colony morphology did not seem to be a stable characteristic of these iron-oxidizing bacteria, as spreading colonies on subsequent streaking could yield small pin-point colonies, and vice versa.

Identification

All the iron-oxidizing bacterial isolates, were short Gram-negative rods occurring singly or in pairs. They oxidized both iron and sulphur. These characteristics agree with those for *T. ferrooxidans* in Bergey's Manual of Systematic Bacteriology (Kelly and Harrison, 1989). The isolates were therefore considered to be this species.

Isolation and Identification of Heterotrophic Organisms from Enrichment Cultures

Isolation

Stable enrichment cultures from duff, coal waste, and drainage water, when tested by streaking on ASM plates, showed the presence of various heterotrophic contaminants or associated bacteria or fungi (including a yeast in the case of one enrichment culture derived from drainage water). Representative single colonies were isolated and purified by successive single colony streaking on ASM plates.

Identification

Bacteria. All bacterial isolates from the various iron-oxidizing HJJ enrichment cultures were short, motile, Gram-negative rods, occurring singly or in pairs. They were weakly catalase-positive and oxidase-negative. All the strains were obligate acidophiles, growing well at pH 2.0 and 2.5, but not at pH 7.0. They were incapable of oxidizing sulphur These properties are consistent with the description of the genus *Acidiphilum* (Harrison, 1981, 1989; Kishimoto *et*

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al.,1995) and the isolates were all assigned to that genus.

The isolates showed differences in pigmentation, with some strains being light pink, and others ranging from mauve to light brown. This indicates that more than one species of *Acidiphilium* might be present as heterotrophic associates to *T. ferrooxidans* in the northern KwaZulu-Natal area. The pigmentation also indicates that these organisms belong to the genus *Acidiphilium* rather than *Acidocella* (Kishimoto *et al.*, 1995)

Ambiguous results from carbon utilization tests have made more accurate description and identification impossible to date.

Yeast. The yeast isolated from the HJJ enrichment culture PB#1 derived from a drainage water culture was ovoid and replicated by budding. Budding was usually slightly subpolar, and cells with multipolar budding were observed. Colonies on ASM plates were white, raised and became surrounded with hyphae on aging. Both mycelium and pseudomycelium were observed, growth becoming progressively more mycelial with aging of the colony. A high degree of polymorphism was observed and yeast cells or pseudomycelium, forming true mycelial hyphae at budding foci, were a common observation.

Both sexual and asexual reproductive structures were observed on the mycelial hyphae. Single conidia formed terminally and were similar in size and shape to the yeast cell. Asci formed laterally on the hyphae and contained numerous spores. Although the asci were extremely loosely connected to the hyphae and could be observed only by placing an undisturbed colony on a Petri dish directly under the microscope, they persisted in the unconnected form.

In the carbon utilization tests the yeast grew on glucose, galactose, sorbose, sucrose, maltose, lactate, succinate, citrate and ethanol, but not on melibiose, lactose, glycerol or mannitol. The organism did not ferment glucose (no gas production) and did not produce extracellular starch-like substances. As growth occurred in the negative control tubes of the nitrogen source utilization tests, these tests were not considered for identification purposes.

Morphologically the organism closely resembled the photographs and description of *Dipodascus macrosporus* in Barnett *et al.* (1990). Its lack of fermentation and most of the carbon source utilization results also agreed with the properties of this species. However, *Dipodascus macrosporus,* as well as the other members of the genus, cannot utilize the disaccharides, sucrose and maltose, which this strain could utilize. The identification of the strain to the species level has therefore not been possible, but on the grounds of morphology, the lack of pigmentation, the presence of asci, and the inability to ferment, it is likely that the organism belongs to the genus *Dipodascus*.

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Filamentous fungi. Filamentous fungi were an important component of the heterotrophic population in enrichment cultures K/E1 and K/G1 derived from coal waste at the Kilbarchan mine and the cultures derived from coal waste in 1995. Only three different colony types were observed among the cultures from the latter samples. They included *Penicillium* isolates as Type 1 and two unidentified forms as Types 2 and 3. These types were similar morphologically, but Type 2 had a darker brown pigmentation than Type 3. Colonies on solid medium had a shiny yeast-like central area and outward radiating strands of growth on the medium surface. The hyphae formed cross-walls and fragmented to form cylindrical spores. Although not identified with certainty, these fungi can be described as *Cladophialophora*-like and closely resemble the drawings and photos of Braun and Feiler (1995).

All of the filamentous fungi detected in the HJJ enrichment cultures derived from drainage water were hyphomycetes, but as fungi formed only a small percentage of the heterotrophic component from these cultures, no further studies were performed on them.

Distribution of Isolated Organisms in Enrichment Cultures from Coal Waste or Mine Drainage Water

Organisms in enrichment cultures from coal waste

The iron-oxidizing cultures from coal waste in HJJ medium yielded no iron-oxidizing isolates because of fungal growth over the isolation media, but yielded associated *Acidiphilium* heterotrophic bacteria and fungi. The CD1 culture from Corby Rock duff and the WC1 culture from coal waste from Kilbarchan mini-dumps yielded bacteria identified as *Acidiphilium*, whereas the K/E1 and K/G1 cultures from Kilbarchan coal waste yielded unidentified filamentous fungi (hyphomycetes).

The distribution of isolated *Acidiphilium* heterotrophic bacteria and fungi among enrichment cultures from the 1995 coal waste samples is shown in Table 17. All samples from which the enrichment cultures were developed, were highly acid, except a single sample from Spring Lake from coal waste which had not acidified. Most of the cultures yielded bacteria, identified as *Acidiphilium* isolates, about half yielded *Penicillium* isolates and most yielded the brown unidentified *Cladophialophora*-like Type 3 fungus. Only one yielded the somewhat similar darker brown Type 2 fungus.

Organisms in enrichment cultures from mine dump drainage water

The enrichment cultures from mine dump drainage water did not produce fungal overgrowth of plates during the isolation of iron-oxidizing bacteria, which were isolated from 8 out of 15

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Table 17. Source mine, sample moisture content and pH, enrichment culture code and isolation of heterotrophic microorganisms from iron-oxidizing enrichment cultures originating from coal waste samples in northern Kwazulu-Natal

Culture Source				Heterotrophic Composition			
				Bacteria	Fungi		
Mine	**Sample moisture (%)[a]**	**Sample pH[b]**	**Culture code**	***Acidiphilium***	**Type 1 *Penicillium***	**Type 2 unidentified**	**Type 3 unidentified**
Ballengeich	9.05	3.33	1A@SILTEL	-	+	-	+
	5.98	2.82	1B@SILTEL	+	-	-	+
Durban Navigation	6.70	3.27	1A@DURNACOL	+	-	+	-
Spring Lake	7.14	6.49	1A@S/L	+	+	-	-
Natal Navigation 4	12.12	2.10	1A@NNC4	+	+	-	+
	9.75	2.50	1B@NNC4	+	-	-	+
Gladstone	12.28	2.41	1A@GLADST	+	-	-	+
	11.50	2.51	1B@GLADST	+	-	-	+
Avoca	9.10	2.42	1A@TALANA	-	-	-	+
	10.86	2.82	1B@TALANA	-	-	-	+
	11.78	2.50	2A@TALANA	+	+	-	+
	16.13	2.88	2B@TALANA	+	+	-	+
Slater Coal	12.51	2.13	1A@SLC	-	+	-	+
	14.73	2.13	1B@SLC	-	+	-	+
Met Mining	9.47	2.60	1A@MM	-	+	-	+
Newcastle Platberg	9.87	2.88	1A@PB	+	-	-	-
	9.21	3.66	1B@PB	+	-	-	+

[a]Mean of two determinations of g moisture/100 g dry coal waste.
[b]Mean pH of duplicate 10 g samples in 25 ml water.

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enrichment cultures. The samples yielding or not yielding these bacteria are shown in Table 18. All of the samples were acid. Acidophilic heterotrophic isolates from these samples are also shown in Table 18. All except one sample yielded heterotrophic bacterial isolates, identified as *Acidiphilium*. However, only three samples yielded filamentous fungi (unidentified hyphomycete isolates), while one yielded a yeast, which appeared to be a *Dipodascus* species.

Interactions Between *T. ferrooxidans* and Fungi from Enrichment Cultures

The growth curves of *T. ferrooxidans*, given as percentage ferrous iron oxidized by each of the combinations of organisms in four of the five sets of media, are shown in Fig. 19. Two growth curves, for *T. ferrooxidans* on its own and in association with *A. organovorum*, served as controls. When no organic compounds (yeast extract) were added to the cultures, very little difference in iron oxidation was observed among the cultures. However as the yeast extract concentration in the medium increased, the following observations were made:

(i) The iron oxidation capacity of *T. ferrooxidans* on its own did not seem to be affected by a yeast extract concentration of 0.5 g/l, but 1.0 g/l showed some inhibition and 5.0 g/l inhibited iron oxidation completely (not shown in Fig. 19).

(ii) The *Penicillium* sp. seemed to have an inhibitory effect on iron oxidation by *T. ferrooxidans* and the inhibition increased with increases in the yeast extract concentration.

(iii) Slight inhibition of iron oxidation seemed to occur in cultures where *A. organovorum* was present at a yeast extract concentration of 0.5 g/l. When the yeast extract concentration showed inhibition of *T. ferrooxidans* at 1.0 g/l, *A. organovorum* seemed to lessen the effect.

(iv) The cultures containing the fungi of the unidentified Types 2 and 3 oxidized the ferrous iron the fastest at all yeast extract concentrations and were best capable of overcoming the limited inhibition of 1.0 g/l yeast extract for *T. ferrooxidans*.

