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

by

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Report to the Water Research Commission on the Project:

"Investigation of the occurrence of bacteria causing acid drainage in the outer layers of coal waste dumps"

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EXECUTIVE SUMMARY

Project Motivation

Drainage from coal mines is typically high in acidity (as sulphuric acid), iron and sulphates (Dugan, 1975; Kleinmann *et al.*, 1981). Sulphuric acid and ferrous 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; Singer and Stumm, 1970). *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 microorganism 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; Director General: Water Affairs and Forestry, 1992-93; 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 reclamation 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), Norris (1990, 1997) and Rawlings (1997) have reviewed evidence that acid formation might be caused not only by *T. ferrooxidans*, but also by several other bacterial species found in mine dumps undergoing acidification. Although *T. ferrooxidans* was confirmed as the most 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. Walsh and Mitchell (1972) postulated a possibly important role for bacteria

of the genus *Metallogenium*, namely, initiating bacterial iron oxidation and acid formation at pH levels too high for the growth of *T. ferrooxidans*. Furthermore, the strictly lithotrophic iron-oxidizing bacteria of the species *T. ferrooxidans* live in close association (consortia) with heterotrophic bacteria in their environment (Johnson and Roberto, 1997). Many of these heterotrophic bacteria have been placed in the genus *Acidiphilium* (Harrison, 1981; 1984, 1989; Johnson *et al.*, 1992; 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). Various other mesophilic, moderately and extremely thermophilic, iron-oxidizing and non-iron-oxidizing bacteria have been isolated from acid mine drainage and similar environments during the past two decades, greatly expanding our knowledge of the potential of these environments for the dissolution of pyrite and other metal sulphides with the generation of acidity (Johnson and Roberto, 1997; Norris, 1997; Rawlings, 1997). 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 creation of anaerobic conditions in the dumps by the covers, together with the inhibition of acid-producing microorganisms and whether similar inhibition could be achieved by a thinner, cheaper cover, was lacking. Also the hydrology of dumps under covers of different types under the conditions of northern KwaZulu-Natal had 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 report, 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.

Project Objectives

The project objectives were:

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

(2) From the results of (1), identification of dump construction or rehabilitation techniques which most successfully limit populations of acid drainage-producing bacteria.

(3) From the results of (1), identification of problem environments in the variously constructed or rehabilitated dumps where acid drainage-producing bacteria flourish and of environments where they are unable to do so.

(4) From (2) and (3), 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.

Literature Survey

A survey of relevant literature on the following topics is presented:

(1) An outline of the chemical and microbial processes involved in acid mine drainage formation, particularly showing how they interact to achieve the rapid dissolution of pyrite and other metal sulphides.

(2) A survey of the microorganisms that may have a role in the formation of acid mine drainage, including newly discovered taxonomic groups encompassing a range of different physiological types.

(3) Discussion of physical and chemical ecological factors affecting acid mine drainage formation in coal waste, especially through their influence on the microbial groups described under (2). Identification of conditions under which acid generation may be promoted or inhibited.

(4) Consideration of an ecological approach to the control of acid drainage from coal waste dumps by using soil and/or clay liners and covers to reduce water infiltration into and out of the coal waste and oxygen infiltration into the waste.

Research Part 1. Abiotic Ecological Determinants (Temperature, Moisture, Oxygen, Carbon Dioxide and pH) and Acid-producing Bacteria in Pilot Scale Coal Waste Dumps in Relation to Covers Used for Dump Rehabilitation

Physical and chemical conditions, acid formation and populations of various groups of bacteria which might produce acid were studied in the outer layers of ten differently constructed pilot scale coal waste dumps (mini-dumps or cells) at the Kilbarchan Mine near Newcastle, KwaZulu-Natal, over a 3- or 4 year period from September 1993. Aerobic conditions, considerable 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 clay (34%) and silt (28%) content 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 covered by uncompacted Avalon soil to give a total cover thickness of 1 m, provide valuable guidelines to the type of soil covers 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. The effectiveness of the double soil covers of Estcourt and Avalon soil in limiting the diffusion of oxygen into the coal waste was, however, not paralled by reduction of the drainage outflow from the waste in the hydrological study of Wates, Meiring and Barnard (Wates and Rykaart, 1999); the greatest reduction of outflow was observed with the double layer of Avalon soil in cell 7, while the 0.5-m single layer of compacted Avalon soil of cell 5 was as effective in reducing outflow as the double soil covers. This superiority of the Avalon soil alone in reducing outflow was not expected from the literature surveyed by Wates and Rykaart (1999), but was ascribed to the high water retention prperties of the Avalon soil coupled with less susceptibility to desiccation cracking than the Estcourt soil..

The presence of a vegetation cover should prove valuable, by preventing the erosion of soil covers on dumps and reducing the movement of water and oxygen to the coal discard, but the advantages were not evaluated in the present study.

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 hand-held 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.

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 thiosulphate- and/or sulphur-oxidizing bacteria. However, the high ferrous iron-oxidizing *T. ferrooxidans* may not have been the dominant iron oxidizer, as population estimates using media with a lower ferrous iron concentration indicated that large numbers of other iron oxidizers with a lower ferrous iron tolerance were often 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.

Research: Part 2. Microorganisms of Iron-Oxidizing Consortia Involved in the Genertation of Acid Mine Drainage in Northern KwaZulu-Natal

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 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 our 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. Our study of the interactions of three fungal isolates with *T. ferrooxidans* in the presence of organic compounds suggest 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.

Recommendations Concerning Coal Waste Dump Rehabilitation Procedures

From the results presented in this report, the following recommendations can be made in connection with procedures for the rehabilitation of coal waste dumps involving the construction of clay and/or soil covers to prevent acid mine drainage formation and other undesirable occurrences, such as spontanenous combustion:

(1) The use of covers consisting of two layers, namely, an underlying less permeable clay or clayey soil layer and an upper topsoil layer, both of a suitable thickness, is recommended. In the present investigation, an underlying layer of 30 or 70 cm compacted Estcourt soil covered by 70 or 30 cm, respectively, of uncompacted Avalon soil (total cover thickness 1 m) was effective in preventing acidificaton of the underlying coal waste over a 4-year period.

(2) Use of a cover of a single soil type, exemplified by Avalon soil in this investigation, is not recommended, even though a 1-m cover of 70 cm compacted and 30 cm uncompacted Avalon soil greatly slowed acidification of the coal waste in the present study and showed the greatest reduction of water outflow. Thirty- and 50-cm-thick covers of Avalon soil had no or little delaying effect on acidification of the coal waste, although the latter was comparable with the double soil covers in limiting water outflow.

(3) Coal waste should be compacted during construction of a dump to counteract spontaneous combustion and soil or clay/soil covers should be vegetated to prevent erosion.

(4) The inhibition of acidification of the coal waste below a cover depends on the effectiveness of the cover as a barrier to oxygen diffusion into the dump. It is recommended that oxygen in the coal waste below covers be monitored routinely as in the present study, using permanently buried probes that extend through the covers into the coal waste. Using an appropriate meter, the oxygen concentration in the atmosphere of the coal waste below each probe can be measured in turn. An immediate result is obtained. Anaerobic conditions (no oxygen) indicate that the cover should be effective in blocking acidification; the presence of oxygen indicates that it is not.

(5) Acidification below covers should also be monitored from time to time by sampling coal waste below the cover by auger and measuring its pH.

(6) 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 under (4) and (5).

Recommendations for Further Research

The following further research is recommended:

(1) Continued monitoring of the oxygen and carbon dioxide concentrations in the Kilbarchan mini-dumps and correlation of the results with rainfall, to check the maintenance of anaerobic conditions beneath the effective covers and in particular the effect of future drought conditions on the entrance of oxygen through dried or cracked covers.

(2) Monitoring of the pH in the coal waste beneath the effective covers (for example, three times per year) to detect acidification if it should occur.

(3) A study of the acidophilic moderate ferrous iron-oxidizing bacteria in uncovered and effectively covered mini-dumps to ascertain whether their populations are better correlated with acidification than those of the acidophilic high ferrous iron-oxidizing bacteria which were the group monitored routinely in the present study. The moderate ferrous iron oxidizers showed greater populations than the high ferrous iron oxidizers in the present study (in a very limited investigation) but their significance is unknown. It may be greater than that of the high ferrous iron oxidizers, exemplified by *T. ferrooxidans*.

(4) Interactions of heterotrophic microorganisms, particularly fungi, with *T. ferrooxidans* and other iron-oxidizing bacteria that appear significant in acidification, with a view to identifying organisms that stimulate or inhibit iron oxidation in the presence of organic matter. The latter group, in particular, may be of value in controlling acid drainage production if the conditions under which they can achieve inhibition in the field are established.

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"Investigation of the occurrence of bacteria causing acid mine drainage in the outer layers of coal waste dumps".

The Steering Committee responsible for this project, consisted of the following persons:

Mr. H.M. du Plessis	Water Research Commission (Chairman)
Dr. M.J. Pieterse	Formerly Water Research Commission (formerly Chairman)
Dr. T. Erasmus	Water Research Commission
Mrs. M.C. Eksteen	Department of Water Affairs and Forestry
Mr. S.A.P. Brown	Formerly Department of Water Affairs and Forestry
Mr. J. Maré	Formerly Department of Water Affairs and Forestry
Prof. F.D.I. Hodgson	University of the Orange Free State
Dr. B.J.H. Janse	University of Stellenbosch
Mr. J.A. Wates	Wates, Meiring and Barnard
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Mr. J.N.J. Viljoen	Trans-Natal Coal Corporation
Mr. J.D. Wells	Digby Wells and Associates, formerly Randgold (deceased)
Mr. D. Huyser	Water Research Commission (Committee Secretary)
Mrs. A.M. du Toit	Water Research Commission (Committee Secretary)
Mr. M.E. Mosia	Water Research Commission (Committee Secretary)

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A. GENERAL INTRODUCTION

A.1. PROJECT MOTIVATION

Drainage from coal mines is typically high in acidity (as sulphuric acid), iron and sulphates (Dugan, 1975; Kleinmann *et al.,* 1981). Sulphuric acid and ferrous iron, resulting from the chemical and biological oxidation of pyrite (FeS_2), 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 (Evangelou and Zhang, 1995; Hutchins *et al.*, 1986; Lundgren and Silver, 1980; Luther, 1987; Moses *et al.*, 1987; Sand *et al.*, 1995; Singer and Stumm, 1970). *Thiobacillus ferrooxidans* and other iron- and sulphur-oxidizing bacteria growing in the aerobic outer layers of coal waste dumps play major roles in the formation of acid drainage by greatly accelerating the oxidation of ferrous to ferric iron as the environment becomes acid and metabolizing sulphur-containing degradation products of pyrite to sulphate (Dugan, 1975; Fernandez *et al.*, 1995; Kleinmann *et al.,* 1981; Mustin *et al.*, 1992; Schippers *et al.*, 1996).

Many of the older coal waste dumps in South Africa are producers of acid drainage (Director General: Water Affairs, 1987–88; Director General: Water Affairs and Forestry, 1992-93; Henzen and Pieterse, 1978; Kemp, 1962). The possibility of controlling acid drainage generation in coal waste by applying antibacterial agents to inhibit iron-oxidizing bacteria such as *T. ferrooxidans* was investigated intensively in laboratory and pilot scale experiments by Loos *et al.* (1990b). A measure of success had been achieved in the U.S.A. by the treatment of wastes with sodium lauryl sulphate, benzoic acid and sorbic acid (Erickson *et al.,* 1985; Kleinmann, 1980; Kleinmann *et al.*, 1981; Kleinmann and Erickson, 1982; Onysko *et al.,* 1984a), even in field trials. These antibacterial agents appeared less promising on the basis of our investigation, particularly because continued retreatment of the wastes would be necessary at a high cost. A technology which would result in permanent inhibition of the iron-oxidizing bacteria without continuing high costs was required.

As *T. ferrooxidans* grows as an aerobic microorganism 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. Recent development 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 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. which construction or rehabilitation procedures are inadequate to prevent bacterial development and do not block the bacterial reactions

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), Norris (1990, 1997) and Rawlings (1997) have reviewed evidence that acid formation might be caused not only by *T. ferrooxidans,* but also by several other bacterial species found in pyritic materials undergoing acidification, as well as consortia of two or more species. Although *T. ferrooxidans* was confirmed as a major and often the most important iron-oxidizing microorganism in the lower mesophilic temperature range, significant roles of the iron-oxidizing *Leptospirillum ferrooxidans* and the sulphur-oxidizing *Thiobacillus thiooxidans* were sometimes indicated (see also Hallmann *et al.*, 1992; Pronk and Johnson, 1992). Walsh and Mitchell (1972) and Walsh (1978) postulated a possible important role for bacteria of the genus *Metallogenium*, namely, initiating bacterial iron oxidation and acid formation at pH levels too high for the growth of *T. ferrooxidans*. Furthermore, the strictly lithotrophic iron-oxidizing *T. ferrooxidans* bacteria live in close association with heterotrophic bacteria in their environment (Harrison, 1981, 1984; Johnson and Kelso, 1983). Many of these heterotrophic bacteria have been placed in the genus *Acidiphilium* (Harrison, 1981, 1984, 1989; 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/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 micro-organisms 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. Subsequently, acting on recommendations of Report WP E-87 of the Director General: Water Affairs (1987-88), the Department of Water Affairs and Forestry undertook to rehabilitate old coal waste dumps under its jurisdiction. The rehabilitation involves collecting all the coal waste of a mine into a well-defined dump, which is then covered with 70 cm 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. The present investigation was designed to scientifically assess the inhibition of acid-producing micro-organisms by the covers and to determine whether similar inhibition could be achieved by thinner, cheaper covers. At the same time, the groups of organisms involved in the generation of acid drainage in the coal wastes of northern KwaZulu-Natal were investigated for comparison of the process with that occurring in other mining areas of the world. The investigation has mainly been conducted using pilot scale coal waste dumps specially constructed without and with different covers by the Department of Water Affairs and Forestry. The same dumps were used

for a parallel hydrological investigation conducted by consulting engineers Wates, Meiring and Barnard (1993, 1995a,b) as project K5/575 of the Water Research Commission.

A.2. PROJECT OBJECTIVES

The project objectives were:

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

(2) From the results of (1), identification of dump construction or rehabilitation techniques which most successfully limit populations of acid drainage-producing bacteria.

(3) From the results of (1), identification of problem environments in the variously constructed or rehabilitated dumps where acid drainage-producing bacteria flourish and of environments where they are unable to do so.

(4) From (2) and (3), 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.

A.3. THE PROJECT AS PART OF A COLLABORATIVE INVESTIGATION

The project objectives were formulated after discussions with the Development of Water Affairs and Forestry, during which northern KwaZulu-Natal was identified as a potential study area. Various old coal waste dumps in this area were being rehabilitated, particularly by the Department of Water Affairs and Forestry, using funds allocated as a result of Report WP E-87 of the Director General: Water Affairs (1987-88). The Department was interested in obtaining an assessment of the effectiveness of its dump rehabilitation technology under local conditions and whether cheaper procedures such as the use of a 1 m or thinner cover comprising a single soil type instead of the 1 m thick clay plus soil cover might be adequate. The present project and project K5/575 of Wates, Meiring and Barnard (Calibration of models for the design of soil covers for open cast mine and waste dumps in South Africa), submitted simultaneously to the Water Research Commission, provided an opportunity for an investigation involving concomitant microbiological and hydrological assessments of dump rehabilitation technologies.

The investigation developed as a collaborative venture involving construction of 10 pilot scale coal waste dumps according to different possible rehabilitation technologies by the Department of Water Affairs and Forestry, the abovementioned scientific assessment and associated studies by the Department of Microbiology of Stellenbosch University and Wates, Meiring and Barnard, funding and co-ordination of the research by the Water Research Commission. The Dundee Office of the Department of Water Affairs and Forestry had a crucial central role in the investigation, being

responsible for the construction and maintenance of the pilot scale dumps on the Kilbarchan Mine near Newcastle and the regular collection of certain data, such as rainfall, gaseous oxygen and carbon dioxide in the coal waste. The objectives under **A.2**, which were formulated for an investigation involving existing mine dumps in the area, could be extended in the controlled environments of the pilot scale coal waste dumps with the intensive data collection, to include detailed studies of important physical and chemical ecological determinants of the bacterial populations in these dumps. These studies, which greatly enhanced the project, were approved by the project Steering Committee.