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Table 18. Source mine, sample pH, enrichment culture code and isolation of iron-oxidizing bacteria and heterotrophic microorganisms from iron-oxidizing enrichment cultures originating from mine drainage in northern Kwazulu-Natal

Culture source and code			Iron-oxidizing bacteria	Heterotrophic isolates	
Mine	**Sample pH**	**Culture code**	**Number of isolates (*T. ferrooxidans*)**	**Bacteria (*Acidiphilium*)**	**Fungi (F) / Yeast (Y)**
Ballengeich	2.50	SILTEC#1	0	+	-
	4.55	SILTEC#2	0	+	-
Durban Navigation	5.90	DURNACOL#1	0	-	-
Spring Lake	2.75	S/L#1	0	+	-
Natal Navigation	1.95	NNC4#2	0	+	-
Gladstone	2.71	GLADST#2	2	-	-
Avoca	2.27	TALANA#1	1	+	F
	2.60	TALANA #2	1	+	F
	2.42	TALANA #3	3	+	-
Natal Steam Coal	2.18	NS#1	1	+	-
Slater Coal	3.55	SLC#1	3	+	-
	2.28	SLC#2	3	+	F
Met Mining	4.65	MM#1	0	+	-
Newcastle Platberg	2.38	PB#1	3	+	Y
	2.72	PB#2	0	+	-

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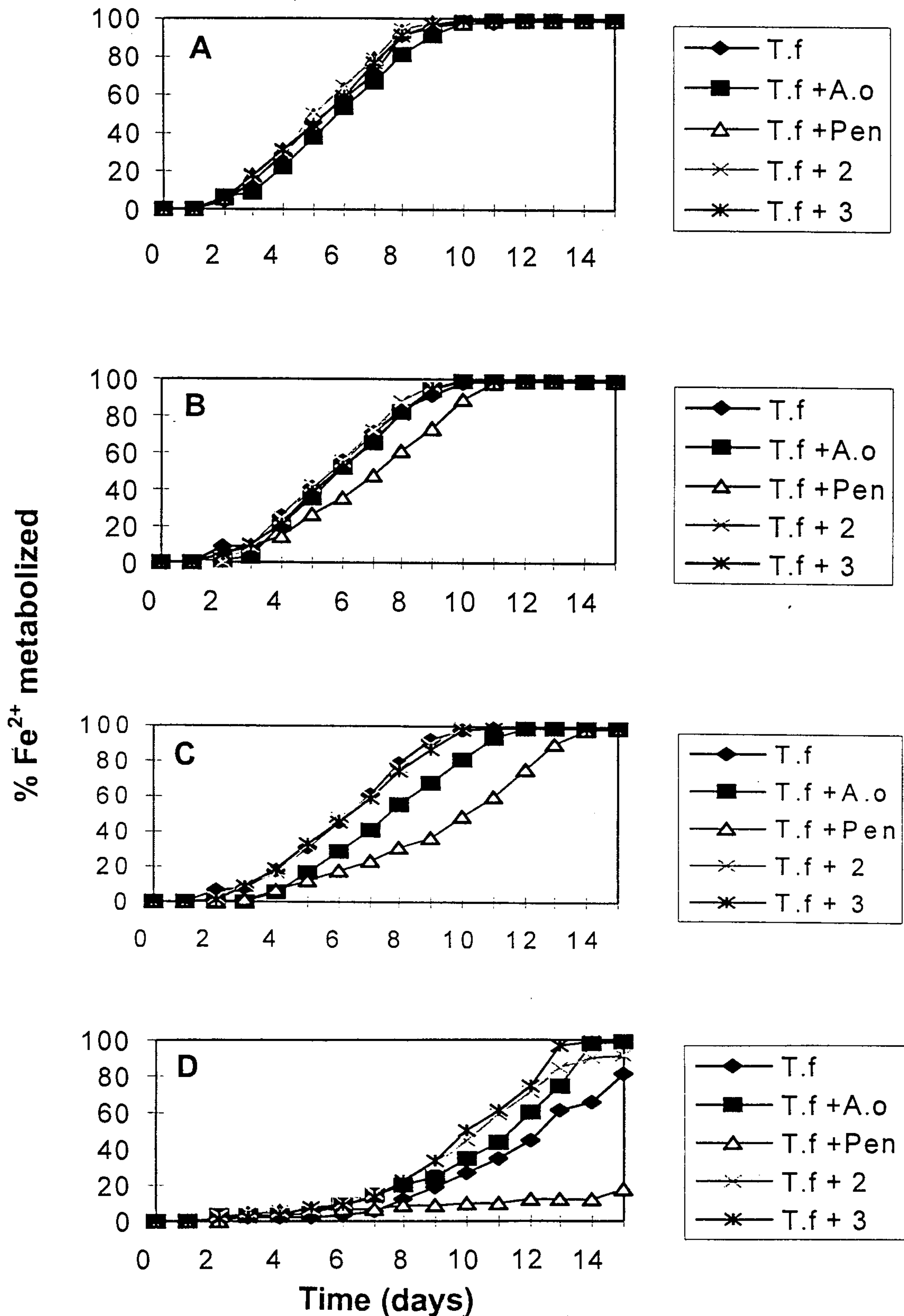

Fig. 19. Metabolism of ferrous iron by *T.ferrooxidans* in JLFe medium without organic supplementation (A) or supplemented with 0.25, 0.50 or 1.00 g/l yeast extract (B, C and D, respectively), alone (T.f) or in association (T.f +) with *A.organovorum* (A.o), *Penicillium* sp. (Pen) or unidentified fungus of Type 2 or 3 (2 and 3, respectively).

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DISCUSSION

Microbial Consortia in Iron-oxidizing Enrichment Cultures

The oxidation of ferrous ions to ferric ions is the rate-limiting step in the oxidation of pyrite and the subsequent formation of acid mine drainage in pyritic mineral environments, as ferric ions are the main agent of the oxidation (Kleinmann *et al.*, 1981; Luther, 1987; Moses and Herman, 1991; Moses *et al*, 1987; Sand *et al.*, 1995). The two well known lithotrophic bacterial species capable of oxidizing ferrous iron in acidic mineral environments, *T. ferrooxidans* and *L. ferrooxidans*, are known to live in close association with acidophilic heterotrophic organisms (especially bacteria of the genus *Acidiphilium*) which share their environment (Hallmann *et al.*, 1992, Harrison, 1984). It has been proposed that the association with heterotrophic organisms in consortia benefits the lithotrophs by removing substances inhibitory to them or by aiding their attachment to solid surfaces (Hallmann *et al.*, 1992; Harrison, 1984; Johnson, 1995a). The heterotrophs remain closely associated with the lithotrophs in iron-oxidizing enrichment cultures, which were therefore used to study the composition of the consortia in coal waste and mine dump drainage in the Klip River Coalfield.

Cultures from coal waste

The analyses of the iron-oxidizing consortia in the enrichment cultures started in 1995 from many different mines in the Klip River Coalfield, showed that both heterotrophic bacteria and fungi remained associated with the iron-oxidizing lithotrophic bacteria throughout the enrichment procedure. As in the older cultures, the bacterial isolates could all be assigned to the genus *Acidiphilium*. The fungi comprised at least two distinct major types, namely, strains of *Penicillium* and fungi of uncertain taxonomy (Types 2 and 3) with a resemblance to *Cladophialophora*.

Coal waste is a solid mineral environment, comparable to soil. It is also the site where primary oxidation of pyrite and subsequent acidification take place. As the coal waste is a soil-like environment and fungi account for most of the biomass and metabolic activity in soils (Allen, 1957; Anderson and Domsch, 1975), the presence of fungi could be expected. That only three types of fungi (two major types) were isolated from the 1995 samples could suggest either a semispecific association between these fungi and the iron-oxidizing bacteria in the enrichment cultures or that only these three types were capable of surviving the enrichment procedure. However, the abundance of fungi (especially the unidentified Type 3) in these samples suggests a possible ecological role for these organisms in the coal waste environment.

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As the enrichment cultures in H and L media were unstable and lost viability, no microbiological analysis of these cultures could be performed, although active iron oxidation was initially observed in the cultures. A study of the organisms that developed in H medium, in particular, might have yielded valuable information on groups of iron-oxidizing bacteria other than *T. ferrooxidans,* which is the species normally enriched in HJJ medium.

Cultures from mine dump drainage water

Drainage water from coal mine dumps is generally of low pH and contains high amounts of dissolved minerals (including iron). It is therefore ideal for the proliferation of lithotrophic iron-oxidizing bacteria. As oxidation of pyrite, the production of acidity and the mobilization of minerals occur in coal waste itself, the iron-oxidizing populations of the drainage water environments can be considered as secondary populations, benefitting from processes occurring in the adjacent waste dumps. Dissolved iron compounds in these environments might be involved in cyclic oxidation-reduction processes yielding ferrous iron as an energy source and ferric iron as an electron acceptor for different bacterial groups in the environments (Johnson, 1995a).

The drainage water is also an aqueous environment (in contrast to the mineral soil-like environment of waste dumps), which can explain why fungi were isolated from so few enrichment cultures as a heterotrophic component. However, the low occurrence of fungi in the cultures from mine dump drainage made the isolation of strains of iron-oxidizing bacteria possible. All of these isolates were Gram-negative rods oxidizing both iron and sulphur and could therefore be classified as *T. ferrooxidans*. *Leptospirillum ferrooxidans* was probably not obtained because HJJ medium favours *T. ferrooxidans.* A medium such as the JLFe medium with its lower concentration of ferrous iron, which gave greatly increased MPN counts of iron-oxidizing organisms in the pilot scale dump study of **EXPERIMENTAL PART 1,** might yield a wider range of iron-oxidizing bacterial species (Johnson, D.B., 1995, personal communication).

All the cultures, except Gladst # 2 which contained no detectable heterotrophic component, contained bacteria of the genus *Acidiphilium,* suggesting an important ecological role for these bacteria in the mine drainage environment which is not too different from that of HJJ-medium.

Both hyphomycete fungi and a yeast tentatively identified as a strain of *Dipodascus* were found in a few cultures, suggesting that fungi might also play an ecological role in iron oxidation in the mine drainage water environment.

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Interaction Studies between *T. ferrooxidans* and Fungi from Enrichment Cultures

The interaction studies between *T. ferrooxidans*, that was subjected to organic compound stress, and fungi isolated from enrichment cultures derived from coal waste suggest that interactions between the iron-oxidizing bacteria and the fungi that occur in acid mine drainage-generating environments in northern Kwazulu-Natal may form an integral part of the ecology of acid mine drainage generation. The obvious stimulatory effect of fungi of the unidentified Types 2 and 3 may be as a result of the consumption of inhibitory organic compounds. Bosch (1990) and Loos *et al.* (1990b) reported the degradation of SLS (a known inhibitor of *T. ferrooxidans*) by yeasts isolated from a pilot scale coal waste dump rehabilitation experiment in the Witbank area of Mpumalanga. It therefore seems that yeasts and fungi may stimulate the growth of *T. ferrooxidans* in a similar way to that of heterotrophic bacteria (see Harrison, 1984; Johnson, 1995a,b). It is noteworthy that the Type 2 and 3 fungi seemed to have a greater stimulatory effect under appropriate experimental conditions than *A. organovorum*, a member of the genus *Acidiphilium* that is well documented for enhancing the growth of iron-oxidizing bacteria (Johnson, 1995a, b). It is unclear why the *Penicillium* sp. had such an inhibitory effect on iron oxidation at elevated yeast extract concentrations. One possible explanation is that during growth on yeast extract this organism produced metabolites that were strongly inhibitory to *T. ferrooxidans*. Such fungi might play an important role in the inhibition of *T. ferrooxidans* below soil covers, by converting organic compounds of the soil to inhibitory compounds. The role of the interactions between fungi and iron-oxidizing bacteria in acid mine drainage-generating environments warrants further investigation.

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GENERAL DISCUSSION AND CONCLUSIONS

An important aim of the present investigation, namely, determination of the effectiveness of various types and depths of soil cover material in creating conditions in coal waste dumps unfavourable for the growth of acid-generating microbial populations, has supplied valuable information over 4 years with their cycles of seasons that can serve as a basis for establishing effective procedures for coal waste dump rehabilitation.

Aerobic conditions, notable decreases in pH and moderately high populations of iron-oxidizing bacteria were observed in uncovered cells and in the coal waste beneath a 0.3-m cover of uncompacted Avalon soil. The moderately high content of clay (30-34%) and silt (26-29%) was obviously not adequate to create unfavourable conditions for iron-oxidizing bacterial populations beneath Avalon soil of that thickness and hence could not prevent the formation of acid mine drainage in the underlying waste. A compacted Avalon soil cover of 0.5 m thick was also not adequate to create permanently anaerobic conditions in the coal waste and prevent acid mine drainage generation. The Avalon soil cover consisting of 0.7-m compacted underlying 0.3-m uncompacted soil, created apparently anaerobic conditions in the coal waste most of the time (becoming aerobic temporarily after prolonged drought conditions) but could not prevent slow acidification of the waste.

The results with the other covers comprising 0.3 or 0.7 m of compacted Estcourt soil (33% clay and 20 % silt) covered by uncompacted Avalon soil to give a total cover thickness of 1 m, provide valuable guidelines to the types of soil cover that can be used to rehabilitate coal waste dumps. These covers created anaerobic conditions in the coal waste and were effective in preventing acidification during the 4-year experimental period. However, these covers showed a shortcoming under drought conditions in 1995, when they developed cracks allowing the entrance of oxygen, so that conditions became aerobic in the coal waste. Surprisingly, the aerobic conditions persisted through the 1995/1996 rainy season, but returned to anaerobic in July 1996. Increasing fluctuation of pH during 1995/1996 among samples from previously anaerobic mini-dumps may indicate that some acidification may have taken place in localized pockets, but there is no evidence of general acidification. Based on these studies, the dump rehabilitation procedures followed by the Department of Water Affairs and Forestry are correct, while 'short cuts' involving the use of a single soil layer with a thickness of 1 m or less would probably be ineffective. The results with cell 8 suggest that a 30-cm clay layer covered by less than 70 cm of topsoil could be investigated as a possible cheaper cover. It is hoped that the final report by Wates, Meiring and Barnard on the hydrology of the pilot scale dumps (project K5/575

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of the Water Research Commission) will help to clarify the differences in effectiveness between the clay/soil and single soil covers.

Valuable methodology for monitoring the success of soil covers in preventing aerobic conditions and acidification in underlying coal waste was demonstrated in this investigation. The gas atmosphere of the coal waste was analysed immediately in the field using permanently buried stainless steel probes, through which gas could be drawn for analysis in a portable oxygen/carbon dioxide meter. Samples of coal waste were extracted by auger for analysis of moisture, pH and microbial populations. The analyses of oxygen and pH can be recommended for the routine monitoring of rehabilitated waste dumps, as they show very quickly whether conditions in the coal waste are favourable for acidification and whether acidification is actually occurring.

Bacteriological investigations for assessment of the effectiveness of covers in controlling acid mine drainage generation cannot be recommended. They are labour-intensive and time-consuming and do not give a reliable indication of whether acidification is taking place extensively or not, as the numbers of iron-oxidizing bacteria can vary dramatically according to their micro-environment which may be quite different from the general macro-environment. The numbers of these organisms will be influenced strongly by environmental conditions, such as oxygen supply and pH, which can be monitored much more quickly and cheaply as recommended in the previous paragraph.

The presence of a vegetation cover should prove valuable, by preventing the erosion of soil covers on dumps and reducing the diffusion of oxygen to the coal discard, but further advantage could not be evaluated in the present study.

The quantitative studies of the various microbial groups possibly associated with the generation of acidity in the coal waste of the pilot scale dumps at the Kilbarchan Mine indicate the dominance of acidophilic iron-oxidizing bacteria rather than sulphur-oxidizing bacteria. However, the high ferrous iron-oxidizing *T. ferrooxidans* may not have been the dominant iron-oxidizer, as population counts using media with a lower ferrous iron concentration indicated that large numbers of other iron oxidizers with a lower ferrous iron tolerance were present. These populations require further investigation. The populations of the high ferrous iron-oxidizing bacteria were particularly affected by pH, tending to be high in acidified samples and low in non-acidified samples.

Investigations of microbial populations forming iron-oxidizing consortia in enrichment cultures from a wide range of coal waste and acid mine drainage samples from northern KwaZulu-Natal, showed the presence of *T. ferrooxidans*, the heterotrophic bacterial genus *Acidiphilium*, fungi of

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the genus *Penicillium*, unidentified filamentous fungi, including *Cladophialophora*-like morphological types, and a yeast of the genus *Dipodascus*. Except for the fungi, which have not been studied in detail as components of iron-oxidizing consortia elsewhere, the results of these microbiological studies agree with those elsewhere, indicating that appropriate conclusions from acid mine drainage research in other parts of the world can be applied in KwaZulu-Natal. The study of the interactions of three fungal isolates with *T. ferrooxidans* in the presence of organic compounds suggests that fungi may have important roles in determining the iron-oxidizing activity of *T. ferrooxidans* in coal waste dumps; on the one hand they may alleviate inhibition of the bacteria by utilizing inhibitory organic substrates, but on the other hand, they may themselves produce active inhibitors. These possibilities require further investigation.

Stellenbosch University http://scholar.sun.ac.za/

REFERENCES

Acuña J., J. Rojas, A.M. Amaro, H. Toledo, and C. A. Jeres. 1992. Chemotaxis of *Leptospirillum ferrooxidans* and other acidophilic chemolithotrophs: Comparison with the *Escherichia coli* chemosensory system. FEMS Microbiol. Lett. **96:** 37-42.

Allen, O.N. 1957. Experiments in soil bacteriology, 3rd ed. Burgess Publishing Co., Minneapolis.

American Public Health Association, American Waterworks Association and Water Pollution Control Federation. 1955. Standard methods for the examination of water, sewage and industrial wastes, 10th ed. American Public Health Association, Washington, D.C.

Anderson, J. P. E., and K. H. Domsch. 1975. Measurement of bacterial and fungal contributions to respiration of selected agricultural soils. Can. J. Microbiol. **21:** 314-322.

Andrews, G. F. 1988. The selective adsorption of thiobacilli to dislocation sites on pyrite surfaces. Biotechnol. Bioeng. **31:** 378-381.

Atlas, R. M., and R. Bartha. 1993. Microbial ecology - Fundamentals and applications, 3rd ed. Benjamin Cummings Publishing Company, Redwood City, California.

Bagdigan, R. M., and A. S. Myerson. 1986. The adsorption of *Thiobacillus ferrooxidans* on coal surfaces. Biotechnol. Bioeng. **28:** 467-479.

Barnett, J. A., R. W. Payne, and D. Yarrow. 1990. Yeasts: Characteristics and identification, 2nd ed. Cambridge University Press, Cambridge.

Bell, K., and M. R. Spurr. 1986. The Klip River Coalfield of Northern Natal, p. 2033-2045. *In* C. R. Anhausser and S. Maske (ed.), Mineral deposits of Southern Africa, vol. 2. Geological Society of South Africa, Johannesburg.

Stellenbosch University http://scholar.sun.ac.za/

Belly, R. J., and T.D. Brock. 1974. Ecology of iron-oxidizing bacteria in pyritic materials associated with coal. J. Bacteriol. **117:** 726-732.

Blake, R. C., E. A. Shute, and G. T. Howard. 1994. Solubilization of minerals by bacteria: Electrophoretic mobility of *Thiobacillus ferrooxidans* in the presence of iron, pyrite, and sulfur. Appl. Environ. Microbiol. **60:** 3349-3357.

Bos, P., R. Meulenberg, J. T. Pronk, W. Hazeu, and J. G. Kuenen. 1994. *Thiobacillus ferrooxidans*, a versatile mineworker. Proc. 6th European Congress on Biotechnology, Florence, p. 1161-1168.

Bosch, C. 1990. Distribution and inhibition of iron-oxidizing bacteria in relation to acid drainage from coal mine dumps in the southern Transvaal. M.Sc. thesis, University of Stellenbosch.

Braun, U., and U. Feiler. 1995. *Cladophialophora* and its teleomorph. Microbiol. Res. **150:** 81-91.

Brierley, C. L. 1978. Bacterial leaching. CRC Crit. Rev. Microbiol. **6:** 207-262.

Brierley, C. L. 1982. Microbiological Mining. Sci. Am. **247**(2): 42-51.

Brierley, J. A.. 1978. Thermophilic iron-oxidizing bacteria found in copper leaching dumps. Appl. Environ. Microbiol. **36:** 523-525.

Brierley, J. A., and S. J. Lockwood. 1977. The occurrence of thermophilic iron-oxidizing bacteria in a copper leaching system. FEMS Microbiol. Lett. **2:** 163-165.

Brown, A. D., and J. J. Jurinak. 1989. Mechanism of pyrite oxidation in aqueous mixtures. J. Environ. Qual. **18:** 545-550.

Caruccio, F. T. 1970. The quantification of the reactive pyrite by grain size. Proc. 3rd. Symposium on Coal Mine Drainage Research, Mellon Institute, Pittsburgh, p.123-131.

Chakraborty, R., and P. Roy. 1992. Chemotaxis of chemolithotrophic *Thiobacillus ferrooxidans* toward thiosulfate. FEMS Microbiol. Lett. **98:** 9-12.

Stellenbosch University http://scholar.sun.ac.za/

Cleghorn, C. 1997. Effect of soil covers on coal waste dumps in KwaZulu-Natal on abiotic factors and bacteria causing acid mine drainage. M.Sc. thesis, University of Stellenbosch.

Colmer, A. R., and M. E. Hinkle. 1947. The role of microorganisms in acid mine drainage: A preliminary report. Science **106:** 253-256.

Colmer, A. R., K. L. Temple, and M. E. Hinkle. 1950. An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. J. Bacteriol. **59:** 317-328.

Daniel, D. E., and Y. K. Wu. 1993. Compacted clay liners and covers for arid sites. J. Geotech. Eng. **119:** 223-237.

De Man, J. C. 1983. MPN tables, corrected. Eur. J. Appl. Microbiol. Biotechnol. **17:** 301-305.

Director General: Water Affairs. 1987-88. Report on the proposed water pollution control works at abandoned coal mines in northern Natal [WP E-87]. Government Printer. Pretoria.

DiSpirito A. A., M. Silver, L. Voss, and O. H. Tuovinen. 1982. Flagella and pili of iron-oxidizing thiobacilli isolated from an uranium mine in Northern Ontario, Canada. Appl. Environ. Microbiol. **43:** 1196-1200.