Throughout the investigation there was close collaboration among the partners, including attendance of Steering Committee meetings of both projects by members of the Steering Committee of either project. This enlarged the pool of valuable ideas and criticisms for both projects. The availability of much of the physical and chemical ecological data for the present project resulted directly form the collaboration with Wates, Meiring and Barnard and the Department of Water Affairs and Forestry; in addition, various analyses of the coal waste were conducted by organizations represented on the Steering Committees. Relevant data and research reports were made available routinely to all partners. This final report for project K5/454 and that of Wates and Rykaart (1999) for project K5/575 cover the research of the collaborative investigation to 1997.

A.4. STRUCTURE OF THE PROJECT AND REPORT

A **Literature Survey** of topics relevant to project K5/454 has been compiled. It includes:

(1) An outline of the chemical and microbial processes involved in acid mine drainage formation.

(2) A survey of the microorganisms that may have a role in the formation of acid mine drainage.

(3) Discussion of physical and chemical ecological factors influencing bacterial acid mine drainage formation in coal waste.

(4) Consideration of an ecological approach to the control of acid drainage from coal waste dumps by using soil and/or clay liners and covers to reduce water infiltration into and out of the coal waste and oxygen infiltration into the waste.

The research of the project has been divided into two parts. The main part of the research, concerned with objectives (1)-(4), is described under **Research: Part 1. Abiotic Ecological Determinants (Temperature, Moisture, Oxygen, Carbon Dioxide and pH) and Acid-producing Bacteria in Pilot Scale Coal Waste Dumps in Relation to Covers Used for Dump Rehabilitation.** This research includes:

(1) Description of the experimental pilot scale coal waste dumps (mini-dumps or cells) near the Kilbarchan Mine, including analyses of the coal waste and soils used to construct the dumps.

(2) Monitoring of the abiotic ecological determinants, temperature (above and in the dumps), 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 through a 3- or 4-year period. The last four determinations were made in the coal waste to a depth of 15-30 cm in covered cells or between depths of 15 and 30 cm in uncovered cells (outer layer of the coal waste).

(3) Monitoring of bacterial population sizes in the samples from the outer layer of the coal waste in the cells. The bacterial populations included:

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

(ii) Acidophilic and non-acidophilic chemolithotrophic thiosulphate-oxidizing bacteria.

(iii) Moderately acidophilic bacteria oxidizing very low concentrations of ferrous iron (presumptive *Metallogenium*).

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

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

(vi) Acidophilic bacteria oxidizing moderate concentrations of ferrous iron and sulphur (S^0).

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

The bacteria under (i) were monitored in every sample over a 3-year period and the groups under (ii) to (vii) 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.

(4) In the final section of part 1, the report critically discusses the research results, searching for causal relationships among the various abiotic factors monitored and the populations of the various bacterial groups in relation to acid drainage formation, but in particular evaluates the potential of the various pilot scale dump treatments for controlling the production of acid mine drainage as shown by their effects on the various ecological factors and bacteria that were 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 part 1 of this investigation were not studied as far as establishing their identity, this aspect of objective (1) was addressed under **Research: Part 2. Microorganisms of Iron-oxidizing Consortia Involved in the Generation of Acid Mine Drainage in Northern KwaZulu-Natal.** The research comprised:

(1) Development of stable iron-oxidizing enrichment cultures, especially in the selective medium for acidophilic chemolithotrophic bacteria oxidizing high concentrations of ferrous iron, 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).

(2) Development of stable iron-oxidizing cultures in the selective medium for acidophilic chemolithotrophic bacteria oxidizing high concentrations of ferrous iron, 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.

(3) A study of the effects of heterotrophic fungi isolated under (1) and (2) on growth and iron oxidation by *T. ferrooxidans.*

(4) A discussion of the various microorganisms that were isolated and their possible roles in the development of acid mine drainage as components of iron-oxidizing microbial consortia.

Under **Discussion, Conclusions and Recommendations,** the most useful findings of the research in respect of the control of acid mine drainage by soil covers are highlighted, and recommendations on cover requirements for successful control in rehabilitated coal waste dumps are made. A cost-effective procedure is recommended for monitoring the success of dump rehabilitation for the control of acid mine drainage. Proposals for further research are made.

The section **References** contains the references for the entire report.

Two **Appendices** complete the report. The first shows a computed table used for most probable number estimates of bacterial populations, with 95% confidence limits which could not be obtained from published tables. The second contains a full record of the moisture contents, pH and bacterial population sizes (including theoretical 95% confidence limits) of individual coal waste samples from the pilot scale dumps, presented in a series of Appendix tables, each giving the data for a different sampling date.

B. LITERATURE SURVEY

B.1. CHEMICAL AND MICROBIAL PROCESSES INVOLVED IN ACID MINE DRAINAGE FORMATION

B.1.1. Oxidation of Pyrite and Other Metal Sulphides

B.1.1.1. Overall pyrite oxidation processes

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 and ferric iron, a complex oxidation process occurs. This process is a combination of abiotic and bacterially catalysed oxidation reactions (Dugan, 1975; Evangelou and Zhang, 1995; Kleinmann *et al.*, 1981; Lundgren and Silver, 1980; Luther, 1987; Moses and Herman, 1991; Moses *et al.*, 1987; Mustin *et al.*, 1992; Sand *et al.*, 1995; Schippers *et al.*, 1996). The phenomena involved in the process include oxidation-reduction processes, as well as solid-solution equilibria, that involve ions and intermediate sulphur-containing compounds. Furthermore, the semiconducting properties of pyrite also play a role.

Abiotic and biotic processes involved in the oxidation of pyrite, according to Lundgren *et al.* (1972), Kleinmann *et al.* (1981) and Evangelou and Zhang (1995) are summarized in the following equations:

$$2FeS_2 + 2H_2O + 7O_2 \longrightarrow 2FeSO_4 + 2H_2SO_4 \quad \text{(A)}$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad \text{(B)}$$

$$Fe_2(SO_4)_3 + 6H_2O \longrightarrow 2\underline{Fe(OH)}_3 + 3H_2SO_4 \quad \text{(C)}$$

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

Process (A), which occurs at a high pH (pH > 4.5), is basically an abiotic process resulting in the spontaneous auto-oxidation of pyrite (Kleinmann *et al.*, 1981). A drop in pH results from the formation of ferrous sulphate and sulphuric acid. In process (B), ferrous sulphate is oxidized abiotically to ferric sulphate at pH levels higher than pH 4.5, but decline of the pH to lower than pH 4.5 causes the abiotic process to slow down and change to a biotic one (Kleinmann and Crerar, 1979; Kleinmann *et al.*, 1981). At pH 2.5 and higher the ferric sulphate product hydrolyses and precipitates as ferric hydroxide, as shown in (C), with further production of acidity. This ferric hydroxide, also called "yellow boy", is indicative of acid mine drainage. Below pH 2.5, the ferric sulphate remains as ions in solution. The biotic process (B) is most prominent below pH 2.5, where its rate is significantly higher than the abiotic oxidation rate (Kleinmann *et al.*, 1981). Electrons, released during the oxidation of ferrous iron in the biotic reaction, are used for the electron transport system and energy metabolism of the responsible bacteria such as *T. ferrooxidans* (Ingledew, 1982). The ferric iron product of process (B) oxidizes pyrite in the basically abiotic process (D). The

sulphuric acid and additional hydrogen ions produced by processes (A), (C) and (D) create the low pH environment that promotes the biotic oxidation of ferrous iron, while process (B) generates the ferric iron for the abiotic attack on pyrite. Process (B) is the rate-limiting step in the oxidation of pyrite (Singer and Stumm, 1970), hence ferrous iron-oxidizing bacteria causing this oxidation play a crucial role in determining the pyrite oxidation rate.

B.1.1.2. Abiotic oxidation of pyrite

During the past two decades, details of the mechanisms underlying processes (A) and (D), by which pyrite is attacked abiotically, have become clearer. Models explaining these mechanisms need to take into account the following observations (Lowson, 1982; Luther, 1987; Moses and Herman, 1991; Moses *et al.*, 1987; Sand *et al.*, 1995; Schippers *et al.*, 1996).

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

(2) 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 in both abiotic and biotic pyrite oxidation systems.

Using molecular orbital theory, Luther (1987) deduced that dissolved ferric iron in the form of ferric hexahydrate could be the oxidizing agent for the attack on pyrite. This deduction 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.*, 1994a,b). Schippers *et al.* (1996) used silver ions to prove conclusively that thiosulphate was the first intermediate in the oxidation of pyrite. 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. A schematic diagram 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:

$$FeS_2 + 6Fe(H_2O)_6^{3+} + 3H_2O \longrightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_2O)_6^{2+} + 6H^+ \quad (E)$$

Conversion (E) forms part of process (D), as the thiosulphate still has to be oxidized to sulphate. Moses *et al.* (1987) postulated a mechanism involving the addition of two hydroxyl groups followed by the removal of water to add each of the oxygens to the one pyrite sulphur atom but it is not clear how the addition of the last two hydroxyl 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 hydroxyl ions (OH^-) may be through an inner sphere electron transfer mechanism where OH^- and an electron exchange simultaneously between the ferric complex $Fe(H_2O)_5OH^{2+}$ and the disulphide.

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

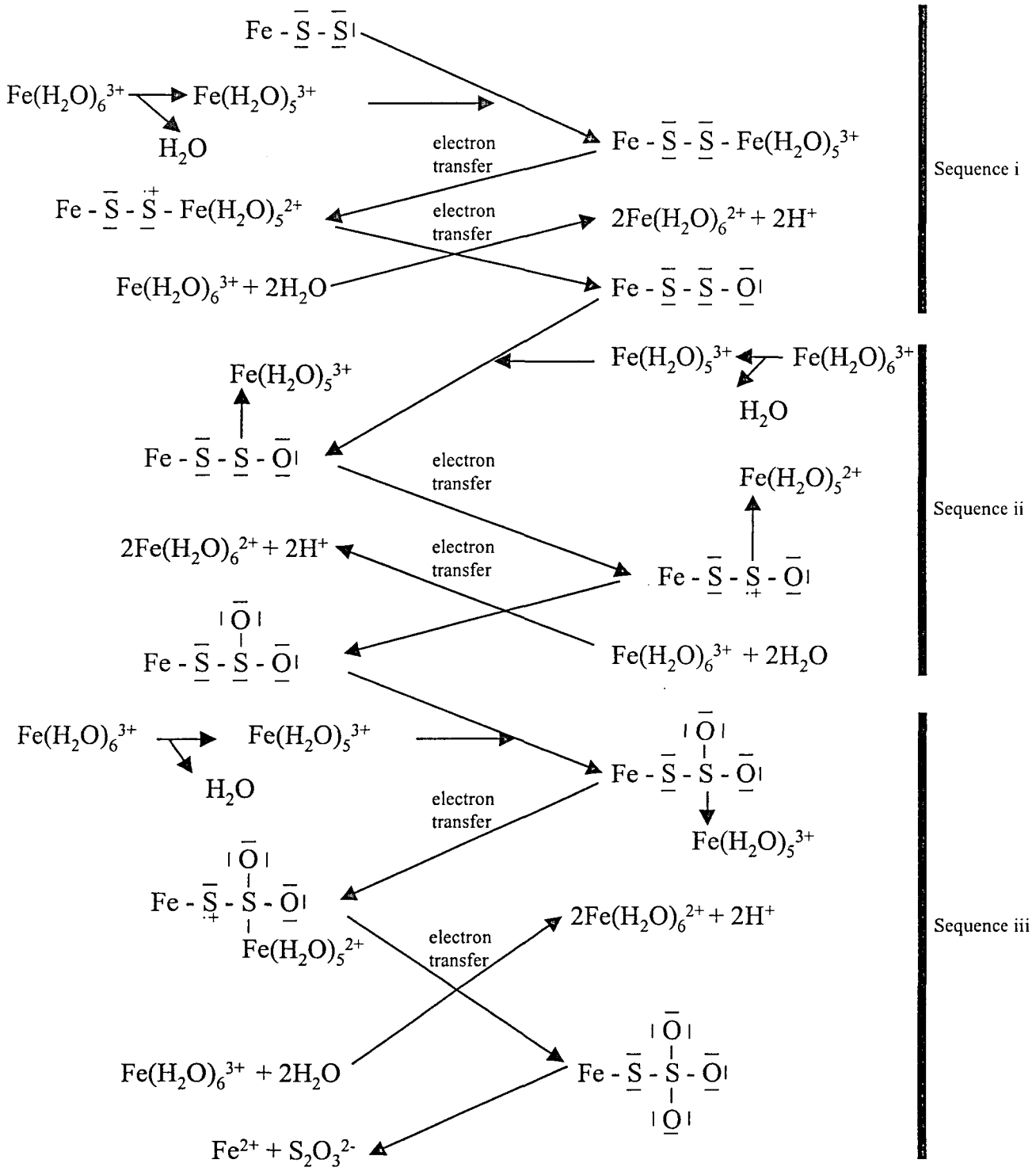

Fig. 1. Schematic diagram of the oxidation of pyrite by ferric hexahydrate, which by repetitive transfers of electrons to ferric iron from one of the pyrite sulphurs leads to the dissolution of pyrite as ferrous iron and thiosulphate. (Adapted from Evangelou and Zhang, 1995; Luther, 1987.)

This differs from the mechanisms of Luther (1987) and Moses *et al.* (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 (F), 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 complexed with the ferrous product.

$$Fe\text{-}S\text{-}S\text{-}OH + Fe(H_2O)_5OH^{2+} \rightleftharpoons Fe\text{-}S\text{-}S\text{-}O + Fe(H_2O)_6^{2+} \qquad (G)$$

Repetition of reactions (F) and (G) 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 the initial stage of process (A) 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 cyclically oxidized and reduced while acting as a conduit for electrons travelling from pyrite to dissolved oxygen (Fig. 2).

Although the mechanism of pyrite oxidation may not be entirely clear and may vary according to the pH at which the reaction proceeds, it is generally agreed that pyrite oxidation proceeds via hydro- or hydroxyl-complexed iron electron carriers and that thiosulphate is the first sulphur 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.

B.1.1.3. *Abiotic oxidation of pyrite oxidation products*

As discussed under section **B.1.1.1**, the ferrous iron produced during the oxidation of pyrite is oxidized to ferric iron abiotically at circumneutral pH as shown in equation (B), but at a declining rate as the pH drops to lower levels. The ferric iron may then be re-used for the further oxidation of pyrite. However, in circumneutral to low acid environments, it readily precipates as ferric hydroxide as shown in equation (C).