Dubinina, G. A. 1970. Untersuchungen über die Morphologie von *Metallogenium* und die beziehungen zu *Mycoplasma.* Zeitschrift für Allg. Mikrobiologie **10:** 309-320.

Dugan, P.R. 1975. Bacterial ecology of strip mine areas and its relationship to the production of acid mine drainage. Ohio. J. Sci. **75:** 266-279.

Dugan, P. R., and D. G. Lundgren. 1964. Acid production by *Ferrobacillus ferrooxidans* and its relation to water pollution. Dev. Ind. Microbiol. **5:** 250-257.

Duncan D. W., J. Landesman, and C. C. Walden. 1967. Role of *Thiobacillus ferrooxidans* in the oxidation of sulfide minerals. Can. J. Microbiol. **13:** 397-403.

Erickson, P.M. 1985. Oxygen content of unsaturated coal mine waste. Bureau of Mines Information Circular I.C. 9027, U. S. Dept. of the Interior, Pittsburgh, p. 19-24.

Stellenbosch University http://scholar.sun.ac.za/

Espejo, R. T., and P. Ruiz. 1987. Growth of free and attached *Thiobacillus ferrooxidans* in ore suspension. Biotech. Bioeng. **30:** 586-592.

Evangelou. V. P., and Y. L. Zhang. 1995. A review: Pyrite oxidation mechanisms and acid mine drainage control. Crit. Rev. Environ. Sci. Technol. **25:** 141-199.

Ewart, D. K., and M. N. Hughes. 1991. The extraction of metals from ores using bacteria. Adv. Inorg. Chem. **36:** 103-135.

Fernandez M. G. M., C. Mustin, P. de Donato, O. Barres, P. Marion, and J. Berthelin. 1995. Occurrences at mineral-bacteria interface during oxidation of arsenopyrite by *Thiobacillus ferrooxidans*. Biotech. Bioeng. **46:** 13-21.

Fornaseiro, D., V. Eijt, and J. Ralston. 1992. An electrokinetic study of pyrite oxidation. Colloids Surf. **62:** 63-73.

Gehrke, T., R. Hallmann, and W. Sand. 1995. Importance of exopolymers from *Thiobacillus ferrooxidans* and *Leptospirillum* ferrooxidans for bioleaching, p. 1-19. *In* T. Vargas, C. A. Jerez and H. Toledo (ed.) Biohydrometallurgical processing. University of Chile Press, Santiago.

Ghauri, M. A., and D. B. Johnson. 1991. Physiological diversity amongst some moderately thermophilic iron-oxidizing bacteria. FEMS Microbiol. Ecol. **85:** 327-334.

Good, D. M., V. T. Ricca, and K. S. Shumate. 1970. The relation of refuse pile hydrology to acid production. Proc. 3rd Symposium on Coal Mine Drainage Research, Mellon Institute, Pittsburgh, p. 145-151.

Goodman, A.E., T. Babij, and A.I.M. Ritchie. 1983. Leaching of sulphide ore by *Thiobacillus ferrooxidans* under anaerobic conditions, p. 361-376. *In* G. Rossi and A.E. Torma (ed.), Recent progress in biohydrometallurgy. Associazone Mineria Sarda, Iglesias, Italy.

Gray, T. R. G., and S. T. Williams. 1971. Soil micro-organisms. Longman, London.

Guay, R., and M. Silver. 1975. *Thiobacillus acidophilus* sp. nov.: Isolation and some physiological characteristics. Can. J. Microbiol. **21:** 281-288.

Stellenbosch University http://scholar.sun.ac.za/

Hallmann, R., A. Friedrich, H.-P. Koops, A. Pommerening-Röser, K. Rohde, C. Zenneck, and W. Sand. 1992. Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and physicochemical factors influence microbial metal leaching. Geomicrobiol. J. **10:** 193-206.

Harrison, A. P. 1978. Microbial succession and mineral leaching in an artificial coal spoil. Appl. Environ. Microbiol. **38:** 861-869.

Harrison, A. P. 1981. *Acidiphilium cryptum* gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. Int. J. Syst. Bacteriol. **31:** 327-332.

Harrison, A. P. 1982. Genomic and physiological comparisons between heterotrophic thiobacilli and *Acidiphilium cryptum, Thiobacillus versutus.*, sp. nov., and *Thiobacillus acidophilus* nom. rev. Int. J. Syst. Bacteriol. **33:** 211-217.

Harrison, A. P. 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. Annu. Rev. Microbiol. **38:** 265-292.

Harrison, A. P. 1989. The genus *Acidiphilium* Harrison, p.1863-1868. *In* J. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams and Wilkins, Baltimore.

Harrison, A. P., B. W. Jarvis, and J. L. Johnson 1980. Heterotrophic bacteria from cultures of autotrophic *Thiobacillus ferrooxidans*: Relationships as studied by means of deoxyribonucleic acid homology. J. Bacteriol. **143:** 448-454.

Harrison, A. P., and P. R. Norris. 1985. *Leptospirillum ferrooxidans* and similar bacteria: Some characteristics and genomic diversity. FEMS Microbiol. Lett. **30:** 99-102.

Henzen, M.R., and M.J. Pieterse. 1978. Acid mine drainage in the Republic of South Africa. Progr. Water Technol. **9:** 981-992.

Hutchins, S. R., M. S. Davidson, J. A. Brierley, and C. L. Brierley. 1986. Microorganisms in the reclamation of metals. Annu. Rev. Microbiol. **40:** 311-336.

Stellenbosch University http://scholar.sun.ac.za/

Ingledew, W.J. 1982. *Thiobacillus ferrooxidans*: The bioenergetics of an acidophilic chemolithotroph. Biochim. Biophys. Acta **683:** 89-117.

Johnson, D. B. 1995a. Acidophilic microbial communities: Candidates for bioremediation of acidic mine effluents. International Biodeterioration and Biodegradation **35:** 41-58.

Johnson, D. B. 1995b. Selective solid media for isolating and enumerating acidophilic bacteria. J. Microbiol. Methods **23:** 205-218.

Johnson, D. B., M. A. Ghauri, and M. F. Said. 1992. Isolation and characterization of an acidophilic, heterotrophic bacterium capable of oxidizing ferrous iron. Appl. Environ. Microbiol. **58:** 1423-1428.

Johnson, D. B., and W. I. Kelso. 1983. Detection of heterotrophic contaminants in cultures of *Thiobacillus ferrooxidans* and their elimination by subculturing in media containing copper sulphate. J. Gen. Microbiol. **129:** 2969-2972.

Johnson, D. B., J. H. M. MacVicar, and S. Rolfe. 1987. A new solid medium for the isolation and enumeration of *Thiobacillus ferrooxidans* and acidophilic heterotrophic bacteria. J. Microbiol. Methods **7:** 9-18.

Johnson, D. B., and S. McGinness. 1991a. A highly efficient universal solid medium for growing mesophilic and moderately thermophilic, iron-oxidizing, acidophilic bacteria. J. Microbiol. Methods **13:** 113-122.

Johnson, D. B., and S. McGinness. 1991b. Ferric iron reduction by acidophilic heterotrophic bacteria. Appl. Environ. Microbiol. **57:** 207-211.

Johnson, D. B., and L. Rang. 1993. Effects of acidophilic protozoa on populations of metal-mobilizing bacteria during the leaching of pyritic coal. J. Gen. Microbiol. **139:** 1417-1423.

Kelly, D. P., and A. P. Harrison. 1989. The genus *Thiobacillus* Beijerinck, p. 1842-1858. *In* J. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams and Wilkins, Baltimore.

Stellenbosch University http://scholar.sun.ac.za/

Kelly, D. P., and O. H. Tuovinen. 1972. Recommendation that the names *Ferrobacillus ferrooxidans* Leathen and Braley and *Ferrobacillus sulfooxidans* Kinsel be recognised as synonyms of *Thiobacillus ferrooxidans* Temple and Colmer. Int. J. Syst. Bacteriol. **22:** 170-172.

Kemp, P. H. 1962. Acidic drainage from coal mines with special reference to the Natal coal fields. C.S.I.R. Special Report No. W. 12, C.S.I.R. National Unit for Water Research, Durban.

Kinsel, N. A. 1960. New sulfur oxidizing iron bacterium: *Ferrobacillus sulfooxidans* sp. n. J. Bacteriol. **80:** 628 - 632.

Kishimoto, N., Y. Kosako, N. Wakao, T. Tano, and A. Hiraishi. 1995. Transfer of *Acidiphilium facilis* and *Acidiphilium aminolytica* to the genus *Acidocella* gen. nov., and emendation of the genus *Acidiphilium*. System. Appl. Microbiol. **18:** 85-91.

Kleinmann, R. L. P. 1979. The biogeochemistry of acid mine drainage and a method to control acid formation. Ph. D. thesis, Princeton University.

Kleinmann, R.L.P., and D.A. Crerar. 1979. *Thiobacillus ferrooxidans* and the formation of acidity in simulated coal mine environments. Geomicrobiol. J. **1:** 373-388.

Kleinmann, R.L.P., D.A. Crerar, and R.R. Pacelli. 1981. Biogeochemistry of acid mine drainage and a method to control acid formation. Mining Eng. **33**: 300-305.

Lane, D. J., A. P. Harrison, D. A. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace. 1992. Evolutionary relationships among sulphur- and iron-oxidizing eubacteria. J. Bacteriol. **174:** 269-278.

Lane, D. J., D. A. Stahl, G. J. Olsen, D. J. Heller, and N. R. Pace. 1985. Phylogenetic analysis of the genera *Thiobacillus* and *Thiomicrospira* by 5S rRNA sequences. J. Bacteriol. **163:** 75-81.

Leathen W. W., N. A. Kinsel, and S. A. Braley. 1956. *Ferrobacillus ferrooxidans*: A chemosynthetic autotrophic bacterium. J. Bacteriol. **72:** 700-704.

Stellenbosch University http://scholar.sun.ac.za/

Leduc, L. G., and G. D. Ferroni. 1994. The chemolithotrophic bacterium *Thiobacillus ferrooxidans*. FEMS Microbiol. Rev. **14:** 103-120.

Leduc, L. G., J. T. Trevors, and G. D. Ferroni. 1993. Thermal characterization of different isolates of *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. **108:** 189-194.

Le Roux, N. W., D. S. Wakerley, and S. D. Hunt. 1977. Thermophilic thiobacillus-type bacteria from Icelandic thermal areas. J. Gen. Microbiol. **100:** 197-201.

Loos, M.A., J.M. Conradie, P.A. Whillier, J. Maré, and C. Bosch. 1990a. Research on the inhibition of bacterial oxidation of pyrite and the concomitant acid mine drainage: Part 1. Investigations on gold mine sand dumps. WRC Report no. 132/1/90, Water Research Commission, Pretoria.

Loos, MA., C. Bosch, and J. Maré. 1990b. Research on the inhibition of bacterial oxidation of pyrite and the concomitant acid mine drainage: Part 2. Investigations on coal waste dumps. WRC Report No. 132/2/90, Water Research Commission, Pretoria.

Loos, M. A., C. Cleghorn, and H. Mödinger. 1997. Occurrence of bacteria causing acid mine drainage in the outer layers of coal waste dumps in relation to abiotic ecological determinants and soil covers used for dump rehabilitation. Report to the Water Resaerch Commission on the Project: K5/454. Department of Microbiology, University of Stellenbosch, Stellenbosch.

Lowson, R. T., 1982. Aqueous oxidation of pyrite by molecular oxygen. Chem. Rev. **82:** 461-497.

Lundgren, D. G., and D. Silver. 1980. Ore leaching by bacteria. Annu. Rev. Microbiol. **34:** 263-283.

Luther, G. W. 1987. Pyrite oxidation and reduction: Molecular orbital theory considerations. Geochim. Cosmochim. Acta **51:** 3193 -3199.

Mackintosh, M. E. 1978. Nitrogen fixation by *Thiobacillus ferrooxidans*. J. Gen. Microbiol. **105:** 215-218.

Stellenbosch University http://scholar.sun.ac.za/

Markosyan, G. E. 1972. [A new acidophilic iron bacterium *Leptospirillum ferrooxidans* gen. et sp. nov.] Biol. Zh. Arm. **25:** 26-33. [In Russian.]

Meynell, G.C., and E. Meynell. 1970. Theory and practice in experimental bacteriology. Cambridge University Press, Cambridge.

McGinness, S., and D. B. Johnson. 1992. Grazing of acidophilic bacteria by a flagellated protozoan. Microb. Ecol. **23:** 75-86.

Mishra, A. K., and P. Roy. 1979. A note on the growth of *Thiobacillus ferrooxidans* on solid medium. J. Appl. Bacteriol. **47:** 289-292.

Moses, C. O., and J. S. Herman. 1991. Pyrite oxidation at circumneutral pH. Geochim. Cosmochim. Acta **55:** 471-482.

Moses, C. O., D. K. Nordstrom, J. S. Herman, and A. L. Mills. 1987. Aqueous pyrite oxidation by dissolved oxygen and by ferric iron. Geochim. Cosmochim. Acta **51:** 1561-1571.

Mustin, C., J. Berthelin, P. Marion, and P. De Donato. 1992. Corrosion and electrochemical oxidation of a pyrite by *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **58:** 1175-1182.

Mustin, C., P. de Donato, J. Berthelin, and P. Marion. 1993. Surface sulphur as promoting agent of pyrite leaching by *Thiobacillus ferrooxidans*. FEMS Microbiol. Rev. **11:** 71-78.

Norris, P. R. 1990. Acidophilic bacteria and their activity in mineral sulfide oxidation, p. 3-27. *In* H. L. Ehrlich and C. L. Brierley (ed.), Microbial mineral recovery. McGraw-Hill Publ. Co., New York.

Norris, P. R., and D. W. Barr. 1985. Growth and iron oxidation by acidophilic moderate thermophiles. FEMS Microbiol. Lett. **28:** 221-224.

Norris, P. R., and D. P. Kelly. 1982. The use of mixed microbial cultures in metal recovery, p. 443-474. *In* A.T. Bull and J. H. Slater (ed.), Microbial interactions and communities. Academic Press, London.

Stellenbosch University http://scholar.sun.ac.za/

Onysko, S.J., P.M. Erickson, and R.L.P. Kleinmann. 1984. Control of acid drainage from fresh coal refuse: Food preservatives as economical alternatives to detergents. 1984 Symposium on Surface Mining, Sedimentology and Reclamation, University of Kentucky, Lexington, p. 35-42.

Pronk, J.T., and D.B. Johnson. 1992. Oxidation and reduction of iron by acidophilic bacteria. Geomicrobiol. J. **10:** 153-171.

Pronk, J.T., K. Liem, P. Bos, and J.G. Kuenen. 1991. Energy transduction by anaerobic ferric iron respiration in *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **57:** 2063-2068.

Pronk, J.T., R. Meulenberg, W. Hazeu, P. Bos, and J.G. Kuenen. 1990. Oxidation of reduced inorganic sulphur compounds by thiobacilli. FEMS Microbiol. Rev. **75:** 293-306.

Razzell, W. E., and P. C. Trussell. 1963. Isolation and properties of an iron-oxidizing *Thiobacillus*. J. Bacteriol. **85:** 595-603.

Rickard, P.A.D., and D.G. Vanselow. 1978. Investigations into the kinetics and stoichiometry of bacterial oxidation of covellite (CuS) using a polarographic oxygen probe. Can. J. Microbiol. **24:** 998-1003.

Roberto, F.F., D.F. Bruhn, A.M. Wilhite, and T.E. Ward. 1993. Phylogenetic and biochemical characterization of acidophilic bacteria. FEMS Microbiol. Rev. **11:** 31-36.

Rodrigues–Leiva, M., and H.Tributsch. 1988. Morphology of bacterial leaching patterns by *Thiobacillus ferrooxidans* on synthetic pyrite. Arch. Micobiol. **149:** 401- 405

Rojas, J., M. Giersig, and H. Tributsch. 1995. Sulphur colloids as temporary energy reservoirs for *Thiobacillus ferrooxidans* during pyrite oxidation. Arch. Microbiol. **163:** 352-356.

Rupela, O. P., and P. Tanro. 1973. Isolation and characterization of *Thiobacillus* from alkali soils. Soil Biol. Biochem. **5:** 891-897.

Stellenbosch University http://scholar.sun.ac.za/

Sand, W., T. Gerke, R. Hallmann, and A. Schippers. 1995. Sulfur chemistry, biofilm, and the (in)direct attack mechanism - a critical evaluation of bacterial leaching. Appl. Microbiol. Biotechnol. **43:** 961-966.

Sand, W., K. Rohde, B. Sobotke, and C Zenneck. 1992. Evaluation of *Leptospirillum* for leaching. Appl. Environ. Microbiol. **58:** 85-92.

Schippers, A., P-G. Jozsa, and W. Sand. 1996. Sulfur chemistry in bacterial leaching of pyrite. Appl. Environ. Microbiol. **62:** 3424-3431.

Shrader, J. A., and D. S. Holmes. 1988. Phenotypic switching of *Thiobacillus ferrooxidans*. J. Bacteriol. **170:** 3915-3923.

Silver, M., and D. G. Lundgren. 1968. The thiosulfate-oxidizing enzyme of *Ferrobacillus ferrooxidans* (*Thiobacillus ferrooxidans*). Can J. Biochem. **46:** 1215-1220.

Silverman, M.P. 1967. Mechanism of bacterial pyrite oxidation. J. Bacteriol. **94:** 1046-1051.

Silverman, M.P., and H.L. Ehrlich. 1964. Microbial formation and degradation of minerals. Adv. Appl. Microbiol. **6:** 153-206.

Silverman, M.P., and D.G. Lundgren. 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. **77:** 642-647.

Soil Classification Working Group. 1991. Soil classification. A taxonomic system for South Africa. Department of Agricultural Development, Pretoria.

Steudel, R., G. Holdt, T. Göbel, and W. Hazeu. 1987. Chromatographic separation of higher polythionates $S_nO_6^{2-}$ (n=3...22) in cultures of *Thiobacillus ferrooxidans*; molecular composition of bacterial sulfur secretions. Angew. Chem. Int. Ed. Engl. **26:** 151-153.

Stellenbosch University http://scholar.sun.ac.za/

Sugio, T., C. Domatsu, O. Munakata, T. Tano, and K. Imai. 1985. Role of a ferric iron-reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **49:** 1401-1406.

Sugio, T., T. Hirose, A. Oto, K. Inagaki, and T. Tano. 1990. The regulation of sulfur use by ferrous iron in *Thiobacillus ferrooxidans*. Agric. Biol. Chem. **54:** 2017-2022.

Sugio, T., T. Katagiri, M. Moriyama, Y. L. Zhen, K. Inagaki, and T. Tano. 1988. Existence of a new type of sulfite oxidase which utilizes ferric ions as an electron acceptor in *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **54:** 153-157.

Sugio, T., W. Mizunashi, K. Inagaki, and T. Tano. 1987. Purification and some properties of sulfur:ferric iron oxidoreductase from *Thiobacillus ferrooxidans*. J. Bacteriol. **169:** 4916-4922.

Suzuki, I., T.L. Takeuchi, T.D. Yuthasastrakosol, and J.K. Oh. 1990. Ferrous iron and sulfur oxidation and ferric iron reduction activities of *Thiobacillus ferrooxidans* are affected by growth on ferrous iron, sulfur or a sulfide ore. Appl. Environ. Microbiol. **56:** 1620-1626.

Taylor, B. E., M. C. Wheeler, and D. K. Nordstrom. 1984a. Oxygen and sulphur composition of sulphate in acid mine drainage: Evidence for oxidation mechanism. Nature **308:** 538-541.

Taylor, B.E., M.C. Wheeler, and D.K. Nordstrom. 1984b. Stable isotope geochemistry of acid mine drainage: Experimental oxidation of pyrite. Geochim. Cosmochim. Acta **48:** 2669-2678.

Temple, K. L., and A. R. Colmer. 1951. The autotrophic oxidation of iron by a new bacterium, *Thiobacillus ferrooxidans*. J. Bacteriol. **62:** 605-611.

Tuovinen, O. H., and D. P. Kelly. 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. Microbiol. **88:** 285-298.

Tuovinen, O. H., S. I. Niemela, and H. G. Gylenberg. 1971. Tolerance of *Thiobacillus ferrooxidans* to some metals. Antonie van Leeuwenhoek **37:** 489-496.

Stellenbosch University http://scholar.sun.ac.za/

Tuttle, J.H., and P.R. Dugan. 1976. Inhibition of growth, iron, and sulfur oxidation in *Thiobacillus ferrooxidans* by simple organic compounds. Can. J. Microbiol. **22:** 719-730.

Tuttle, J.H., C.I. Randles, and P.R. Dugan. 1968. Activity of microorganisms in acid mine waters. I. Influence of acid water on aerobic heterotrophs of a normal stream. J. Bacteriol. **95:** 1495-1503.

Visca, P., E. Bianchi, M. Polidoro, V. Buonfiglio, P. Valenti, and N. Orsi. 1989. A new solid medium for isolating and enumerating *Thiobacillus ferrooxidans*. J. Gen. Appl. Microbiol. **35:** 71-81.