Thiosulphate is unstable in acidic environments and decomposes to form various sulphur compounds, including various 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 mechanism for thiosulphate (Fig. 3), which includes intermediate sulphur compounds detected during the oxidation of pyrite by ferric iron under sterile conditions. This mechanism is cyclical and involves both ferric iron and oxygen as electron acceptors. Sulphate is an important product. The details of the proposed thiosulphate oxidation mechanism are as follows:

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

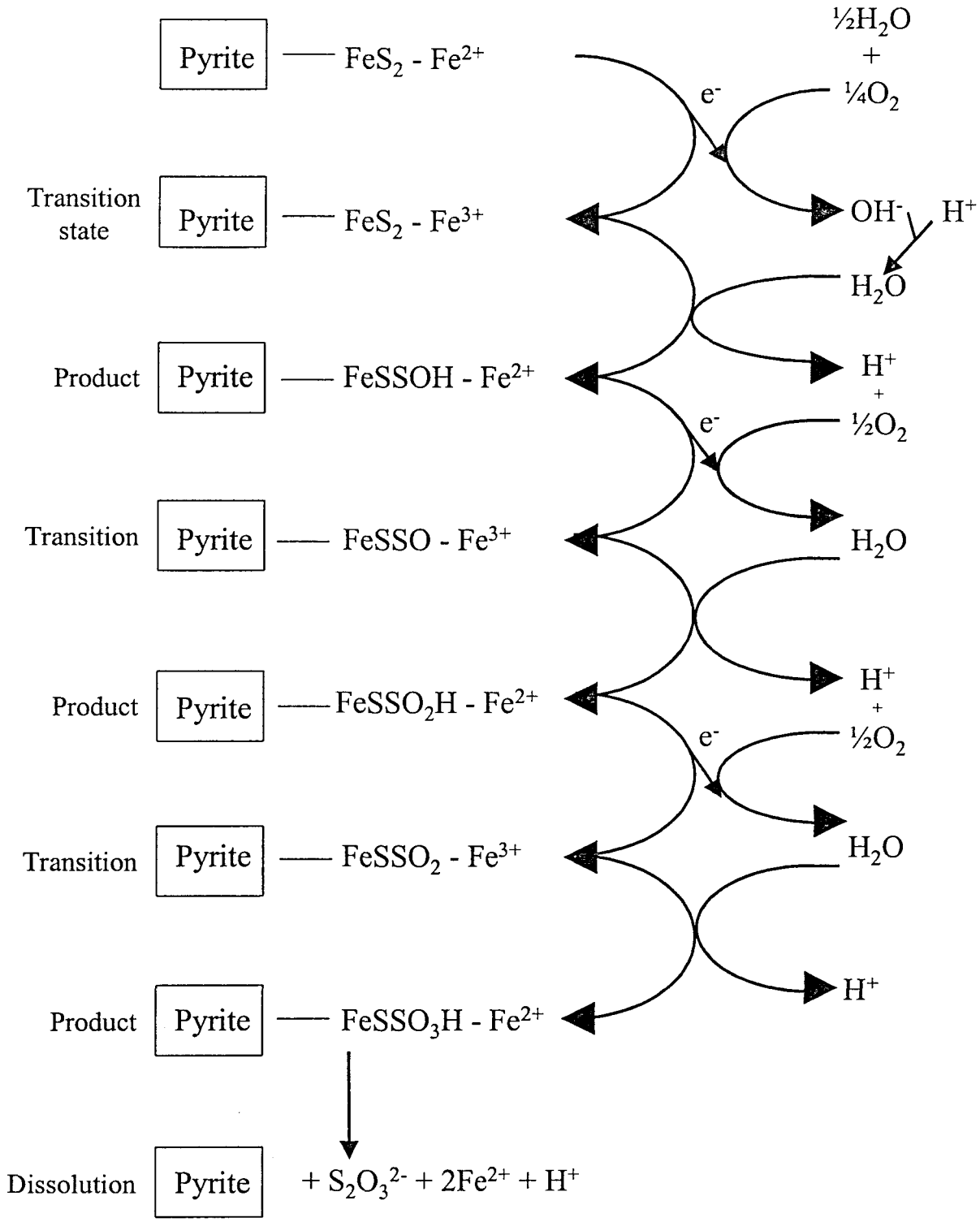

Fig. 2. Schematic model of pyrite oxidation at circumneutral pH, involving ferrous iron, adsorbed to the pyrite surface, giving up electrons to oxygen. Repeated electron transfers between oxygen and pyrite via the adsorbed iron conduit lead to the dissolution of pyrite. The adsorbed and liberated iron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

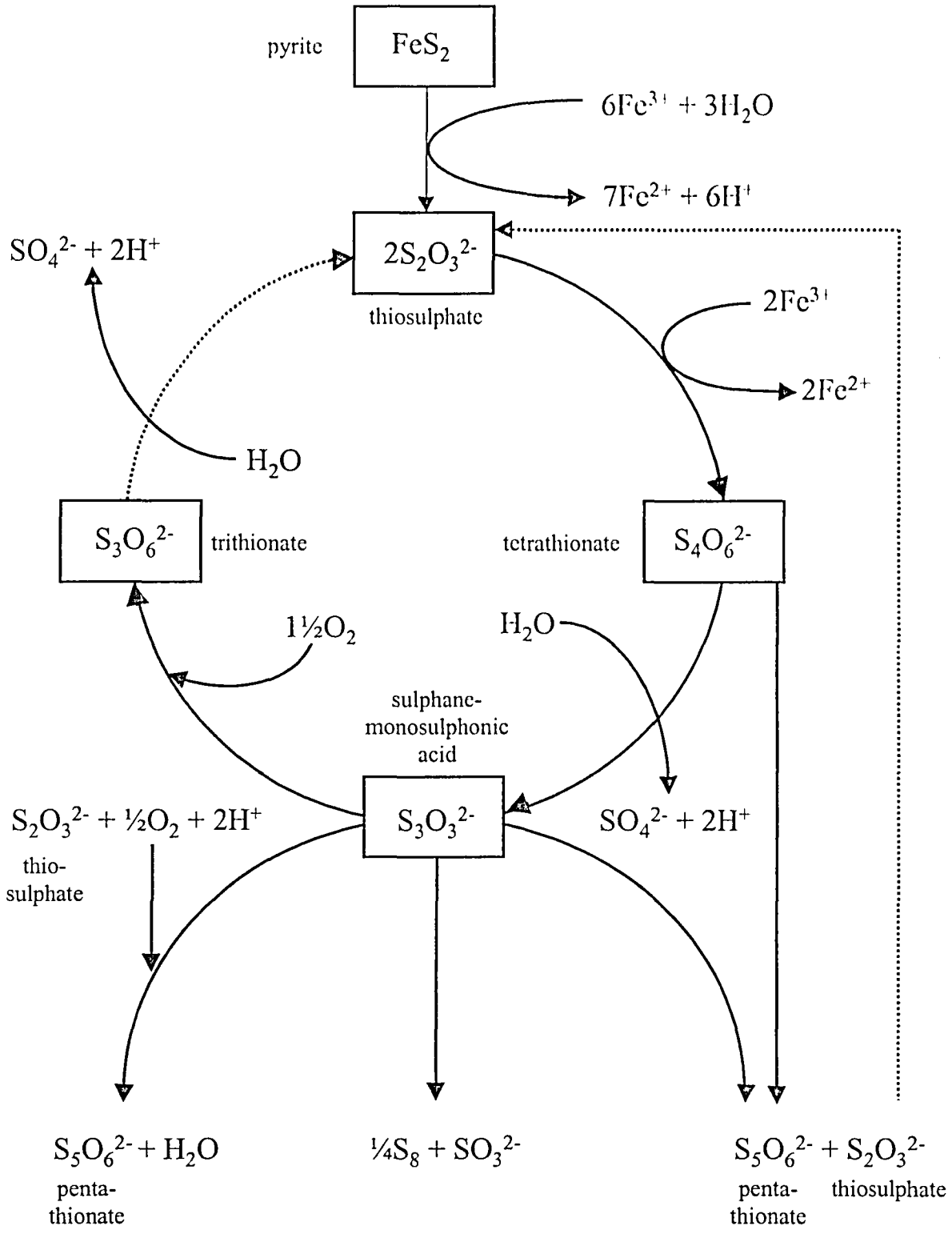

Fig. 3. Cycle of reactions involved in pyrite oxidation during chemical and/or bacterial leaching. Dotted lines indicate where thiosulphate may enter the cycle again. (Adapted from Pronk *et al.*, 1990; Schippers *et al.*, 1996).

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

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

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

Disulphane monosulphonic acid may react in several ways to form elemental sulphur, sulphite, thiosulphate, trithionate, pentathionate (reactions J to M) and various other polythionates.

$$4S_3O_3^{2-} \longrightarrow S_8 + 4SO_3^{2-} \quad (J)$$

$$S_3O_3^{2-} + S_4O_6^{2-} \longrightarrow S_2O_3^{2-} + S_5O_6^{2-} \quad (K)$$

$$2S_3O_3^{2-} + 3O_2 \longrightarrow 2S_3O_6^{2-} \quad (L)$$

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

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

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

This sequence of reactions appears to be catalysed by the pyrite surface as vigorous shaking was required for the reactions to proceed in experiments where no bacterial catalyst was available. Reaction (J) seemed to be the dominant side–reaction of disulphane monosulphonic acid, as S^0 was the dominant minor sulphur product after the main product, sulphate (Schippers and Sand, 1999).

B.1.1.4. Biotic oxidation of pyrite oxidation products

Various groups of acidophilic bacteria able to oxidize ferrous iron, sulphur and inorganic sulphur compounds play important catalytic roles in the oxidation of products of the abiotic oxidation of pyrite. These bacteria, which are discussed comprehensively in section **B.2,** are mainly obligate or facultative chemolithotrophs obtaining energy for growth from the inorganic oxidations. The most intensively studied acid mine drainage organisms in respect of their metabolism are the *Thiobacillus* species, *T. ferrooxidans*, which oxidizes both iron and sulphur substrates, and *T. thiooxidans,* which oxidizes only sulphur substrates (Hooper and Dispirito, 1985; Ingledew, 1982; Rawlings, 1997).

The processes used by iron- and sulphur-oxidizing chemolithotrophic bacteria to oxidize pyrite oxidation products and the relationship of these processes to the abiotic pyrite oxidation processes are outlined in Fig. 4. The bacteria obtain energy from these processes by the production of protons on the outside of the cytoplasmic membrane and their uptake on the inside of the membrane, thereby increasing the pH gradient across the membrane. The accumulation of protons on the outside of the membrane drives the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) by ATP synthase, which is located in the membrane (Madigan *et al.*, 1997). The ATP is the main energy-rich compound driving the biosynthetic reactions of the bacteria.

Details of the ferrous iron oxidation mechanism are available in the reviews of Ingledew (1982) and Rawlings (1997). The transfer of an electron to cytochrome c, external to the cytoplasmic

membrane, does not generate protons, but these are available in abundance because of the low environmental pH required by the acidophilic iron-oxidizing bacteria and because of the generation of protons by other reactions involved in the pyrite degradation process, such as the abiotic reactions described in previous sections. The uptake of protons on the inside of the membrane occurs during the reduction of oxygen to water (Fig. 4).

Biotically produced ferric iron serves as an oxidant for the abiotic oxidation of pyrite, but also for the oxidation of sulphur and sulphur compounds, such as thiosulphate in reaction (H), sulphur to sulphite and sulphite to sulphate:

$$S^0 + 4Fe^{3+} + 3H_2O \longrightarrow SO_3^{2-} + 4Fe^{2+} + 6H^+ \qquad (O)$$

$$SO_3^{2-} + 2Fe^{3+} + H_2O \longrightarrow SO_4^{2-} + 2Fe^{2+} + 2H^+ \qquad (P)$$

Reaction (H) occurs abiotically and as the initial reaction in the cyclic mechanism proposed by Pronk *et al.* (1990) and Schippers *et al.* (1996) for the oxidation of thiosulphate by thiobacilli. Reaction (O) is a biotic reaction of *T. ferrooxidans* catalysed by sulphur:ferric iron oxidoreductase (Sugio *et al.*, 1985, 1987), subsequently renamed hydrogen sulphide:ferric iron oxidoreductase (Pronk and Johnson, 1992; Sugio *et al.*, 1990). Reaction (P) occurs both as an abiotic reaction (Sugio *et al*, 1985) and as an enzyme reaction of *T. ferrooxidans* (Sugio *et al.*, 1988). Reactions (O) and (P) contribute substantially to the pool of protons and hence reduction of the pH on the outside of the cytoplasmic membrane.

Various other reactions involved in the oxidation of sulphur and the sulphur compounds indicated in Fig. 4 have been found in thiobacilli. The cyclic system for the oxidation of thiosulphate shown in Fig. 3 was postulated as both an abiotic and a biotic system (Pronk *et al.*, 1990; Schippers *et al.*, 1996). Other reactions included in a review by Hooper and Dispirito (1985) are shown in Fig. 5. These reactions are extracytoplasmic and cytoplasmic membrane-associated oxidations of sulphur and sulphur compounds to sulphate as the final product. They yield electrons which reduce oxygen to water on the cytoplasmic side of the membrane (with the uptake of protons) and protons in the extracytoplasmic pool, thereby drivng ATP synthesis.

Sulphite which is transported into the cytoplasm of thiobacilli or which is produced there from transported thiosulphate (Fig. 5) may be oxidized to sulphate by the following reactions (Hooper and Dispirito, 1985) involving the sequential formation of adenosine phosphosulphate (APS) and ADP from adenosine monophosphate (AMP):

$$SO_3^{2-} + AMP \longrightarrow APS + 2e^- \qquad (Q)$$

$$APS + HPO_4^{2-} \longrightarrow ADP + SO_4^{2-} \qquad (R)$$

Reaction (Q) is catalysed by APS reductase and reaction (R) by ADP sulphurylase. The conversion of AMP to ADP represents an energy gain for the cell. However, this pathway has been found in only a few *Thiobacillus* species (Madigan *et al.*, 1997).

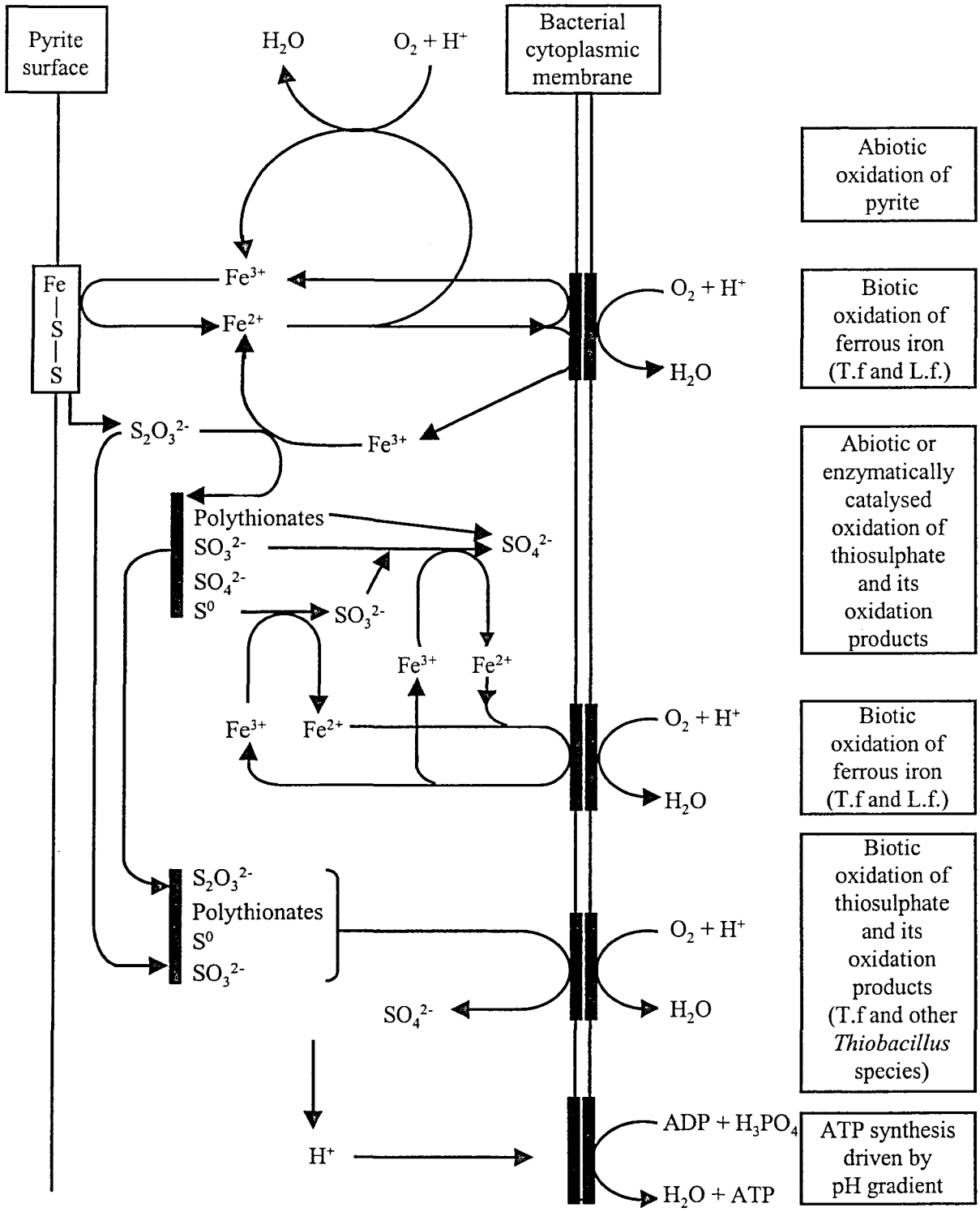

Fig. 4. Outline of abiotic and biotic oxidation processes involved in the oxidation of pyrite to sulphate, ferrous and ferric iron. The biotic oxidations occur by the transfer of electrons via carriers at the outer surface of and within the cytoplasmic membrane to the terminal electron acceptor, oxygen, inside the membrane. The production of protons on the outside of the membrane and their uptake on the inside of the membrane create a pH gradient which drives the synthesis of ATP to provide energy for the cell. The linked chemical transformations in this diagram are not balanced. T.f. = *Thiobacillus ferrooxidans* and L.f. = *Leptospirillum ferrooxidans.*