Vogel, A. I. 1951. A textbook of quantitative inorganic analysis, 2nd ed. Longmans, Green and Co., London.

Wakao, N., K. Hanada, A. Takahashi, Y. Sakurai, and H. Shiota. 1991. Morphological, physiological, and chemotaxonomical characteristics of iron- and sulfur-oxidizing bacteria isolated from acid mine drainage waters. J. Gen. Appl. Microbiol. **37:** 35-48.

Waksman, S.A., and J.S. Joffe. 1922. Microorganisms concerned in the oxidation of sulfur in the soil. II. *Thiobacillus thiooxidans* a new sulfur-oxidizing bacteria isolated from the soil. J. Bacteriol. **7:** 239-256.

Walsh, F. 1978. Biological control of mine drainage, p. 377-389. *In* R. Mitchell (ed.), Water pollution microbiology, vol. 2. John Wiley and Sons, New York.

Walsh, F., and R. Mitchell. 1972. A pH-dependent succession of iron bacteria. Environ. Sci. Technol. **9:** 809-812.

Walsh, F., and R. Mitchell. 1973. Differentiation between *Gallionella* and *Metallogenium*. Arch. Microbiol. **90:** 19-25.

Wates, Meiring and Barnard. 1993. Calibration of models for the design of soil covers for opencast mine and waste dumps in RSA. Experimental programme and procedure. Report No. 2564/761/2/G for the Water Research Commission. Wates, Meiring and Barnard, Marshalltown.

Stellenbosch University http://scholar.sun.ac.za/

Wates, Meiring and Barnard. 1995a. Kilbarchan Dump Rehabilitation Experiment Progress Report No. 2534/1081/1/G for the Department of Water Affairs and Forestry. Wates, Meiring and Barnard, Halfway House.

Wates, Meiring and Barnard. 1995b. Calibration of models for the design of soil covers for opencast mine and waste dumps in South Africa. Progress Report No. 2564/1122/4/G for the Water Research Commission. Wates, Meiring and Barnard, Halfway House.

Wichlacz, P.L., and R.F. Unz. 1981. Acidophilic, heterotrophic bacteria of acidic mine waters. Appl. Environ. Microbiol. **41:** 1254-1261.

Wichlacz, P. L., R. F. Unz, and T. A. Langworthy. 1986. *Acidiphilium angustum* sp. nov., *Acidiphilium facilis* sp. nov., and *Acidiphilium rubrum* sp.nov.: Acidophilic heterotrophic bacteria isolated from acidic coal mine drainage. Int J. Syst. Bacteriol. **36:** 197-201.

Zobell, C. E. 1943.The effect of solid surfaces upon bacterial activity. J. Bacteriol. **46:** 39-59.

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APPENDIX

DETAILS OF pH, MOISTURE, MPN DETERMINATIONS AND PLATE COUNTS OF MICROBIAL POPULATIONS OF COAL WASTE SAMPLES FROM THE 10 EXPERIMENTAL PILOT SCALE DUMPS CONSTRUCTED NEAR THE KILBARCHAN MINE

Appendix Table	Sampling date	Microbial population investigated besides acidophilic high ferrous iron-oxidizing microorganisms (26°C)[a]
1	27/2/95	Highly acidophilic high ferrous iron-oxidizing (37°C)
2	10/4/95	Highly acidophilic high ferrous iron-oxidizing (37°C)
3	24/9/95	Highly acidophilic high ferrous iron-oxidizing (37°C)
4	20/11/95	———————
5	21/1/1996	Highly acidophilic high ferrous iron-oxidizing (37°C)
6	18/3/1996	Acidophilic bacteria by plate count (26°C)
7	6/5/1996	Acidophilic moderate ferrous iron-oxidizing (26°C)
7a	6/5/1996	Acidophilic moderate ferrous iron- and sulphur-oxidizing (26°C)
8	3/6/1996	Acidophilic moderate ferrous iron-oxidizing (26°C)
8a	3/6/1996	Acidophilic moderate ferrous iron- and sulphur-oxidizing. as well as acidophilic bacteria by plate count (26°C)
9	2/9/1996	Acidophilic moderate ferrous iron-oxidizing (26°C)
9a	2/9/1996	Acidophilic moderate ferrous iron-and sulphur-oxidizing; also thiosulphate oxidizing strains of this group (26°C)
10	21/10/1996-7/8/1997	pH and sampling positions of samples taken between 21/10/1996 and 7/8/1997

[a] Microbial populations able to oxidize ferrous iron in HJJ medium at 26 °C were counted by MPN in all samples until 2 September 1996. Counts of these organisms in the 27/2/95 and 10/4/95 samples have been recorded by Cleghorn (1997).

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Appendix Table 1. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing microbial populations able to grow in L medium at 37°C (including category and 95% confidence limits), of coal waste samples obtained on 27 February 1995 from the 10 experimental mini-dumps constructed near the Kilbarchan mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in L-medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/W5	2.53	9.65	13158	3	3290-39474
K1/N7	2.68	9.56	16434	1	3287-41633
K2/N9	3.18	9.27	82	1	19-217
K2/E6	3.68	9.23	1016	1	197-3932
K3/N10	4.24	8.72	360	1	90-1080
K3/E1	2.87	10.63	262[e]	-	96-1041
K4/N2	2.96	7.64	3767	0	969-10118
K4/N8	4.71	9.13	38	2	10-103
K5/N1	5.16	9.28	47	1	10-198
K5/W7	3.04	8.95	50117	1	9806-205720
K6/N6	5.47	12.42	3	1	1-11
K6/W2	5.26	14.14	5	1	1-21
K7/S8	5.12	8.95	47	1	10-197
K7/W3	4.94	8.74	25	1	5-102
K8/S1	5.39	11.67	402	0	101-1050
K8/E7	5.53	11.53	26	1	6-105
K9/S2	5.67	7.06	2462	1	535-10064
K9/W4	5.88	12.68	16902	1	3380-42818
K10/S6	4.79	10.34	>110000	-	-
K10/N5	4.22	12.01	1042	1	202-4032

[a] N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

[b] pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

[c] Mean of duplicate determinations.

[d] MPN, category and 95% confidence limits according to De Man (1983).

[e] MPN according to American Public Health Association *et al.* (1955); no category; 95 % confidence limits according to Dr. J. H. Randall (Biometrician, Faculty of Agricultural Sciences, University of Stellenbosch, personal communication).

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Appendix Table 2. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing microbial populations able to grow in L medium at 37°C (including category and 95% confidence limits), of coal waste samples obtained on 10 April 1995 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/E6	3.25	13.94	855	1	194-2267
K1/N9	2.76	11.27	5118	1	1001-22031
K2/E1	2.86	11.55	51313	1	10040-220869
K2/W5	2.83	10.26	25	1	6-104
K3/E4	3.08	12.04	4258	1	1008-11652
K3/E10	2.67	11.53	266	1	56-1048
K4/E5	2.74	9.43	25	1	5-103
K4/N7	3.52	8.53	25	1	5-102
K5/N6	6.01	8.71	10	1	2-39
K5/N3	2.92	8.22	24891	1	5411-101727
K6/S8	6.05	9.29	0.3	1	0.1-1
K6/N5	6.23	9.26	0.4	1	0.1-2
K7/E2	6.16	8.57	2	0	0.3-4
K7/W2	5.66	7.54	2	1	1-10
K8/E3	6.03	7.92	16	1	3-41
K8/N10	6.14	8.24	2	1	1-10
K9/S2	4.46	7.00	1498	2	428-3745
K9/W2	6.03	9.81	1	2	0.1-2
K10/N1	6.45	7.87	100	1	19-388
K10/S9	6.57	16.79	1752	1	350-4438

a N=North side, 1-10 east to west (with 1 m interval)
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

b pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

c Mean of duplicate determinations.

d MPN, category and 95% confidence limits according to De Man (1983).

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Appendix Table 3. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing microbial populations able to grow in HJJ medium at 26°C or L medium at 37 °C (including category and 95% confidence limits), of coal waste samples obtained on 24 September 1995 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ medium			Organisms growing in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]	MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/W3	2.67	8.36	21673	0	5418-41178	47	0	10-196
K1/N2	2.51	6.28	478260	1	95652-1923668	46	1	10-192
K2/S10	2.60	6.48	308799	3	95834-1054177	99	1	19-383
K2/W9	2.70	6.50	1172	3	426-3728	46	3	10-193
K3/N8	2.70	6.71	2454	1	534-10030	10	1	2-38
K3/W6	2.58	7.45	24712	1	5372-100999	31	3	10-101
K4/W4	3.64	7.17	99666	1	19290-385804	15	2	4-38
K4/N5	2.87	7.56	10003	1	1936-38720	31	3	10-101
K5/E7	3.42	9.18	101536	1	19652-393 041	4	1	1-11
K5/E10	2.75	8.57	467	1	98-1965	1629	1	326-4 126
K6/N1	3.22	9.37	4702716	1	984289-19795155	25	1	5-103
K6/W1	6.90	9.46	3	1	1-10	10	1	2-39
K7/S2	5.06	7.73	463231	1	96955-1949881	3	1	1-10
K7/N7	4.29	8.18	21636	2	5409-40174	<0.3	-	-
K8/S1	6.10	9.84	1648	1	330-4174	22	2	5-42
K8/W2	6.27	10.51	254166	1	55253-1005613	<0.3	-	-
K9/E1	3.48	9.19	163785	1	32757-414921	4 695	1	983-19 763
K9/S8	4.05	8.81	468	1	98-1969	41	1	10-113
K10/E4	4.27	8.11	45406[e]	-	9730-112434	3	1	1 – 10
K10/S4	4.89	8.77	13052	3	3 263-39 157	5	1	1 – 20

[a] N=North side, 1-10 east to west (with 1 m interval)
S=South side, 1-10 east to west (with 1 m interval).
E=East side, 1-10 north to south (with 1 m interval).
W=West side, 1-10 north to south (with 1 m interval).

[b] pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

[c] Mean of duplicate determinations.

[d] MPN, category and 95% confidence limits according to De Man (1983).

[e] MPN according to American Public Health Association *et al.* (1955); no category; 95 % confidence limits according to Dr. J. H. Randall (Biometrician, Faculty of Agricultural Sciences, University of Stellenbosch, personal communication).