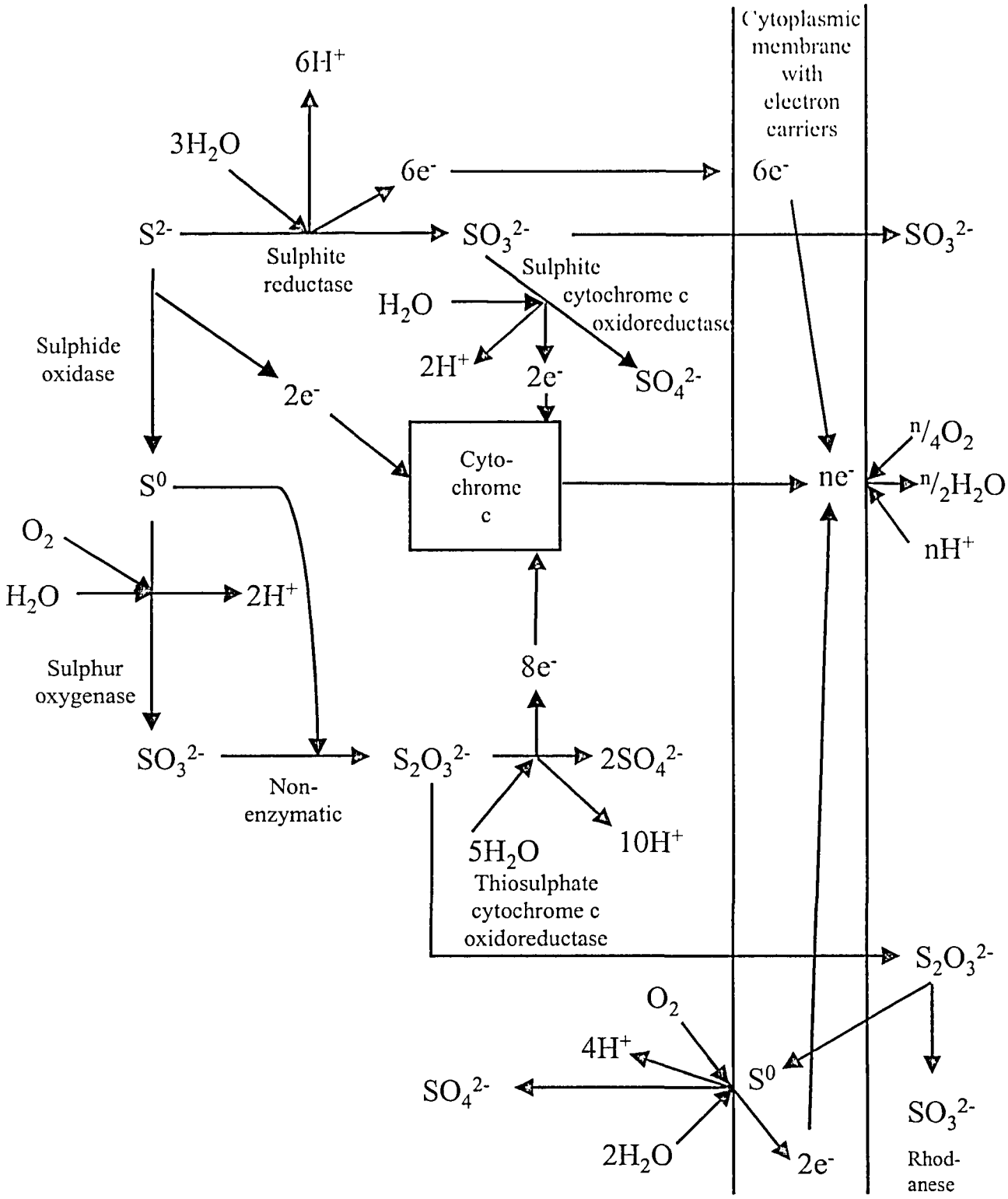

Fig. 5. Extracytoplasmic and cytoplasmic membrane-associated oxidations of sulphur and sulphur compounds by thiobacilli. (Adapted from Hooper and Dispirito, 1985).

B.1.1.5. Oxidation of other metal sulphides

Two distinct abiotic mechanisms for the dissolution of different metal sulphides in an acidic solution of ferric iron were recently demonstrated by Schippers and Sand (1999). Molybdenite (MoS_2) was degraded like pyrite mainly to sulphate, presumably via thiosulphate (see sections **B.1.1.2** and **B.1.1.3**), with the production of sulphur, tetrathionate and pentathionate as minor products. Both pyrite and molydenite, as well as the disulphide of tungsten (WS_2), occur as acid-insoluble crystals and it was postulated that their dissolution required oxidative attack by ions such as ferric iron resulting in initial oxidation of the sulphur moiety to thiosulphate.

The second mechanism was observed with the acid-soluble sulphides hauerite (MnS_2), sphalerite (ZnS), chalcopyrite ($CuFeS_2$), galena (PbS), orpiment (As_2S_3) and realgar (As_4S_4). These sulphides produced sulphur (S_8) as the major product, with sulphate, tetrathionate and pentathionate in some cases as minor products. Schippers and Sand (1999) postulated initial attack on these sulphides by protons, followed by the formation of polysulphides as follows:

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

$$H_2S + Fe^{3+} \longrightarrow H_2S^{\cdot+} + Fe^{2+} \qquad (T)$$

$$H_2S^{\cdot+} + H_2O \longrightarrow H_3O^+ + HS^{\cdot} \qquad (U)$$

$$2HS^{\cdot} \longrightarrow H_2S_2 \qquad (V)$$

$$H_2S_2 + Fe^{3+} \longrightarrow H_2S_2^{\cdot+} + Fe^{2+} \qquad (W)$$

$$H_2S_2^{\cdot+} + H_2O \longrightarrow H_3O^+ + HS_2^{\cdot} \qquad (X)$$

$$HS_2^{\cdot} + HS^{\cdot} \longrightarrow H_2S_3 \qquad (Y)$$

$$2HS_2^{\cdot} \longrightarrow H_2S_4 \qquad (Z)$$

Further analogous reactions give higher polysulphides, which degrade to sulphur as follows:

$$H_2S_n + 2Fe^{3+} \longrightarrow S_8 + 2Fe^{2+} + 2H^+ \text{ or } H_2S \qquad (ZZ)$$

In natural or mine environments where iron- and sulphur-oxidizing bacteria are present, they will maintain the supply of ferric irons for catalysis of the sulphide degradation processes and oxidize the sulphur products to sulphate as described in section **B.1.1.4.**

B.2. MICROORGANISMS INVOLVED IN ACIDIFICATION OF METAL SULPHIDES

Microorganisms involved in the degradation of metal sulphides and which have application or potential application for the recovery of metals from ores by bioleaching, have recently been comprehensively reviewed by Rawlings (1997), Norris (1997) and Johnson and Roberto (1997). These organisms may also generate unwanted acid mine drainage from mine wastes. They occur in both prokaryote domains, namely, the *Bacteria* and *Archaea*, comprising eubacteria and archaebacteria (= archaeobacteria, Staley *et al.*, 1989), respectively (Brock *et al.*, 1994). Some have not yet been isolated from mine environments, but only from acidic iron-, sulphur- and sulphide-containing natural environments, such as hot springs, mudpots and soils of solfatara fields. However, selected isolates (thermophiles) among them have efficiently degraded sulphide ores in the laboratory (Brierley, C.L., 1978, 1982; Brierley, J.A., 1990; Norris, 1997) and they or similar organisms may yet be encountered in high temperature bioleaching systems or self-heating mine waste dumps.

B.2.1. Acidophilic Iron-oxidizing Bacteria

In the presence of water and oxygen, these bacteria are capable of oxidizing ferrous iron to ferric iron, which catalyses the converson of pyrite to thiosulphate and ferrous iron (section **B.1.1.2**) and the degradation of other metal sulphides as outlined in section **B.1.1.5**. Their activity thus increases sulphide degradation rates and the associated formation of acid mine drainage in exposed sulphide-containing ores.

B.2.1.1. *Thiobacillus ferrooxidans*

Thiobacillus ferrooxidans is the best characterized member of the iron-oxidizing eubacteria involved in bioleaching and the formation of acid mine drainage and was for many years considered to be the main organism involved (Evangelou and Zhang, 1995; Hutchins *et al.*, 1986; Kleinmann *et al.*, 1981; Lundgren and Silver, 1980). Colmer and co-workers first isolated *T. ferrooxidans* from bituminous coal mines in 1947–1951 (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). Some strains possess a flagellum or flagella and some form pili (DiSpirito *et al.*, 1982). The species is an obligate chemolithotroph capable of growth on ferrous iron, sulphur and a range of inorganic sulphur compounds, including pyrite, thiosulphate, tetrathionate and sulphite (Kelly and Harrison, 1989). It obtains its energy for growth from the oxidation of these substrates (Ingledew, 1982; Pronk *et al.*, 1990). Cellular carbon is obtained by the fixation of carbon dioxide (Brierley, C.L., 1978, 1982; Hutchins *et al.*, 1986;

Lundgren *et al.*, 1972; Tuovinen and Kelly, 1973, 1974). Although *T. ferrooxidans* is classified as an aerobic species (Kelly and Harrison, 1989), Brock and Gustafson (1976), Sugio *et al.* (1985), Pronk and Johnson (1992) and Pronk *et al.* (1991, 1992) have reported that it is capable of growth on elemental sulphur under anaerobic conditions, using ferric iron instead of oxygen as an electron acceptor. Ferrous iron-grown cells of *T. ferrooxidans* exhibited significant rates of ferric iron-dependent sulphur oxidation (Pronk *et al.*, 1992; Suzuki *et al.*, 1990). Sulphuric acid produced by the growth of *T. ferrooxidans* on elemental sulphur creates the acidic environment necessary to sustain high populations of *T. ferrooxidans*. These results suggest that ferric iron may be an important electron acceptor for the oxidation of sulphur compounds in acidic environments.

Mackintosh (1978) demonstrated nitrogen fixation by *T. ferrooxidans;* however, the organism prefers to grow on fixed nitrogen, with ammonium salts being the best source (Tuovinen *et al.*, 1979).

Thiobacillus ferrooxidans is mesophilic, with a growth range of 10-37°C and an optimum of 30-35°C, according to Kelly and Harrison (1989). However, some strains do not grow at 37°C (Norris, 1990; Silverman and Lundgren, 1959). Leduc *et al.* (1993) found psychrotrophic strains that grew from 2°C to 35°C and Norris (1990) recorded strains that grew at 40°C. The species is an obligate acidophile with a pH range for growth of approximately pH 1.3-4.5 (Kelly and Harrison, 1989), but the range and/or optimum may vary according to the substrate on which it is growing under laboratory conditions. The pH range for growth by the oxidation of ferrous iron is approximately pH 1.3-3.5 and the optimum about pH 2.0 (Ingledew, 1982), but on thiosulphate, growth occurred only between pH 3.6 and pH 4.7, with the optimum at pH 3.6 (Tuovinen and Kelly, 1974). Growth on tetrathionate occurred between pH 1.3 and pH 4.4, with optimal growth at pH 2.3-2.5.

Thiobacillus ferrooxidans is highly susceptible to inhibition by organic compounds, as is clearly illustrated by the difficulty of growing this organism on media containing organic gelling agents such as agar or agarose (Johnson, 1995b; Mishra and Roy, 1979; Tuovinen and Kelly, 1973; Visca *et al.*, 1989). Tuttle and Dugan (1976) found that ferrous iron and sulphur oxidation, as well as growth of *T. ferrooxidans,* were entirely or partially inhibited by a wide range of organic compounds, including C_1–C_6 alkanoic acids, citric acid cycle acids and certain 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. The relative electronegativity of the organic inhibitor appeared 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. The complexing agent ethylenediaminetetraacetic acid (EDTA) is inhibitory (Silver and Lundgren, 1968), as are various anionic detergents, such as sodium lauryl sulphate (Dugan, 1975; Dugan and Lundgren, 1964; Loos *et al.*, 1990a,b; Onysko *et al.*, 1984b) and the antimicrobial benzoic and sorbic acids (Loos *et al.*, 1990a,b; Onysko *et al.*, 1984b).

Acid mine drainage is an environment with high concentrations of sulphate and heavy metal ions (Silverman and Ehrlich, 1964), making heavy metal 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) and is tolerant to zinc, nickel, cobalt, manganese and aluminium salts with metal concentrations exceeding 10 000 mg/l or copper salts with at least 1 000 mg/l copper (Tuovinen *et al.*, 1971). Heavy metal tolerance is strain dependent and varies according to the growth substrate, with cells growing on ferrous iron exhibiting the highest tolerance to the heavy metal ions.

Different strains of *T. ferrooxidans* vary considerably in respect of temperature range and optimum for growth, 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) studied 23 strains from various geographical locations and found that they belonged to seven different DNA homology groups that correlated with their physiological characteristics. Although the strains of two of these groups were unable to oxidize sulphur and were later found to be morphologically and/or phylogenetically far removed from the thiobacilli (Kelly and Harrison, 1989; Lane *et al.*, 1985, 1992), the DNA of the remaining five homology groups had base compositions ranging from 56 to 62 mol % G + C (Harrison, 1982; Kelly and Harrison, 1989). The results of the DNA analyses suggest that *T. ferrooxidans* is a phenospecies rather than a genospecies.

B.2.1.2. *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 eubacteria, especially *L. ferrooxidans,* may play as important a role in catalysing the process (Hallmann *et al.*, 1992; Hutchins *et al.*, 1986; 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 requirements for growth as *T. ferrooxidans* (Hallmann *et al.*, 1992; Harrison and Norris, 1985; Sand *et al.*, 1992), it is conceivable that *L. ferrooxidans* is as widely distributed as *T. ferrooxidans*.

Morphologically, *L. ferrooxidans* cells are characterized by a spiral or vibrioid 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 inorganic 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 concentration 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.

Leptospirillum ferrooxidans is a mesophilic organism which grows well between 20°C and 40°C and optimally between 28°C and 30°C. However, with decrease of temperature below 20°C its growth rate declines 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*, with various records in the range 52-57 mol % (Harrison and Norris, 1985; Kelly and Harrison, 1989) and phylogenetically show no close relationship to any known bacteria on the basis of 16S rRNA analyses (Lane *et al.*, 1992). The *L. ferrooxidans* strains analysed by Lane *et al.* (1992) appeared closely related to one another, but Hallmann *et al.* (1992) 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.

B.2.1.3. Moderately acidophilic very low ferrous iron-oxidizing bacteria (genus *Metallogenium)*

Metallogenium is a genus of aerobic pleomorphic eubacteria, forming coccoid cells, usually in clusters, and filaments that are heavily encrusted with manganese oxide (Zavarzin, 1989). These bacteria oxidize manganese compounds and multiply by means of a budding process.

The oxidation of sulphidic materials such as pyrite results in a decrease in pH, with the possibility of sequential domination by organisms with progressively lower pH maxima and optima for growth. It has been proposed that *Metallogenium* may be the dominant iron-oxidizing organism in ferrous iron-oxidizing environments until the pH drops below pH 3.5, after which the more acid-tolerant *T. ferrooxidans* would take over (Walsh, 1978; Walsh and Mitchell, 1972). It was postulated that *Metallogenium* was responsible for catalysing pyrite oxidation in the pH range 3.5-4.5, with an optimum pH of 4.1. *Metallogenium* tolerates a much lower free iron concentration (>150 mg/l becomes inhibitory) than *T. ferrooxidans* and may be active in catalysing the iron oxidation-reduction cycle in those regions of a coal mine where abiotic oxidation is slow and where environmental conditions limit the activity of *T. ferrooxidans*.

Walsh and Mitchell (1972) concluded that *Metallogenium* is of environmental significance because it creates the pH environment necessary for *T. ferrooxidans* to grow and flourish. Their work suggested the possibility of curbing acid mine drainage generation by creating conditions that are unfavourable for the growth of *Metallogenium* and *T. ferrooxidans*. They demonstrated that partly neutralized mine water (pH 4.0-4.5) that was also rich in ferrous iron could result in a reduction of acidity and soluble iron production from coal waste when added to material with high acidity and *T. ferrooxidans* populations. Their explanation was that the rise in pH would have been unfavourable

for the rapid growth of *T. ferrooxidans*, while the activity of *Metallogenium* would also have been inhibited by the high concentrations of ferrous iron in its immediate surroundings.

Kleinmann and Crerar (1979) could not prove beyond any reasonable doubt that the presence of *Metallogenium* was necessary for the initial acidification process or that it played a significant role in the initial stages of pyrite oxidation. *Thiobacillus ferrooxidans* appeared able to adapt to a neutral macro-environment (pH 6.9), perhaps by establishing itself in an acid micro-environment and changing it sufficiently for its own growth and survival.