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Appendix Table 4. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing microbial populations able to grow in HJJ medium at 26°C (including category and 95% confidence limits), of coal waste samples obtained on 20 November 1995 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/W6	3.16	17.28	27	1	6-110
K1/W2	2.99	15.31	496	1	196-2295
K2/E4	2.99	14.96	107	1	21-414
K2/WS	3.10	18.68	110	1	21-427
K3/E9	4.18	15.27	127	2	46-403
K3/W4	4.81	17.02	1755	1	468-4447
K4/W4	3.30	15.43	3347	3	1039-11428
K4/E6	2.97	10.04	473	1	99-1992
K5/N3	4.06	12.88	429	1	102-1174
K5/S3	3.34	7.86	25	1	5-101
K6/W8	6.33	9.91	25	1	6-103
K6/S10	6.35	9.81	82	1	19-219
K7/NIO	3.57	8.63	261	1	54-1021
K7/E7	5.75	9.14	47	1	10-198
K8/N7	5.20	11.89	168	1	34-425
K8/S7	6.26	9.07	47	1	10-197
K9/E3	4.54	12.04	104	1	20-403
K9/N1	6.28	14.50	26	1	6-108
K10/N6	5.22	9.56	47	1	10-198
K10/W9	6.41	14.71	3	1	1-11

a N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval),
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

b pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

c Mean of duplicate determinations.

d MPN, category and 95% confidence limits according to De Man (1983).

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Appendix Table 5. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing microbial populations able to grow in HJJ medium at 26 °C or L medium at 37 °C (including category and 95% confidence limits), of coal waste samples obtained on 21 January 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ medium			Organisms growing in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]	MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/N2	2.94	13.67	49	1	10-206	307	0	102-1069
K1/S9	2.95	12.68	39	0	10-106	1352	3	338-4056
K2/E4	3.06	12.63	17	1	5-43	5	1	1-20
K2/W9	2.89	13.57	17	1	5-43	1056	1	204-4088
K3/W5	4.43	13.18	170	3	57-430	32	3	10-106
K3/S2	4.28	13.65	182	3	57-432	318	3	102-1068
K4/N5	2.53	12.79	169	1	34-429	846	1	192-2245
K4/N6	2.50	11.14	1034	1	200-4 001	1667	1	333-4223
K5/E3	3.14	14.20	106	1	21-411	1062 [e]	-	407-4269
K5/S7	2.83	15.35	107	1	21-415	70	0	14-196
K6/N7	6.87	16.85	27	1	6-110	5	1	1-21
K6/S3	6.23	15.45	87	1	20-230	27	1	6-109
K7/S10	3.07	15.45	231	2	58-439	173	1	21-416
K7/W3	6.11	15.41	323	3	104-1085	23[e]	-	5-44
K8/W4	4.22	13.79	330	3	102-1 127	106	1	20-410
K8/E1	5.72	16.05	32	3	10-110	108	1	21-418
K9/N9	6.76	16.39	27	1	6-110	27	1	6-109
K9/S4	5.74	14.06	262	1	57-1072	106	1	21-411
K10/N4	6.54	17.31	109	1	21-422	11	1	2-32
K10/W6	4.34	15.16	86	1	20-230	173	1	35-438

[a] N=North side, 1-10 east to west (with 1 m interval)
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

[b] pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

[c] Mean of duplicate determinations.

[d] MPN, category and 95% confidence limits according to De Man (1983).

[e] MPN according to American Public Health Association *et al.* (1955); no category; 95 % confidence limits according to Dr. J. H. Randall (Biometrician, Faculty of Agricultural Sciences, University of Stellenbosch, personal communication).

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Appendix Table 6. Details of pH, moisture, MPN determinations of acidophilic high ferrous iron-oxidizing microbial populations able to grow in HJJ medium at 26°C (including category and 95% confidence limits) and plate counts (with 95% confidence limits) of acidophilic bacterial populations, of coal waste samples obtained on 18 March 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ-medium			Acidophilic bacteria by plate count on FeSo plates	
			MPN/g[d]	Category[d]	95% Confidence limits[d]	Plate count[e]	95% Confidence Limits[f]
K1/W4	3.10	12.60	105	1	20-405	236460	178069-294851
K1/E10	3.18	10.88	1663	1	333-4213	ND	-
K2/S2	3.02	11.15	2557	1	556-10448	311220	244665-377775
K2/W6	2.99	11.48	1037	1	200-5013	1036764	915108-11584201
K3/N1	3.09	13.89	239	2	34-456	102501	55148-149854
K3/S7	3.17	13.41	2608	1	567-10661	ND	-
K4/N6	5.74	15.06	26	1	6-108	9205	5522-12887
K4/W10	2.84	13.00	8475	1	1921-22448	644100	547559-740641
K5/N2	4.09	15.72	4976	1	1042-20946	266156	203355-328957
K5/E7	4.96	17.61	1764	1	353-4469	70566	20426-110492
K6/W7	6.04	16.52	50	1	10-211	ND	-
K6/W8	6.19	14.80	26	1	6-108	ND	-
K7/N7	5.65	12.42	4834	1	1012-20348	314776	247460-382092
K7/S3	5.52	16.56	1748	1	350-4429	ND	-
K8/E5	5.99	15.86	50	1	10-210	6565	3444-9686
K8/W2	4.89	14.81	17221	1	3444-43627	68886	58822-78950
K9/N4	6.14	12.02	10	1	2-39	16243	10331-22155
K9/S9	6.25	19.50	111	1	22-430	ND	-
K10/S10	6.39	26.91	55	1	11-230	38073	13199-62947
K10/W3	6.29	20.74	519	1	109-2185	54333	18835-89831

a N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval).
W=West side, 1-10 north to south (with 1 m interval).

b pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

c Mean of duplicate determinations.

d MPN, category and 95% confidence limits according to De Man (1983).

e ND = not determined.

f Confidence limits (95%) from the theoretical standard error for the total count over all replicate plates, assuming a Poisson distribution, according to Meynell and Meynell (1970).

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Appendix Table 7. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing and moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in, respectively, HJJ and JLFe-medium (including category and 95% confidence limits), of coal waste samples obtained on 6 May 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ-medium			Organisms growing in JLFe-medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]	MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/S7	2.98	13.57	1056	1	204-4088	2612	1	568-10675
K1/N7	2.79	15.20	50	1	20-229	265	1	58-1083
K2/S3	3.15	16.85	5025	1	1 986-23253	18	3	6-44
K2/N6	3.08	1625	50	1	20-231	337	3	105-1151
K3/E5	3.58	13.45	2382	2	340-4538	2609	1	567-10664
K3/N10	3.43	10.01	319	3	99-1089	4730	1	990-19911
K4/W3	3.51	8.81	1632	1	326-4135	2503	1	544-10228
K4/N2	3.10	8.93	1634	1	327-4139	10130	1	1961-39215
K5/W6	3.45	16.05	2437	2	348-4642	406	0	104-1091
K5/W2	3.42	10.19	231	2	33-440	23139	2	3 306-44074
K6/W4	6.29	13 .12	3	1	0.6-11	<0.3	-	-
K6/N5	6.42	11.59	167	1	10-202	480	1	100-2020
K7/S10	3.29	14.53	2291	2	573-4352	106512	1	20615-412306
K7/E6	6.10	11.11	103	1	20-400	16	2	4-39
K8/E7	6.19	11.81	26	1	6-105	168	1	34-425
K8/N8	5.42	10.91	2551	1	555-10426	1664	3	550-4210
K9/E4	6.09	12.98	486	1	102-2045	226	2	56-429
K9/S8	3.17	12.40	1686	1	337-4271	483	1	101-2034
K10/E2	5.26	11.80	26	1	6-105	157	2	45-391
K10/E7	3.80	12.81	43	1	10-117	49	1	10-204

[a] N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval).
W=West side, 1-10 north to south (with 1 m interval).

[b] pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

[c] Mean of duplicate determinations.

[d] MPN, category and 95% confidence limits according to De Man (1983).

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Appendix Table 7a. Details of pH, moisture and MPN determinations of acidophilic moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in JLFe-medium (see Appendix Table 7) and subsequently in S^0 medium (including category and 95% confidence limits), of coal waste samples obtained on 6 May 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in S^0-medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]
K1/S7	2.98	13.57	34	2	10-114
K1/N7	2.79	15.20	<0.3	-	-
K2/S3	3.15	16.85	7	2	2-19
K2/N6	3.08	1625	<0.3	-	-
K3/E5	3.58	13.45	4	1	0.2-19
K3/N10	3.43	10.01	<0.3	-	-
K4/W3	3.51	8.81	<0.3	-	-
K4/N2	3.10	8.93	12	1	4-38
K5/W6	3.45	16.05	50	1	10-210
K5/W2	3.42	10.19	4	1	0.2-19
K6/W4	6.29	13 .12	<0.3	-	-
K6/N5	6.42	11.59	<0.3	-	-
K7/S10	3.29	14.53	<0.3	-	-
K7/E6	6.10	11.11	10	1	2-39
K8/E7	6.19	11.81	<0.3	-	-
K8/N8	5.42	10.91	10	1	2-39
K9/E4	6.09	12.98	4	1	0.2-19
K9/S8	3.17	12.40	33	3	10-106
K10/E2	5.26	11.80	4	1	0.2-19
K10/E7	3.80	12.81	17	1	5-43

a N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

b pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

c Mean of duplicate determinations.

d MPN, category and 95% confidence limits according to De Man (1983).

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Appendix Table 8. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing and moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in, respectively, HJJ and JLFe medium (including category and 95% confidence limits), of coal waste samples obtained on 3 June 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ medium			Organisms growing in JLFe medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]	MPN/g[d]	Category[d]	95% Confidence Limits[d]
KI/N2	3.06	8.56	10096	1	1954-39082	2497	1	543 –10205
KI/W6	3.10	8.88	250	1	98-1132	21776	2	5444 –41374
K2/W2	3.38	7.85	377	0	97-1014	22649	2	3236 – 43142
K2/W4	3.04	8.35	2275	1	542-4334	47675[e]	-	18412 – 190700
K3/W9	3.56	8.80	316	3	98-1077	16320	1	3264 –41345
K3/S8	3.66	9.76	25244	1	5488-103170	47195	1	9 878-198 657
K4/N1	3.22	9.68	10200	1	1974-39484	102000	1	19742 – 394837
K4/N5	3.75	9.80	395	0	98-1032	10212	0	1976 – 39484
K5/N4	5.76	10.15	47	1	10-199	47	1	10 –199
K5/E10	3.45	9.07	82	1	19-217	17452	0	3272 – 41447
K6/S10	6.15	12.49	10	I	2-39	105	1	20 – 405
K6/E9	6.37	10.23	2535	1	551-10361	474	1	99 –1995
K7/W1	4.34	9.65	164	1	33-417	4715	1	987 –19847
K7/E9	4.67	10.33	103	1	20-397	474	1	99 –1997
K8/N3	6.04	10.65	103	1	20-398	476	1	100 – 2003
K8/E4	5.33	10.39	10267	1	1987-39741	15455	2	4416 – 38637
K9/W8	6.36	12.74	485	1	101-2041	846	1	192 – 2244
K9/E3	3.19	11.85	3132	3	1007-10514	10402	1	2013-40265
K10/S9	6.45	20.31	4	1	0.2-20	18	1	5 – 46
K10/S1	3.12	10.13	2974	0	991-10352	16520	0	3304 –41850

[a] N=North side, 1-10 east to west (with 1 m interval)
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

[b] pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

[c] Mean of duplicate determinations.