B.2.1.4. 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 eubacteria. These organisms could oxidize ferrous iron and catalyse the oxidation of pyrite (Hutchins *et al.*, 1986). Since then similar organisms with temperature optima of approximately 45-50°C have been isolated from various sources, including acid drainage, coal piles and mineral-processing operations (Norris, 1990, 1997). These bacteria are short to long rods, with certain isolates exhibiting filamentous growth and/or endospores (Brierley J.A., 1978; Brierley and Lockwood, 1977; Ghauri and Johnson, 1991). Some have been classified in the species *Sulfobacillus thermosulfidooxidans* and *Sulfobacillus acidophilus*, which contain, respectively, the acid drainage strain BC1 and coal waste strain ALV of Norris and Barr (1985), while others including strain TH3 from a copper leach dump have been classified in the species *Acidimicrobium ferrooxidans* (Clark and Norris, 1996; Norris, 1997; Norris *et al.*, 1996).

These organisms prefer to grow mixotrophically, on ferrous iron or pyrite and yeast extract, but certain isolates appeared to grow chemo-autolithotrophically on iron or pyrite, as well as heterotrophically on yeast extract (Ghauri and Johnson, 1991; Norris and Barr, 1985). At least some of the moderately thermophilic facultatively lithotrophic iron-oxidizing bacteria oxidize sulphur (Norris, 1997) and some seem to require reduced forms of sulphur for biosynthesis; however, a strain that could utilize sulphate as sulphur source has been isolated (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 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). Strains of all three species grew under microaerophilic to anaerobic conditions using ferric iron as electron acceptor and glycerol as the electron donor; some strains could also use tetrathionate as the electron donor (Bridge and Johnson, 1998). A strain of *S. acidophilus* growing on glycerol under these conditions caused the reductive dissolution of ferric hydroxide, jarosite and goethite to ferrous iron.

Phylogenetically (based on 16S rRNA sequence analysis) the three moderately thermophilic facultatively lithotrophic iron-oxidizing bacterial strains (TH3, BC and ALV) tested by Lane *et al.* (1992), grouped with 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 strain TH3 in the high and strains BC and ALV in the low G + C sub-division of these bacteria.

B.2.1.5. Mesophilic heterotrophic iron-oxidizing bacteria

Johnson *et al.* (1992) isolated from streamer growth in acid mine drainage 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. In various respects it resembled members of the *Sphaerotilus-Leptothrix* group of filamentous eubacteria, but was acidophilic (able to grow at pH 2.0) with 62 mol % G + C in its DNA, whereas *Sphaerotilus* and *Leptothrix* strains are neutrophilic and have 69-72 mol % G + C in their DNA. A second heterotrophic iron-oxidizing bacterium (T-21) isolated at the same laboratory (Pronk and Johnson, 1992), grew as short rods and did not form any macroscopic growth in liquid media; it also grew at pH 2.0 (Bacelar-Nicolau and Johnson, 1999).

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 organism 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).

Bacelar-Nicolau and Johnson (1999) studied pyrite degradation by strain T-21 and other superficially similar isolates from mineral sulphide-oxidizing environments. Pyrite was oxidized by one isolate (T-24) in a basal salts medium and by most of the others where the medium was supplemented with a low concentration of yeast extract. Some isolates were shown to cause pyrite oxidation synergistically in association with *T. thiooxidans* or *T.acidophilus* in the pyrite-basal salts medium without yeast extract, apparently using organic compounds synthesized by these *Thiobacillus* species for their growth and consequent iron oxidation. The ferric iron would oxidize the pyrite, producing sulphur substrates for the thiobacilli. In 16S rDNA sequence analysis, five of the isolates grouped in a tight cluster near the point of divergence of Gram-negative and Gram-positive bacteria, with a close relationship to the moderately thermophilic iron-oxidizing *A. ferrooxidans*. However they seemed to represent more than one species. The species name *Ferromicrobium acidophilus* was proposed for isolate T-23 (Johnson and Roberto, 1997) but this name awaits official confirmation (Bacelar-Nicolau and Johnson, 1999).

B.2.1.6. Thermophilic iron-oxidizing archaea

Thermoacidophilic archaea (archaebacteria) able to oxidize ferrous iron, sulphur and metal sulphides occur in the genera *Sulfolobus*, *Acidianus* and *Metallosphaera* (Norris, 1997). These genera have unusual lobed or irregular coccoid cells, which grow aerobically as facultative or obligate chemolithotrophs by the oxidation of elemental sulphur or inorganic sulphur compounds (Fuchs *et al.*, 1995; Huber *et al*, 1989; Stetter, 1989). Some strains oxidize ferrous iron as a source of electrons. *Sulfolobus* also oxidizes sulphur anaerobically, using Fe^{3+} or MnO_4^{2-} as electron acceptor, while

Acidianus grows anaerobically by the oxidation of hydrogen gas using sulphur as electron acceptor. *Metallosphaera* appears not to grow anaerobically. Growth occurs in the pH range 1-4.5 or 6 and at temperatures of at least 45°C (*Acidianus*) or 50-55°C (*Sulfolobus* and *Metallosphaera*). The G + C contents of the DNA of strains in the three genera show clear differences, namely, *Acidianus* about 31 mol %, *Sulfolobus* 36-38 mol % and *Metallosphaera* 45-46 mol %.

The iron- and/or sulphur-oxidizing archaea have been isolated mainly from acid hot springs, mud-holes and solfataras (Huber *et al.*, 1989; Huber and Stetter, 1991; Stetter, 1989), but a *Sulfolobus metallicus* strain was found in association with self-heating coal mine waste (Marsh and Norris, 1983; Norris, 1997) and *Metallosphaera prunae* in self-heating uranium mine waste (Fuchs *et al.*, 1995). Strains of *Sulfolobus acidocaldarius, S. metallicus, Acidianus brierleyi, Metallasphaera sedula, M. prunae* and *Sulfolobus*-like organisms released metals from sulphide minerals at high temperatures very effectively and have potential for use in the bioleaching of ores (Brierley, 1990; Huber *et al.*, 1989; Huber and Stetter, 1991; Norris, 1997). They appear to have the potential to establish themselves in suitable coal and ore wastes, for example, in heaps undergoing self-heating, and thereby to contribute to acid mine drainage formation.

B.2.2. Acidophilic Sulphur-oxidizing Bacteria

During the oxidation of pyrite and other metal sulphides by ferric iron, sulphur and a range of inorganic sulphur-containing compounds are formed (see section **B.1.1**). Although the capacity of organisms to oxidize sulphur or inorganic sulphur compounds does not enable them to catalyse directly the oxidation of pyrite to form acid mine drainage, they assist the formation of the acid drainage by oxidizing these substances to sulphuric acid (Evangelou and Zhang, 1995).

This section will focus mainly on the members of the genus *Thiobacillus* that have been found in acid drainage and other sulphide mineral dissolution environments, including several that grow under moderately acid to neutral conditions and are classified as neutrophiles and not as acidophiles (Kelly and Harrison, 1989). Certain iron-oxidizing eubacteria, such as *T. ferrooxidans,* strains of moderately thermophilic facultatively lithotrophic iron-oxidizing bacteria and the thermophilic iron-oxidizing archaea also possess the capacity to oxidize inorganic sulphur compounds. These organisms can therefore catalyse both the primary oxidation of pyrite (via ferric iron formation) and the downstream oxidation of sulphur-containing intermediates. As these organisms have been described under the iron-oxidizing bacteria, descriptions of them will not be repeated.

B.2.2.1. Mesophilic obligately lithotrophic *Thiobacillus* species

Acidophiles in this group, besides *T. ferrooxidans*, are *T. thiooxidans* and *Thiobacillus albertis* (Kelly and Harrison, 1989). *Thiobacillus thiooxidans* was first isolated by Waksman and Joffe (1922) and has since been isolated from various acid environments where inorganic sulphur substrates are available, including acid mine drainage (Kelly and Harrison, 1989). *Thiobacillus albertis* was isolated by Bryant *et al.* (1983) from acid soil adjacent to a sulphur stock pile. Both are short Gram-negative rods (*T. thiooxidans*, 0.5 x 1.0-2.0 μm; *T. albertis*, 0.45 x 1.2-1.5 μm) which are motile by means of a polar flagellum (*T. thiooxidans*) or a tuft of polar flagella (*T. albertis*).

Both *T. thiooxidans* and *T. albertis* are obligately aerobic chemolithotrophs and autotrophs (Kelly and Harrison, 1989). They are capable of oxidizing elemental sulphur and various inorganic sulphur compounds, including thiosulphate and tetrathionate. They can therefore utilize products of pyrite degadation, but *T. thiooxidans* cannot oxidize pyrite itself.

Thiobacillus thiooxidans grows between 10°C and 37°C, with optimal growth at 28-30°C, and at pH 0.5-5.5 with optimal growth at pH 2.0-3.0 (Kelly and Harrison, 1989). *Thiobacillus albertis* grows optimally at 28-30°C and pH 3.5-4.0, with growth possible at pH 2.0-4.5.

The G + C content of *T. thiooxidans* DNA is 52-53 mol % and that of *T. albertis* 61.5 mol % (Kelly and Harrison, 1989; Rawlings, 1997). The DSM 612 *Thiobacillus* strain, designated *T. thiooxidans*, from acidic sulphate soil, has DNA with G + C 62 mol % and a low degree of homology with *T. thiooxidans* DNA; it may be similar to *T. albertis* (Harrison, 1982, 1984). Phylogenetically, on the basis of 5S and 16S rRNA analyses, *T. thiooxidans* and *Thiobacillus* DSM 612 are closely related to the type and other strains of *T. ferrooxidans* (Lane *et al.*, 1985, 1992; Rawlings, 1997).

Bacteria which may have included the neutrophilic *Thiobacillus thioparus* were detected by Sand *et al.* (1995) in uranium mine waste dumps. This species is an aerobic obligate chemolithotroph and autotroph which oxidizes sulphur and a range of inorganic sulphur compounds (Kelly and Harrison, 1989). It grows at pH 4.5-7.8 or (some strains) even pH 10, and optimally at pH 6.6-7.2 and 28°C.

B.2.2.2. Mesophilic facultatively lithotrophic *Thiobacillus* species

Several acidophilic and neutrophilic mine waste-associated *Thiobacillus* species are capable of heterotrophic growth on various organic substrates (Kelly and Harrison, 1989). The acidophilic *Thiobacillus acidophilus* was isolated initially from a culture of *T. ferrooxidans* (Guay and Silver, 1975). The neutrophilic *Thiobacillus delicatus* was isolated from mine water (Mizoguchi *et al.*, 1976). Neutrophilic thiobacilli, which may have been *Thiobacillus novellus, Thiobacillus intermedius* or *T. thioparus* (see section **B.2.2.1**), were detected in large numbers in uranium mine waste dumps (Sand *et al.*, 1995). Optimum growth conditions for the facultatively lithotrophic species (Kelly and Harrison, 1989) are: *T. acidophilus*, 27-30°C and pH 2.5-3.0; *T. delicatus*, 30-35°C and pH 5.5-6.0; *T. novellus*, 25-30°C and pH 7.0; *T. intermedius*, 30-35°C and pH 5.5-6.0. The pH ranges for growth are pH 1.5-5.5, pH 5.0-7.0, pH 5.7-9.0 and pH5.0 (or lower)-7.5, respectively.

These facultatively chemolithotrophic thiobacilli are short Gram-negative rods. They are capable of growing lithotrophically on sulphur and/or certain inorganic sulphur compounds, but pyrite is not recorded as a substrate (Kelly and Harrison, 1989). They can also grow heterotrophically on various organic substrates. *Thiobacillus intermedius* fails to grow in media containing only a single organic carbon source and no thiosulphate, grows poorly on yeast extract alone, but well in media containing yeast extract, thiosulphate and an additional suitable organic carbon source. The organisms are aerobes, but *T. delicatus* also respires anaerobically using the reduction of nitrate to nitrite as an electron-accepting reaction (Katayama-Fujimura *et al.*, 1984).

The facultatively lithotrophic *Thiobacillus* species may stimulate the oxidation of pyrite and acid mine drainage generation in two ways. Firstly, they oxidize sulphur-containing 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 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).

B.2.2.3. Moderately thermophilic *Thiobacillus* species

Thiobacillus caldus is a moderately thermophilic acidophilic non-iron-oxidizing chemolithotrophic sulphur-oxidizing species (Hallberg and Lindström, 1994). The species description is based on studies of two isolates, strains KU and BC13 from coal wastes. The two strains show 100 % DNA-homology with each other, insignificant homology with other *Thiobacillus* species, but a close relationship by 16S rRNA analysis with *T. ferrooxidans* and *T. thiooxidans*. The G + C content of the DNA is 63-64 mol %. *Thiobacillus caldus* grows aerobically by the oxidation of sulphur, thiosulphate, tetrathionate, sulphide and molecular hydrogen, but not pyrite. It can grow mixotrophically on tetrathionate plus yeast extract or glucose, but not heterotrophically on organic compounds alone. Growth occurs at 32-52°C (optimally at 45°C) and pH 1.0-3.5 (optimally at pH 2.0-2.5).

Strain BC13 is a well studied strain with possible use in the bioleaching of ores (Norris, 1990, 1997). It and other strains of *Thiobacillus caldus* may have potential as a partner for *L. ferrooxidans* in the bioleaching of chalcopyrite or zinc-lead-iron ore concentrates at 35-40°C.

B.2.2.4. Thermophilic sulphur-oxidizing archaea

The thermophilic sulphur-oxidizing archaea of greatest interest in respect of their abilities to release metals from sulphidic ores are those which also oxidize iron and are discussed in detail in section **B.2.1.6.** However, these groups are primarily sulphur oxidizers and not all strains in the groups oxidize iron (Kelly and Harrison, 1989). On account of their more limited lithotrophic oxidation abilities, the strains oxidizing sulphur and inorganic sulphur compounds but not iron seem to have received little attention as potential bioleaching bacteria. A strain designated *Sulfolobus* strain B6-2, which does not oxidize iron, has received some attention, but on the basis of its electrophoretic protein profile it did not appear to be closely related to other *Sulfolobus* species or *Acidianus brierleyi* (Norris, 1990; Norris *et al.*, 1986).

B.2.3. Acidophilic Heterotrophic Bacteria

In this section, only acidophilic heterotrophic bacteria which do not oxidize inorganic iron and sulphur compounds or sulphur, but which may play a role in the generation of acid by mineral-oxidizing bacteria, will be considered.

Acidophilic heterotrophic bacteria occur in acid mine drainage water and have been found in cultures of *T. ferrooxidans* (Harrison, 1981; Johnson and Roberto, 1997; Wichlacz and Unz, 1981), as well as in geothermal sulphur-containing environments. The obligately chemolithotrophic iron-oxidizing bacteria that catalyse the oxidation of pyrite and the subsequent formation of acid mine drainage are very sensitive to inhibition by organic substances (Harrison, 1984; Hallmann, *et al.*, 1992; 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 (*Acidiphilium* sp.) could stimulate the oxidative dissolution of pyrite ores in mixed culture with *L. ferrooxidans,* although not with *T. ferrooxidans.*

B.2.3.1. Mesophilic acidophilic heterotrophic bacteria

Mesophilic acidophilic heterotrophic eubacteria isolated from acid mineral environments and *T. ferrooxidans* cultures have been assigned mainly to the genus *Acidiphilium* (Hallmann *et al.*, 1992; Harrison, 1981, 1984; Johnson and Kelso, 1983; Kishimoto *et al.*, 1993, 1995; Wichlacz *et al.*, 1986) and species of this genus are considered to be important in stimulating the formation of acid mine drainage. Harrison (1989), in Bergey's Manual of Systematic Bacteriology, described members of the genus 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* species 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. The major isoprenoid quinone is ubiquinone with 10 isoprene units and the G + C content of the DNA of *Acidiphilium* species after removal of *Acidocella* (see below) is in the range 63-68 mol % (Kishimoto et al., 1995).

Hallmann *et al.* (1992) found that *L. ferrooxidans* formed flocs in mixed cultures with *Acidiphilium* strains and they suggested that the *Acidiphilium* might also stimulate pyrite oxidation by encouraging adhesion of the iron-oxidizing lithotrophic bacteria to the pyrite surface. Various members of the genus are capable of reducing ferric iron to ferrous iron, which may give them an additional role in the biogeochemical cycling of iron in acid mine drainage environments (Johnson and McGinness, 1991b).