[d] MPN, category and 95% confidence limits according to De Man (1983).

[e] MPN according to American Public Health Association *et al.* (1955); no category; 95 % confidence limits according to Dr. J. H. Randall (Biometrician, Faculty of Agricultural Sciences,University of Stellenbosch, personal communication).

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Appendix Table 8a. Details of pH, moisture and MPN determinations of acidophilic moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in JLFe-medium (see Appendix Table 8) and subsequently in S^0 medium (including category and 95% confidence limits) and plate counts (with 95% confidence limits) of acidophilic bacterial populations, of coal waste samples obtained on 3 June 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in S^0-medium			Acidophilic bacteria by plate count on FeSo plates	
			MPN/g[d]	Category[d]	95% Confidence limits[d]	Plate count[e]	95% Confidence Limits[f]
KI/N2	3.06	8.56	<0.3	-	-	ND	-
KI/w6	3.10	8.88	806	3	131-8511	166949	118703-215195
K2/W2	3.38	7.85	669	3	129- 8342	352310	282556-422064
K2/W4	3.04	8.35	8	2	1-80	657323	355329-959318
K3/W9	3.56	8.80	3	3	0.1-10	232107	52280-411933
K3/S8	3.66	9.76	670	3	132-866	274400	22585-514922
K4/N1	3.22	9.68	22	0	5-42	5209800	2774768-6257448
K4/N5	3.75	9.80	33	3	10-104	874740	524040-1225440
K5/N4	5.76	10.15	<0.3	-	-	ND	-
K5/E10	3.45	9.07	<0.3	-	-	ND	-
K6/S10	6.15	12.49	48	1	10-204	ND	-
K6/E9	6.37	10.23	<0.3	-	-	ND	-
K7/W1	4.34	9.65	22	0	5-42	219300	4386-434214
K7/E9	4.67	10.33	<0.3	-	-	ND	-
K8/N3	6.04	10.65	<0.3	-	-	73767	41437-106096
K8/E4	5.33	10.39	<0.3	-	-	ND	-
K9/W8	6.36	12.74	4	1	0.2-19	ND	-
K9/E3	3.19	11.85	10	1	2-39	ND	-
K10/S9	6.45	20.31	<0.3	-	-	ND	-
K10/S1	3.12	10.13	10	1	2-39	ND	-

a N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval).
E=East side, 1-10 north to south (with 1 m interval).
W=West side, 1-10 north to south (with 1 m interval).

b pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

c Mean of duplicate determinations.

d MPN, category and 95% confidence limits according to De Man (1983).

e ND = not determined.

f Confidence limits (95%) from the theoretical standard error for the total count over all replicate plates, assuming a Poisson distribution, according to Meynell and Meynell (1970).

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Appendix Table 9. Details of pH, moisture and MPN determinations of acidophilic high ferrous iron-oxidizing and moderate ferrous iron-oxidizing microbial populations. able to grow at 26°C in, respectively, HJJ and JLFe medium (including category and 95% confidence limits), of coal waste samples obtained on 2 September 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Organisms growing in HJJ medium			Organisms growing in JLFe medium		
			MPN/g[d]	Category[d]	95% Confidence limits[d]	MPN/g[d]	Category[d]	95% Confidence limits[d]
KI/W3	2.96	8.23	47	1	10-196	11905	1	2165-43290
KI/S7	2.98	7.79	259	1	43-1067	49584	1	9701-213425
K2/E10	3.05	8.92	47	1	10-197	50104	1	9803-215667
K2/S3	3.13	8.71	47	1	10-197	119582	1	21742-434470
K3/S2	5.37	6.71	224	2	32-427	213416	2	53354-405491
K3/W10	3.48	9.59	131	3	33-395	78908	2	13151-186 309
K4/E4	3.45	9.57	164	1	33-416	50404	1	9862-216957
K4/N9	2.89	10.77	266	1	44-1097	166161	1	33232-420941
K5/W9	3.61	10.01	165	1	33-418	121011	1	22002-440038
K5/S1	3.31	10.41	25	1	6-104	121448	1	22081-441629
K6/N5	5.93	10.67	509	1	100-2191	1660	1	332-4 205
K6/N1	6.61	11.12	26	1	6-104	511	1	100-2 200
K7/N2	5.50	10.31	25	1	6-104	102	1	20-397
K7/W5	4.31	9.25	25	1	5-103	251275	1	54625-1026950
K8/N4	5.88	9.94	168	1	33-418	2529	1	550-10334
K8/N7	6.05	9.03	25	1	5-102	5 015	1	981-21588
K9/E7	4.42	9.78	263	1	44-1 087	25248	1	54894-103189
K9/W4	5.98	11.82	168	1	34-425	2572	1	559-10511
KIO/E6	4.83	9.42	263	1	44-1083	252	1	55-1029
K10/S8	5.93	15.16	26	1	6-108	2649	1	576-10825

a N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval).
W=West side, 1-10 north to south (with 1 m interval).

b pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

c Mean of duplicate determinations.

d MPN, category and 95% confidence limits according to De Man (1983).

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Appendix Table 9a. Details of pH, moisture and MPN determinations of acidophilic moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in JLFe medium (see Appendix Table 9), and subsequently in S^0 medium and then Starkey's medium (including category and 95% confidence limits), of coal waste samples obtained on 2 September 1996 from the 10 experimental mini-dumps constructed near the Kilbarchan Mine

		Moisture in sample	Organisms growing in S^0 medium			Organisms growing in Starkey's medium		
Mini-dump/ sample[a]	pH[b]	(g/100 g dry mass)[c]	MPN/g[d]	Category[d]	95% Confidence limits[d]	MPN/g[d]	Category[d]	95% Confidence limits[d]
KI/W3	2.96	8.23	17	3	5-41	10	1	2-38
KI/S7	2.98	7.79	4958	1	970-21340	4	1	0.2-18
K2/E10	3.05	8.92	32	3	10-102	25	1	5-102
K2/S3	3.13	8.71	1196	1	217-4348	8	1	1-22
K3/S2	5.37	6.71	800	1	181-2123	117	3	43-373
K3/W10	3.48	9.59	2301	1	548-4384	25	1	5-103
K4/E4	3.45	9.57	1205	1	219-4383	47	1	10-198
K4/N9	2.89	10.77	2326	1	554-4431	510	1	100-2193
K5/W9	3.61	10.01	15	2	4-39	15	2	4-39
K5/S1	3.31	10.41	8	2	1-19	4	1	0.2-19
K6/N5	5.93	10.67	830	1	122-2202	7	0	1-19
K6/N1	6.61	11.12	48	1	10-201	10	1	2-39
K7/N2	5.50	10.31	103	1	20-397	17	1	4-42
K7/W5	4.31	9.25	120170	1	21849-436980	<0.3	-	-
K8/N4	5.88	9.94	506	1	99-2177	8	1	1-20
K8/N7	6.05	9.03	1635	1	327-4143	<0.3	-	-
K9/E7	4.42	9.78	23	1	5-44	<0.3	-	-
K9/W4	5.98	11.82	1230	1	224- 4473	4	1	0.2-19
KIO/E6	4.83	9.42	25	1	5-103	<0.3	-	-
K10/S8	5.93	15.16	1267	1	230-4606	<0.3	-	-

[a] N=North side, 1-10 east to west (with 1 m interval).
S=South side, 1-10 east to west (with 1 m interval)
E=East side, 1-10 north to south (with 1 m interval)
W=West side, 1-10 north to south (with 1 m interval).

[b] pH of suspension of 10 g sample in 25 ml distilled water; mean of duplicate determinations.

[c] Mean of duplicate determinations.

[d] MPN, category and 95% confidence limits according to De Man (1983).

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Appendix Table 10. Details of pH of samples taken from the 10 experimental mini-dumps constructed near the Kilbarchan mine. during the period 21 October 1996 to 7 August 1997

Date	21/10/96[a]	09/12/96		02/02/97		24/03/97		12/05/97		07/07/97	
Cell and sample	pH	Position[b]	pH	Position[b]	pH	Position[b]	pH	Position[b]	pH	Position[b]	pH
1 (1)	3.07	E7	3.12	E2	2.99	E4	2.82	E2	3.00	W5	3.23
1 (2)	3.03	E8	3.14	S2	3.06	S4	2.97	W4	2.91	E5	3.21
2 (1)	3.12	N3	3.06	N7	2.99	W8	2.95	S6	2.88	S7	3.15
2 (2)	3.08	W8	3.16	S7	2.90	N3	2.94	N7	2.91	N3	3.19
3 (1)	3.62	W6	2.98	S9	3.98	E8	5.35	S9	3.54	W4	4.32
3 (2)	4.32	W4	3.16	S5	3.90	S6	5.11	S7	6.01	S3	4.41
4 (1)	2.85	S3	3.51	S4	2.84	N2	3.50	S8	2.92	E9	2.99
4 (2)	3.14	S6	3.69	W3	2.76	E7	2.88	W3	3.34	E6	2.76
5 (1)	3.36	W2	3.55	E5	3.26	N5	2.90	N2	3.02	E3	3.23
5 (2)	3.04	S9	3.37	E9	3.00	W3	3.25	S8	2.89	N6	2.98
6 (1)	5.90	E3	5.75	E7	5.75	W7	6.28	E8	6.57	W2	6.65
6 (2)	6.13	S7	6.25	N2	5.86	W2	6.60	N3	6.67	N5	6.79
7 (1)	3.92	S5	3.96	S6	4.40	W9	3.16	W4	3.87	N4	3.65
7 (2)	6.03	E2	5.54	S8	3.93	E5	2.73	S3	3.27	N2	3.56
8 (1)	6.03	N2	6.03	E8	5.60	E9	6.02	E9	6.47	W6	6.29
8 (2)	5.70	N7	5.07	N3	5.73	S3	6.18	E6	6.38	S5	6.21
9 (1)	3.44	E9	3.02	W8	5.74	W6	6.26	W5	6.67	W7	6.66
9 (2)	4.20	S8	5.45	W6	5.81	W4	6.33	E5	6.57	N8	6.73
10 (1)	4.49	N2	4.11	W4	4.43	N8	4.05	N9	5.26	W3	4.05
10 (2)	3.40	N8	5.80	S3	4.85	S2	5.31	W7	6.17	W9	3.53

[a] Position not availiable.

[b] N=North side, 1-10 east to west (with 1 m interval)
S=South side, 1-10 east to west (with 1 m interval).
E=East side, 1-10 north to south (with 1 m interval).
W=West side, 1-10 north to south (with 1 m interval).