Based on 5S and 16S rRNA analyses, *Acidiphilium* spp. are closely related to the facultatively chemolithotrophic *Thiobacillus* species, *T. acidiphilus* (Lane *et al.*, 1985, 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 species *Acidiphilium facilis* and *Acidiphilium aminolytica.* The distinction between the two genera is based on 16S rRNA analysis, as well as differences in pigmentation, including synthesis of photopigments (*Acidocella* spp. are not pigmented), and susceptibility to inhibition by organic acids. The G + C content of *Acidocella* DNA is 58-64 mol %.

Another genus of mesophilic acidophilic obligately heterotrophic bacteria isolated from acid mine drainage is *Acidobacterium*, represented by the species *Acidobacterium capsulatum* (Kishimoto *et al.*, 1991). The bacteria are encapsulated Gram-negative rods (0.3-0.8 μm x 1.1-2.3 μm) with peritrichous flagella. They grow at pH 3.0-6.0 and 25-37°C, but not at 42°C. They are aerobic, oxidizing various sugars and starch, but not sulphur or ferrous iron. The major isoprenoid quinone is menaquinone, with eight isoprene units, which is a major distinguishing characteristic for the genus and species. The G +

C content of the *A. capsulatum* DNA is 60-61 mol % and it hybridized poorly with the DNA of *Acidiphilium* species.

B.2.3.2. Thermophilic acidophilic heterotrophic bacteria

Three unusual acidophilic, thermophilic *Bacillus* species (*Bacillus acidocaldarius, Bacillus acidoterrestris and Bacillus cycloheptanicus*) have been isolated from acidic hot springs and soils in geothermal areas (Darland and Brock, 1971; Deinhard *et al.*, 1987a,b; Johnson and Roberto, 1997). They are Gram-positive, endospore-forming rods, which grow aerobically, heterotrophically and thermophilically, at temperatures of 45-70°C, 35->55°C and 40-53°C, respectively (Wisotzkey *et al.*, 1992). Their pH ranges for growth are pH 2-6, pH 2.2-5.8 and pH 3.0-5.5, respectively. They differ from other *Bacillus* species in their ability to grow under such highly acidic conditions, and also in the presence of unique ω-cyclohexyl or ω-cycloheptyl fatty acids as the major fatty acid components of their cell membranes. The G + C contents of their DNA are 60-62, 51-53 and 54-57 mol %, respectively.

Phylogenetic evidence from 16S rRNA analyses, as well as the unusual phenotypic characteristics distinguishing these thermoacidophiles from other *Bacillus* species, have led to a proposal for their reclassification in a new genus, *Alicyclobacillus* (Wisotzkey *et al.*, 1992). This genus has a relatively close relationship with the moderately thermophilic iron- and mineral sulphide-oxidizing *Sulfobacillus* species (Johnson and Roberto, 1997).

B.2.3.3. Thermophilic acidophilic heterotrophic archaea

Thermophilic acidophilic heterotrophic archaea which do not oxidize inorganic iron and/or sulphur compounds as sources of energy are found in the genera *Thermoplasma* and *Picrophilus* (Johnson and Roberto, 1997). *Thermoplasma acidophilum* has been isolated from various self-heating coal wastes (Langworthy and Smith, 1989) and with or without *Thermoplasma volcanium*, from acidic hot springs, soils and sediments in solfatara fields (Segerer *et al.*, 1988; Yasuda et al., 1995). *Picrophilus* species have been obtained from Japanese solfatara fields from a hot spring and an extremely acid (pH < 0.5), dry, hot (50-60°C) soil heated by solfataric gases (Schleper *et al.*, 1995). *Thermoplasma acidophilum* was isolated from a similar slightly less acid solfatara soil environment.

Thermoplasma species have no cell wall and a pleomorphic morphology typical of mycoplasmas, forming both spheres and filaments (Segerer *et al.*, 1988). The outer layer is an archaebacterial cell membrane containing ether lipids based on 40-carbon isopranoid-branched diglycerol tetraethers. The organisms are facultative anaerobes, with anaerobic growth being strongly enhanced by elemental sulphur which is respired to hydrogen sulphide. They are obligate thermoacidophiles, growing within the ranges 33-67°C (optimum 57-60°C) and pH 0.5-4 (optimum pH 1-2). They have an absolute requirement for extract of yeast, meat, bacteria or archaea, but various sugars may stimulate growth. The G + C content of the DNA of *T. acidophilum* is 46-49 mol % and that of *T. volcanium* about 38 mol % (Segerer *et al.* 1988; Yasuda *et al.*, 1995).

Picrophilus species, like *Thermoplasma* species, are non-lithotrophic and require factors occurring in yeast extract for growth, which occurs at 45-65°C with 60°C optimal; however, they show certain

molecular differences from *Thermoplasma* species, and are ultra-acidophilic, growing at pH 0-3.5 with pH 0.7 optimal (Schleper *et al.*, 1995). The G + C content of *Picrophilus oshimae* DNA is about 36 mol %, which is lower than that of the two *Thermoplasma* species.

The genera *Thermoplasma* and *Picrophilus* are closely related in the kingdom *Euryarchaeota* and both are well separated from *Sulfolobus* which is placed in the family *Crenarchaeota* (Schleper *et al.*, 1995).

B.3. ECOLOGICAL FACTORS INFLUENCING BACTERIAL ACID MINE DRAINAGE FORMATION IN COAL WASTE

B.3.1. Physical Factors

B.3.1.1. Moisture

Moisture in a coal waste dump is determined by infiltration of atmospheric rainfall into the dump and various other factors, for example, the clay, coal and pyrite content of the dump (Dugan, 1975). Good *et al.* (1970) obtained data indicating that 25% of the applied water infiltrating into a dump emerged as base flow during dry periods. Approximately 75% of the applied water came off immediately as runoff. During periods of high water flow following heavy rainfall, acid may be washed out of dumps in a surge resulting in a period of high input of acid into a drainage area or receiving stream (Dugan, 1975). This problem causes major environmental concern world-wide. However, it can be assumed that coal seldom becomes waterlogged and that moisture evaporates quickly from exposed mine dumps in dry weather.

Humidity influences pyrite oxidation. Borek (1994) reported that different pyrite types weathered differently forming different oxidation products at rates which depended on the humidity (as water vapour). She evaluated six types of pyritic materials under four relative humidities (34%, 50%, 70% and 79%) and found that two of the pyrite samples produced no weathering products at any humidity, a third type of pyrite produced hematite at all humidities, while three sedimentary pyrites produced ferrous sulphates after varying times at the different relative humidities.

Harries and Ritchie (1983) found that the moisture content of the upper 1.0 to 1.5 m of surface waste rock dumps changed with the cycle of wet and dry seasons, decreasing during the dry season. However, although the moisture and other measured physicochemical parameters varied significantly with season, it appeared that there was little variation in the pyrite oxidation rate throughout the year.

Good *et al.* (1970) also found that pyrite oxidation proceeded at a fairly constant rate between periods of precipitation, with the oxidation products accumulating in the outer mantle of the waste dumps. Erosion during periods of precipitation constantly renewed this reactive outer mantle. Although chemolithotrophic bacteria were metabolically active over a wide range of moisture contents (12%-35%), optimal pyrite oxidation rates were found at moisture levels of 23% to 35% (Belly and Brock, 1974). *Thiobacillus ferrooxidans* could survive extended periods without rainfall, then showed increased activity after heavy rains. Rainfall would also flush the bacteria out of dumps, then decrease their concentrations in the drainage water by dilution (Tuttle *et al.*, 1968).

However, Kleinmann and Crerar (1979) concluded that *T. ferrooxidans* would not be active in saturated ground-water environments. In experiments conducted in Montana, Olsen *et al* (1981) found that exposed coal spoils supported very little bacterial pyrite oxidation during the dry season. This was probably related to water stress. *Thiobacillus ferrooxidans* was completely inhibited by a water potential of -23 bars.

B.3.1.2. Temperature

The surface temperature of a mine dump depends mainly on atmospheric weather conditions, including air temperature and humidity. The main bacteria involved in the generation of acid mine drainage in the surface layers of dumps which are not undergoing self heating will be mesophiles, for which the temperature ranges and optima for growth are recorded under section **B.2**. The optimum temperatures for the various groups of mesophiles tend to be about 30°C, but different strains of *T. ferrooxidans* grow over the range 2°C (Leduc *et al.*, 1993) to 40°C (Norris, 1990). *Leptospirillum ferrooxidans* favours higher temperatures within this range, growing well at 40°C but not as well as *T. ferrooxidans* at temperatures below 20°C. Thus, in the field studies of Sand *et al.* (1992) at the Ilba mine in Romania, they detected very few or no *L. ferrooxidans* below 20°C, but this species was usually more abundant at temperatures above 20°C. Other mesophilic acidophilic bacteria (iron- or sulphur-oxidizing or heterotrophic) can likewise grow well in the temperature range 20-40°C, hence temperatures from 20°C to 30-40°C should be favourable for the acidification of pyrite. In Wisconsin coal piles, Belly and Brock (1974) found temperatures of 20-30°C to be optimal for autotrophic microbial activity indicated by $^{14}CO_2$ fixation.

Thermophilic bacteria found in coal and other mineral waste dumps, or even not yet found there but known from other acidic sulphide mineral-containing environments (section **B.2**), could catalyse the acidification of self heating dumps. At temperatures between about 40°C and 50°C, moderate thermophiles, such as *Sulfobacillus* species, *A. ferrooxidans* and *T. caldus*, could fill this role, whereas at higher temperatures thermophilic archaea such as species of *Sulfolobus, Acidianus* and *Metallosphaera* could be catalysts. These archaea should be able to function to 70°C or higher (Norris, 1990, 1997). Great interest has been shown in the possible use of moderately thermophilic eubacteria and thermopilic archaea for the bioleaching of metals from mineral sulphides at high temperatures, thereby increasing extraction rates over those achieved with mesophiles (Brierley, C.L., 1978, 1982; Brierley, J.A., 1990; Norris, 1997). The rise in temperature in self heating coal waste can be expected to affect the acidification rate similarly after natural selection of the responsible thermophiles.

Heterotrophic or facultatively lithotrophic bacteria that may benefit the groups of obligately lithotrophic iron- and sulphur-oxidizing bacteria growing over the different temperature ranges by utilizing possibly inhibitory organic compounds, have been found in coal waste dumps or acid drainage. They include mesophilic heterotrophic *Acidiphilium, Acidocella* and *Acidobacterium* species, mesophilic heterotrophic iron-oxidizing bacteria, mesophilic facultatively lithotrophic *Thiobacillus* species, moderately thermophilic mixotrophic or facultatively lithotrophic *Sulfobacillus* and *Acidomicrobium* species, as well as thermophilic archaea in the genera *Sulfolobus*

and *Thermoplasma* (see section **B.2.**) Thus, coal waste dumps have the potential for the efficient generation of acid drainage from near 0°C to about 70°C.

B.3.2. Chemical Factors

B.3.2.1. pH

The pH of coal waste dumps may vary considerably over small distances according to Harrison (1978), from about pH 5.5 to "hot spots" of about of pH 2.0. At neutral pH the rate of chemical oxidation of pyrite is slow, but exceeds the rate of biological oxidation and may result in a lowering of the pH in a particular area where pyrite is exposed to atmospheric oxygen (Kleinmann *et al.*, 1981). Below pH 4.5 the chemical oxidation rate is very slow, causing the bacterial oxidation to dominate. Ferrous iron is chemically stable and almost non-auto-oxidizable in highly acid conditions below pH 4.0 (Wakao *et al.*, 1991).

The mechanisms for pyrite oxidation shown in section **B.1** indicate various abiotic reactions which can occur during the initial phase of the oxidation above pH 4.5 and which produce ferrous and ferric iron, thiosulphate and its oxidation products, including sulphate, and hydrogen ions which lower the pH. Although biotic ferrous iron oxidation by acidophilic bacteria gathers momentum only below about pH 4.5 (section **B.2.1**), the bacterial oxidation of thiosulphate and its oxidation products by neutrophilic *Thiobacillus* species, such as *T. thioparus* (section **B.2.2.1**), *T.delicatus, T.novellus* and *T.intermedius* (section **B.2.2.2**) can occur from this pH to above neutrality. As the pH drops below 4.5, iron-oxidizing bacteria of the genus *Metallogenium*, active at pH 3.5-4.5, could be involved in the acceleration of pyrite oxidation, although their crucial role in initiating biotic acidification as postulated by Walsh and Mitchell (1972) and Walsh (1978) could not be confirmed by Kleinman and Crerar (1979). This postulated role of *Metallogenium* also does not seem necessary, as strains of many of the acidophilic iron-oxidizing, sulphur-oxidizing and heterotrophic bacteria are able to grow at pH 4.5 or higher (section **B.2**). Decline of the pH to about pH 2 by the combined effects of the abiotic and biotic reactions involved in pyrite oxidation under the increasingly acidic conditions, makes the environment approximately optimum for the growth of many of these bacteria and hence for their catalytic activities.

B.3.2.2. Oxygen and carbon dioxide

Several models of oxygen penetration into various parts of coal waste dumps have been proposed. Good *et al.* (1970), Dugan (1975) and Erickson (1985) proposed the concept of an oxygen "barrier" which usually would not allow oxygen to penetrate into the pile beyond a depth of 20-30 cm. This "barrier" is composed of sediments of fine clayey material which have been compacted by water movement resulting from rain and other forms of precipitation (Dugan, 1975). Nicholson *et al.* (1989) noted that molecular diffusion through pores partially filled with gas is the most important mechanism for oxygen transport in mine waste with the rate of diffusion depending largely on the pore size between dump particles. The subsequent rate of oxidation of ferrous sulphides, such as pyrite, with generation of a low pH effluent containing high concentrations of sulphates, ferric iron

and trace metals, is controlled by the availability of oxygen at the sulphide surface (Elberling *et al.*, 1994).

Good *et al.* (1970) reported an oxygen "barrier" 2.5 cm (or more) thick in a coal waste dump, some 10-25 cm below the outer surface of the dump. However, Erickson (1985) reported oxygen concentrations of up to 20% to depths of several metres into a coal refuse dump. She noted that the oxygen content decreased with depth. This profile appeared to be consistent with oxygen diffusion from the atmosphere and oxygen consumption within the refuse pile. Seasonal changes in oxygen profiles were also noted. Higher concentrations of oxygen observed at greater depths in winter than in warmer seasons may have been due to decreased chemical activity of the coal waste in the colder winter months.

Harries and Ritchie (1983) and Goodman *et al.* (1983) have reported on oxygen and carbon dioxide concentrations in two metal sulphide-containing waste rock dumps at Rum Jungle in Australia's Northern Territory. Oxygen and oxidation of the minerals were detected to depths of 5-6 m or greater, but the oxygen concentration declined markedly through the upper 0.5 m. Below 0.5 m the oxygen concentrations in the gas atmosphere were frequently less than 0.1% by volume. However, carbon dioxide concentrations were high, often more than 2% by volume. Carbon dioxide levels were low when oxygen levels were high and *vice versa.* The oxygen content of the waste dumps did not show seasonal changes. This result differs from the seasonal differences observed by Erickson (1985) but the respective studies were conducted in vastly different climate zones, where rainfall and temperature differed, as well as with different waste material in the dumps.

The involvement of gaseous oxygen in the abiotic and biotic oxidation of pyrite, other metal sulphides and their oxidation products is evident from sections **B.1** and **B.2**. The most important responsible bacteria are aerobic chemolitho-autotrophs, with a respiratory metabolism and a requirement for carbon dioxide as their main carbon source. However, *T. ferrooxidans* and various other species among them are able to oxidize sulphur and certain other substrates as sources of energy under anaerobic conditions using ferric iron as the electron acceptor instead of oxygen. These organisms therefore have the potential for survival and possibly limited growth under microaerophilic and anaerobic conditions, hence the low or negligible concentrations of oxygen through most of the dump material (10-100 times lower than those of the atmosphere in the Rum Jungle dumps) may not entirely eliminate acid and sulphate generation or the solubilization of metal sulphides in the presence of ferric iron. The high carbon dioxide levels in the dumps (20-100 times greater than atmospheric) are highly favourable for the growth of autotrophic bacteria, for example, Barron and Lueking (1990) found that a carbon dioxide concentration of 7-8% could be advantageous to the growth of *Thiobacillus* spp., although higher concentrations could be detrimental.

Harries and Ritchie (1983) observed heat production, indicative of oxidation, in the Rum Jungle waste rock dumps at depths which correlated with the transport of oxygen into the dumps, while Goodman *et al.* (1983) found high populations of *T. ferrooxidans* at depths of at least 3 m. This distribution of *T. ferrooxidans* supports the conclusion in the previous paragraph that maintenance or even growth of bacteria catalysing the oxidation of metal sulphides can take place in the

microaerophilic to anaerobic zone of a dump below the well aerated surface layer. However, maximal carbon dioxide uptake ascribed to organisms such as *T. ferrooxidans* occurred within the first 8-10 cm from the air-dump interface in coal refuse material of 3-5 years old in Wisconsin (Belly and Brock, 1974), indicating the favourable effect of aeration on the development of the bacteria. Although Walsh (1978) stated that mine sealing was not effective because the amount of oxygen required for the oxidation-reduction cycles of pyrite degradation was very small, Kleinmann and Crerar (1979) noted that acid drainage could be reduced by flooding a redundant mine or by air-sealing it as suggested by Colmer and Hinkle (1947), thereby reducing the amount of oxygen available.

B.3.2.3. Substrate availability

Thiobacillus ferrooxidans is a chemolithotroph that can utilize the energy released by the oxidation of ferrous to ferric iron as its sole source of energy and an autotroph that uses atmospheric carbon dioxide as its sole source of carbon (section **B.2.1.1**). Iron pyrite, found commonly in sulphide ores and coal waste, serves as an important source of ferrous iron for *T. ferrooxidans*. In bioleaching processes, ferrous iron provides a soluble substrate for growth of *T. ferrooxidans*, whereas minerals such as pyrite and chalcopyrite ($CuFeS_2$) are insoluble substrates (Devasia *et al.*, 1993) from which ferrous iron is released by chemical reactions (section **B.1**).

Sulphur in coal is usually present in organic combination as part of the coal substance and in inorganic combination as pyrite or marcasite and as calcium sulphate in weathered coals (Colmer and Hinkle, 1947). Commercial coals of the Eastern United States contain from 0.5-1.5% sulphur. Olsen *et al.* (1981) noted that the sulphur in two major coal seams at Colstrip, Montana, averaged 1.07% and 1.87%. Coal refuse obtained from New Lexington, Ohio, by Dugan and Apel (1983) for leaching experiments had 6-8% total sulphur.

In bacterial ore leaching experiments, surface area of the ore determined the yields and extraction rates of metals (Lundgren and Silver, 1980). A reduction in size of the ore particles increased the surface area of the ore and also increased the degree of separation of the ore into soluble and insoluble constituents by the percolation of water.

If we assume that intimate contact of bacteria with a sulphide mineral is necessary to catalyse its oxidation, larger particles will be limiting only if all the potential reaction sites are occupied by active bacteria so that excess active bacteria cannot be accumulated on the surface of the mineral (Ehrlich and Fox, 1967). A reduction in the size of particles of low grade metal ores may expose new metal sulphide sites and increase host rock reaction sites, which would affect leaching beneficially.

B.4. AN ECOLOGICAL APPROACH TO CONTROL OF ACID DRAINAGE FROM COAL WASTE DUMPS: REDUCTION OF WATER AND OXYGEN INFILTRATION BY MEANS OF SOIL AND/OR CLAY LINERS AND COVERS

The most efficient way of storing large quantities of coal and coal waste is placement on the ground (Olem *et al.*, 1983). However, water percolating through material containing large populations of acid drainage-causing microorganisms results in serious pollution of not only streams and rivers, but also the surrounding soil. The soil beneath a dump might be protected by the use of an impermeable liner. Benson and Daniel (1994a) evaluated various compacted soil liners to determine the minimum thickness needed to effectively prevent water moving into and out of the enclosed waste material. They defined a compacted soil liner as an earthen barrier used to minimize the movement of liquid into or from a landfill, surface impoundment, or other facility that can contaminate groundwater. For a soil liner to perform adequately it must be physically stable (cover, foundation and sideslopes), permit leakage only after a relatively long time and have a small flux subsequent to the commencement of leakage.

Depending on the soil structure and compaction of the liner, liquid moving through the soil flows in a spatially variable continuum or through relatively large and widely spaced pores called macropores. These macropores are irregular in size, distribution and orientation and although they represent only a small percentage of the total volume of soil, they act as discrete channels for water flow. Both flow processes were modeled. Benson and Daniel (1994b) showed from models and field data that soil liners with a thickness of 30 cm or less displayed greater hydraulic conductivity than liners that were at least 60 cm thick. A minimum thickness for compacted soil liners, that would allow for low vertical water movement, seemed to be 60-90 cm.

Suitable soil and/or clay covers may be used to minimize gas exchange with the atmosphere as well as water infiltration and movement through dumps (Koerner and Daniel, 1992). Soil covers have not been applied extensively on tailings because of high cost and lack of reliable methods for the prediction and evaluation of their effectiveness. In Canada, the problem of acid mine drainage has been estimated to involve some 12 000 ha of tailings with a rehabilitation cost of $3 billion-$5 billion over two decades (Yanful, 1993).

Yanful (1993) conducted a set of experiments to evaluate the performance of a three-layer sand-clay-sand cover on tailings in laboratory columns and in the field. The sands functioned as capillary barriers in preventing the clay from losing moisture by drainage and evaporation. He found that the diffusion of oxygen to the tailings was largely prevented by a 30- or 60-cm clay layer (in the column and field studies, respectively), which reduced the concentration from 20-21% (v/v) in the upper sand layer to 1-2% (v/v) in the lower sand layer. A decrease in the movement of oxygen (over time) with an increase in water saturation was noted in diffusion studies in the laboratory. Another important factor to consider in connection with oxygen diffusion in soils, is the rate of oxygen consumption by chemical and biological processes within the soil mass. Microbial processes in soil are responsible for the greatest uptake of oxygen. Dissolved oxygen played a minor role in the oxidation of tailings. Thus, the elimination or control of gaseous oxygen diffusion

into tailings is the key requirement in any sulphidic tailings management strategy. The effect of water on the clay covers, be it from precipitation or run-off, must also be considered.

Liner and cover systems serve to avoid the transfer of contaminants into the adjacent soil and biosphere, and also prevent the infiltration of rain water into the waste, thereby limiting the risk of ground water and surface water contamination (Melchior *et al.*, 1993). Although there have been many studies performed on the effectiveness of compacted clay liners and covers for landfill and waste pile rehabilitation, there are still problems associated with the usage of these materials as cover systems. Thus, compacted clay will tend to desiccate from above or below and crack unless adequately protected. It is also vulnerable to damage from freezing and must be protected by a suitably thick layer of cover soil. Compacted clay liners are difficult to repair if they desiccate, crack or are penetrated. Woods (1992) agreed that channeling may cause short circuiting in covers on waste material.

Clay not only cracks due to loss of water but also swells when enough water is present. Therefore, the placement of a cover in a condition of high water saturation and close to maximum density is essential if water infiltration into the cover is to be reduced (Yanful, 1993). Environmental conditions such as climate, ground water, drainage, regulation cover, and field permeability determine the actual amount and rate of swell. Yanful (1993) found that a thickness of about 30 cm of clay could greatly reduce the gaseous oxygen flux to tailings and thereby should greatly reduce the acid flux, but 60 cm would give an added margin of safety in the field.

From the studies described in this section, 30 cm of compacted soil or clay are not considered effective enough in the reduction of water and oxygen movement to underlying material to be recommended for use in the field as liners or covers. A thickness of 60-90 cm is recommended for adequate reduction of the infiltration of both. From these basic studies, the cover thickness of 1 m (70 cm clay plus 30 cm top-soil) used by the Department of Water Affairs and Forestry for dump rehabilitation in Northern Kwazulu-Natal would appear to be adequate. Whether this is indeed the case and whether thinner and cheaper covers might provide adequate barriers under the local conditions were investigated in the present study.

The effect of vegetation on soil covers must not be overlooked. In studies performed on landfill covers by Nyhan *et al.* (1990), it was found that fully established vegetation on the covers decreased soil water content during the summer and spring months. Very little leachate was produced at these times. Although vegetation and top-soil covers temporarily store the atmospheric water input, most of the water is subsequently returned to the atmosphere by means of evapotranspiration.

Mentis (1999) has reported on extensive field experiments to evaluate the success of rehabilitation of opencast coal mines on the Mpumalanga highveld by the establishment of grass pastures on the new topsoil-covered surfaces of the mineral areas. Smuts finger grass (*Digitaria eriantha*) and love grass (*Eragrostis curvula*) on suitably limed and fertilized soils established themselves the most successfully to yield pastures which could be grazed (to their benefit) to yield an estimated 140 000 tons of beef annually from the 2000 km^2 which may ultimately be affected by opencast mining.

C. RESEARCH: PART 1. ABIOTIC ECOLOGICAL DETERMINANTS (TEMPERATURE, MOISTURE, OXYGEN, CARBON DIOXIDE AND pH) AND ACID-PRODUCING BACTERIA IN PILOT SCALE COAL WASTE DUMPS IN RELATION TO COVERS USED FOR DUMP REHABILITATION

C.1. INTRODUCTION

The pilot scale study of coal waste dump rehabilitation techniques started in 1993, with the construction of ten mini-dumps (cells) near the Kilbarchan Mine in northern KwaZulu-Natal by the Department of Water Affairs and Forestry. Our part of the study was to investigate the effect of the various treatments and cover compositions on the acidification of coal waste and the microbial populations that could be involved in accelerating the process, in relation to ecological determinants such as moisture, oxygen, carbon dioxide and pH in the coal waste. It was hoped that results from this study would provide guidelines for the rehabilitation of coal waste dumps and the prevention of acid mine drainage.

C.2. MATERIALS AND METHODS

C.2.1. Materials and Construction of Pilot Scale Dumps

C.2.1.1. Coal waste

The coal waste used for the construction of the pilot scale cells, obtained from the Kilbarchan Mine main dump (which has now been rehabilitated), showed a particle size distribution in screen analysis of 4.36% > 3 mm (cross-section), 18.12% 1-3 mm, 39.93% 0.5-1 mm, 34.23% 0.075-0.5 mm and 3.36% < 0.075 mm (data supplied by T. D'Oliviera of Wates, Meiring & Barnard, Marshalltown). A more detailed analysis presented by Wates and Rykaart (1999) with different boundaries for the size fractions showed 10% > 2 mm, 40% 0.425-2 mm, 38% 0.075-0.425 mm and 12% < 0.075 mm with 5% in the 0.002-0.05 mm "silt" fraction and 3% in the <0.002 mm "clay" fraction.

Two samples of the coal waste were analysed by Trans-Natal Laboratories for the calorific value, proximate analysis and sulphur content (Wates, Meiring and Barnard, 1995b; Wates and Rykaart, 1999). The analytical data are shown in Table 1. Additional analysis of the sulphur content of two samples of the coal waste by Mrs. L. Strydom of the Department of Metallurgical Engineering, University of Stellenbosch, showed 4.15% and 0.81% sulphur. The analyses were performed using a Leco Sulphur Analyzer (Leco Corporation, London, U.K.), which combusted the coal in a stream of oxygen to yield sulphur dioxide. The latter was titrated with iodine, produced as required for the titration from potassium iodate, the consumption of which was measured.

Table 1. Calorific value, proximate and sulphur analyses of coal waste used to construct the cells of the pilot scale dump rehabilitation experiment near the Kilbarchan Mine (Wates, Meiring and Barnard, 1995b)

Sample No	Calorific Value (Mj/kg)	Inherent Moisture (%)[a]	Ash Content (%)	Volatile Content (%)	Fixed Carbon (%)	Total Sulphur (%)
1	14.69	1.9	48.1	16.4	33.7	1.54
2	18.27	1.5	40.8	18.8	39.0	0.91

[a] All percentage values are g/100 g dry coal waste.

Table 2. Chemical analyses of coal waste used to construct the cells of the pilot scale dump rehabilitation experiment near the Kilbarchan Mine[a]

Analysis	Water-soluble components (aqueous extract)	Components liberated by H_2O_2 oxidation
Elemental analysis (mg/kg):		
Al	0.0	693.0
B	3.3	0.0
Ba	1.0	0.0
Ca	3380.0	2700.0
Cd	0.0	4.3
Co	0.0	3.5
Cu	0.0	6.3
Fe	0.9	9929.1
K	68.8	135.1
Mg	284.2	290.5
Mn	13.4	48.4
Na	141.5	126.1
Ni	0.0	0.0
Sr	72.9	35.0
Zn	0.7	7.7
Acid potential as $CaCO_3$ (mg/kg)[b]	0	24500
Base potential as $CaCO_3$ (mg/kg)[b]	540	240
pH	7.20	1.90

[a] Analysed in August 1993 by Professor F.D.I. Hodgson and colleagues, Institute for Ground Water Studies, University of the Orange Free State, Bloemfontein.

[b] Acid and base potentials were determined at pH 7.0.

Chemical analyses of the coal waste by Professor F.D.I. Hodgson and colleagues at the Institute for Ground Water Studies, University of the Orange Free State, Bloemfontein, are shown in Table 2. The acid potential which appeared on oxidation of the waste by excess hydrogen peroxide (neutralized by 24 500 mg $CaCO_3$/kg) is equivalent to 0.77% unoxidized sulphur in the untreated coal waste (an additional 0.35% sulphur was present as water-soluble sulphate). This acid potential far exceeded the total base potential (equivalent to 780 mg $CaCO_3$/kg), indicating that chemical and bacterial mechanisms for the generation of acidity from the sulphur would result in the production of acid drainage.

Analyses in April 1995 by Lorentz *et al.* at the Department of Agricultural Engineering, University of Natal (Wates, Meiring and Barnard, 1995a) of the physical characteristics of the coal waste incorporated into the pilot scale dumps are shown in Table 3. More than 90% of the waste was of sand particle size or larger (> 0.05 mm cross-section) and it contained very little material of silt and clay particle size. Cell 1 with the uncompacted coal waste had the lowest bulk density (0.99 g/cm^3) and the highest porosity (0.33 cm^3/cm^3). However, the bulk density of the uncompacted coal waste of cell 3 (1.07 g/cm^3) was almost the same as that of the compacted coal waste in cell 2 (1.06 g/cm^3). The compacted and both uncompacted coal waste samples showed a similar particle density (1.68-1.69 g/cm^3), but against expectation a lower porosity was recorded for cell 3. No clear trends were therefore observed in these physical parameters of the uncompacted and compacted coal waste in spite of differences in the measured values. The low porosity values for the coal waste coupled with low bulk density values are the result of the low particle density of the coal caused by its high carbon content (Wates, Meiring and Barnard, 1995a).

The permeability of uncompacted and compacted coal waste determined *in situ* in the pilot scale dumps (Wates and Rykaart, 1999) is shown in Table 4. The compacted waste showed a lower permeability than the uncompacted waste, but its permeability was still high.

In studies of the hydraulic properties of the coal waste performed by Lorentz *et al.* (Wates, Meiring and Barnard, 1995a,b) it was found that the coal waste retained about 40% of the pore water at a matric pressure head of 100 cm, with the compacted and uncompacted waste showing similar retention characteristics. Also, the saturated hydraulic conductivities of the compacted and uncompacted coal waste were rather similar, although the hydraulic conductivity for the uncompacted waste at the small matric pressure head of 1 cm was several times higher than that of the compacted coal waste. However, with further reduction in water content and increase in matric pressure, the hydraulic conductivity of the compacted and uncompacted coal waste became similar.

C.2.1.2. Soil cover materials

Table 5 shows various physical characteristics of the Estcourt and Avalon soils (MacVicar *et al.*, 1977; Soil Classification Working Group, 1991) used to construct the pilot scale dump covers and the 1-m thick walls of compacted Estcourt soil separating the 10 cells, as determined by Lorentz *et al.* (Wates, Meiring and Barnard, 1995a). Both soils were available at the site of the pilot scale dumps. The two soils had an approximately similar texture, comprising 37-47% sand, 20-29% silt

Table 3. Physical characteristics of coal waste of pilot scale dumps (Wates, Meiring and Barnard, 1995a)

Cell	Layer[a]	% "Sand" and larger (>0.05 mm)	% "Silt" (0.002-0.05 mm)	% "Clay" (<0.002 mm)	Bulk Density (g/cm^3)	Porosity (cm^3/cm^3)	Particle Density (g/cm^3)
1	CU	91	6	3	0.99	0.33	1.69
2	CC	91	6	3	1.06	0.30	1.68
3	CU	93	5	2	1.07	0.25	1.69

[a] CU = uncompacted coal waste;
CC = compacted coal waste.

Table 4. Permeability (saturated hydraulic conductivity) of coal waste of pilot scale dumps measured *in situ* by three infiltration procedures (Wates and Rykaart, 1999)

Cell	Layer[a]	Permeability (cm/sec) measured using		
		Large[b] double ring infiltrometer	Small[c] double ring infiltrometer	Guelph permeameter
1,3[d]	CU	1.00×10^{-2}	6.08×10^{-3}	5.65×10^{-3}
2	CC	2.78×10^{-3}	4.47×10^{-3}	5.00×10^{-3}

[a] CU = uncompacted coal waste;
CC = compacted coal waste.
[b] Diameter 1000 mm.
[c] Diameter 300 mm.
[d] The permeability values are means of the values recorded for these two cells.

and 30-34% clay. The Avalon soil could be classified according to the texture chart of the Soil Classification Working Group (1991) as a clay-loam and the Estcourt soil as a sandy clay-loam (although near the clay-loam boundary).

The lowest bulk density (1.54 g/cm^3) and the highest porosity (0.39 cm^3/cm^3) were recorded for the uncompacted Avalon soil in cell 4 (Table 5). The process of compaction considerably reduced this porosity of the Avalon soil through reduction of the larger pores present in the uncompacted material (Wates, Meiring and Barnard, 1995a). The compacted Estcourt soil had the highest bulk density (1.78 g/cm^3), almost the highest particle density (2.55 g/cm^3) and the lowest porosity (0.30 cm^3/cm^3) of the soils used. Nonetheless, its porosity was similar to that of the compacted coal waste of cell 2 and higher than that of the uncompacted waste of cell 3.

Although the Estcourt soil had no more clay, less silt and more sand than the Avalon soil, it was used as the lower barrier or "clay" layer where both soils were used in a cover and for the walls forming barriers to water movement between the cells. The permeability (saturated hydraulic conductivity) of uncompacted Avalon soil (Table 6) was rather similar to that of compacted coal waste (Table 4) and was one to two orders of magnitude higher than that of the compacted Estcourt soil. The compacted Avalon soil also had a higher permeability than the compacted Estcourt soil, especially in the tests with the small double ring infiltrometer and Guelph permeameter where the difference was about an order of magnitude. Besides this permeability difference, the Estcourt soil also showed higher water retention than the Avalon soil (Wates, Meiring and Barnard, 1995a,b; Wates and Rykaart, 1999). Thus, with a matric pressure head of 6000 cm, the residual water contents of compacted Estcourt and Avalon soils were 90% and 60% of the saturation contents, respectively.

C.2.1.3. Construction of pilot scale dumps

The pilot scale dumps were constructed as ten 10x10x3-m cells separated by 1-m-thick walls of compacted Estcourt soil (Fig. 6, 7). The construction of the dumps, except for the establishment of vegetation (see section **C.2.1.4**), was completed in August 1993.

Cells 1-8 had approximately horizontal tops with a drainage slope of only 1 in 50, whereas cells 9 and 10 had surface slopes of 1 in 10 and 1 in 5, respectively. The construction of the ten cells was as follows (measurements are depths of coal waste and cover materials):

1. 3.0 m uncovered uncompacted coal waste.
2. 3.0 m uncovered compacted coal waste.
3. 3.0 m uncovered uncompacted coal waste which was limed, fertilized and vegetated.
4. 2.7 m uncompacted coal waste covered with 0.3 m uncompacted Avalon soil and vegetated.
5. 2.5 m uncompacted coal waste covered with 0.5 m compacted Avalon soil and vegetated.
6. 2.0 m uncompacted coal waste covered with 0.7 m compacted Estcourt soil and 0.3 m uncompacted Avalon soil and vegetated.

Table 5. Physical characteristics of cover materials of pilot scale coal waste dumps (Wates, Meiring and Barnard, 1995a)

Cell	Layer[a]	% Sand (0.05-2.00 mm)	% Silt (0.002-0.05 mm)	% Clay (< 0.002 mm)	Bulk Density (g/cm^3)	Porosity (cm^3/cm^3)	Particle Density (g/cm^3)
4	AU	38	28	34	1.54	0.39	2.53
8	AU	40	29	31	1.66	0.34	2.51
5	AC	37	29	34	1.64	0.34	2.50
7	AC	44	26	30	1.69	0.35	2.60
6	EC	47	20	33	1.78	0.30	2.55

[a] AU = uncompacted Avalon soil;
AC = compacted Avalon soil;
EC = compacted Estcourt soil.

Table 6. Permeability (saturated hydraulic conductivity) of cover materials of pilot scale coal waste dumps measured *in situ* by three infiltration procedures (Wates and Rykaart, 1999)

Soil layer	Permeability (cm/sec) measured using		
	Large[a] double ring infiltrometer	Small[b] double ring infiltrometer	Guelph permeameter
Avalon soil, uncompacted	2.72×10^{-3}	3.18×10^{-3}	2.28×10^{-3}
Avalon soil, compacted	4.17×10^{-4}	3.63×10^{-4}	3.00×10^{-5}
Estcourt soil, compacted	3.33×10^{-4}	1.22×10^{-5}	5.00×10^{-6}

[a] Diameter 1 000 mm.

[b] Diameter 300 mm.

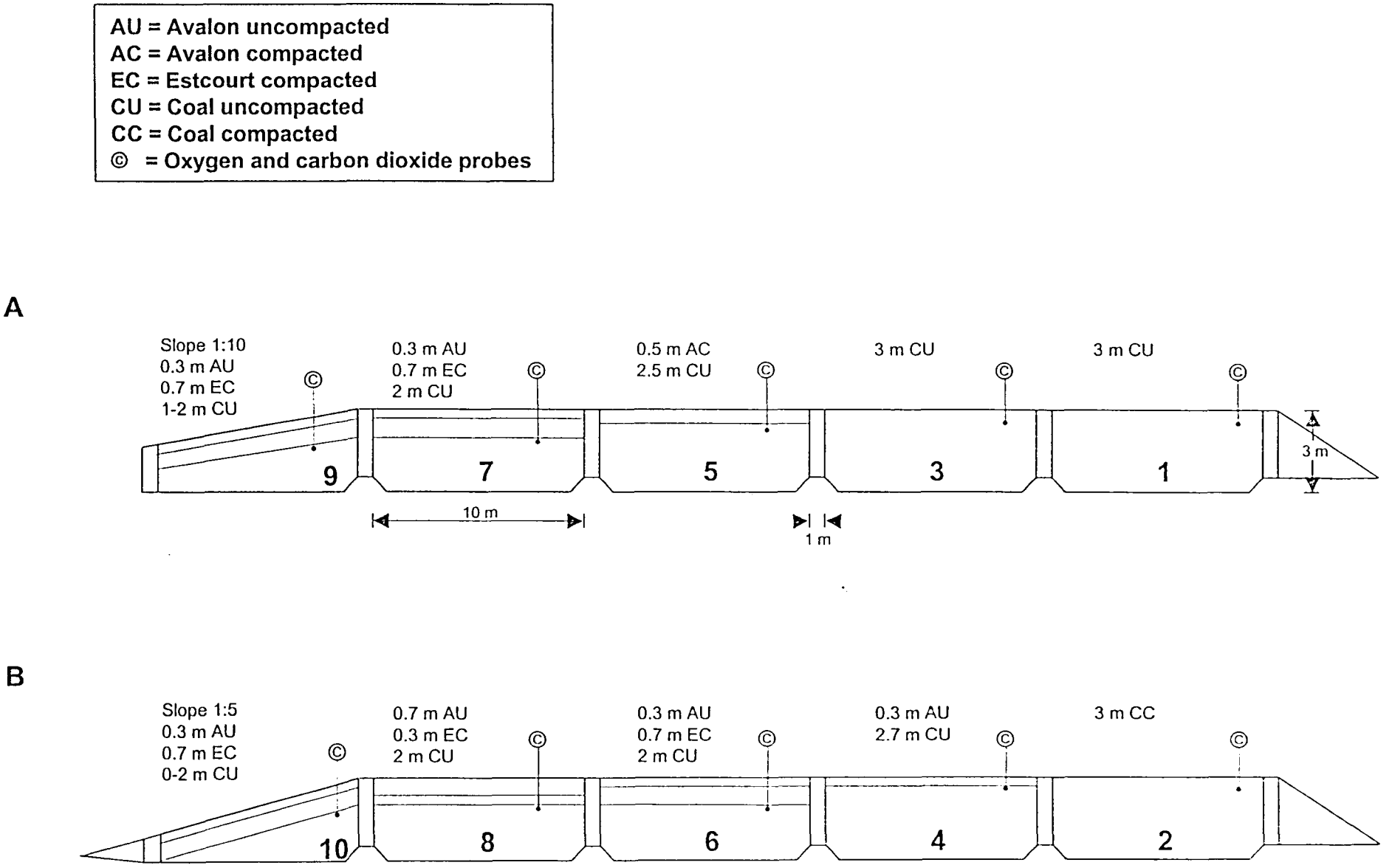

Fig. 6. Cross section through mini-dumps (cells) 1,3,5,7 and 9 (A) and 2, 4, 6, 8 and 10 (B) in pilot scale coal waste dump rehabilitation study.

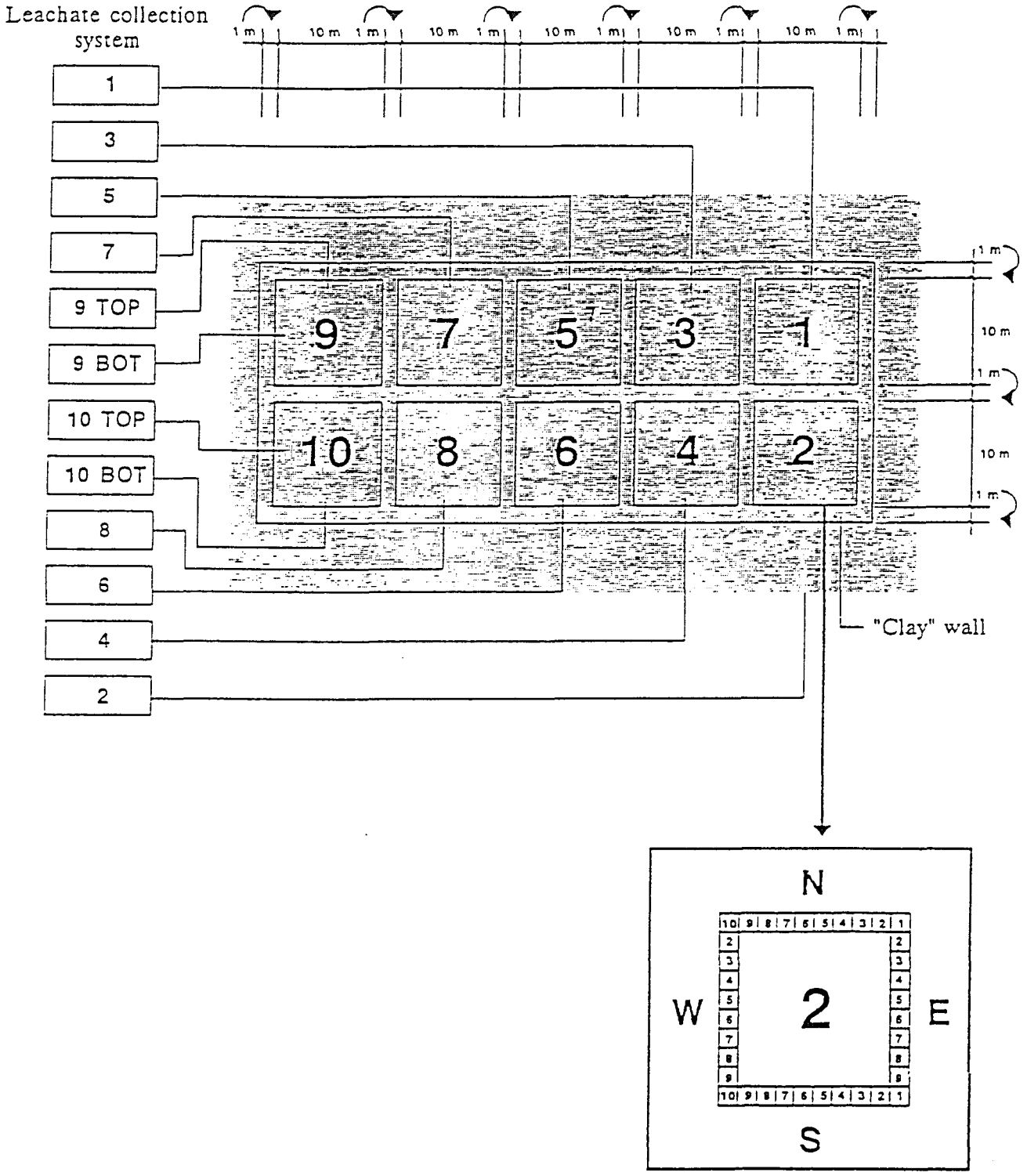

Fig. 7. Plan of mini-dumps (cells) in pilot scale waste dump rehabilitation study with sampling points indicated on cell 2.

7. 2.0 m uncompacted coal waste covered with 0.7 m compacted Avalon plus 0.3 m uncompacted Avalon soil and vegetated.
8. 2.0 m uncompacted coal waste covered with 0.3 m compacted Estcourt soil and 0.7 m uncompacted Avalon soil and vegetated.
9. 1.0-2.0 m uncompacted coal waste covered with 0.7 m compacted Estcourt soil and 0,3 m uncompacted Avalon soil, both sloped at 1 in 10, and vegetated.
10. 0-2.0 m uncompacted coal waste covered with 0.7 m compacted Estcourt soil and 0.3 m uncompacted Avalon soil, both sloped at 1 in 5, and vegetated.

The cells were constructed on plastic lining allowing a hollow for the collection of drainage water and each was supplied with two probes per cell for the measurement of oxygen and carbon dioxide in the upper 15 cm of coal waste, overlapping with the depths from which samples for moisture, pH and microbiological studies were taken. A railway container was placed on the site and pipes were installed during the construction of the cells to convey leachate from the cells to collection drums in the container, for determination of outflow volumes and chemical analyses in project K5/575 (Wates, Meiring and Barnard, 1993, 1995a,b; Wates and Rykaart, 1999). In February 1995, the collection drums were replaced with tipping buckets equipped with counters to measure the outflow volumes.

C.2.1.4. Vegetation of dumps

Since vegetation may play an important role in the stabilisation and prevention of erosion of the cover material and also reduce the flow of water into the coal waste, cells 3-10 were seeded with a grass cocktail in November 1993. The seed cocktail contained the tufted species *Cenchrus ciliaris, Chloris gayana* (which also forms stolons), *Digitaria eriantha, Eragrostis curvula, Eragrostis tef* and *Paspalum notatum,* as well as *Cynodon dactylon* and *Pennisetum clandestinum* which are characterized by stolon formation. Each seed was supplied at 5 kg/ha. No lime or fertilizer was added. Because the vegetation from this seeding never established well, the treatment was repeated in October 1994 by the Chamber of Mines Vegetation Unit. At this seeding cell 3 was limed (2 tons/ha) and the seeds raked in with the lime, but they never germinated fully and subsequently died off. On cells 4-9, the vegetation cover from the reseeding established well, attaining a cover percentage of 90% with an average grass height of 50 cm by February 1995. However, an uneven distribution caused by erosion of seed to the bottom of the slope was noted on cell 10, with the highest (more than 95%) and the lowest (less than 5%) vegetation cover at the bottom and the top of the slope, respectively. The Chamber of Mines Vegetation Unit therefore again seeded the cells in November 1995. All vegetated cells were fertilized with 500 kg/ha 2:3:2 N-P-K fertilizer. Bare areas on the vegetated cells were lightly scarified, seeded with 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. The 1995/1996 rainy season was particularly wet and the grass covers on the cells became well established. The tufted grasses established themselves better than the stoloniferous species, in agreement with experience in

rehabilitated mined areas on the Mpumalanga highveld (Mentis, 1999). Additional details of the grass establishment are provided by Wates and Rykaart (1999).

C.2.2. Studies of Abiotic Ecological Determinants

C.2.2.1. Temperature measurements

C.2.2.1.1. Atmospheric temperature. A weather station erected on the site measured the atmospheric temperature on an hourly basis and logged the data into a computer.

C.2.2.1.2. Soil and coal waste temperatures. Temperatures at the interface of the Avalon and Estcourt layers of the soil cover, as well as in the Estcourt layer and in the coal waste of cell 6 were measured by three probes installed at depths of 0.30 m, 0.72 m and 1.65 m, respectively, from the surface of the cell. The temperatures were recorded every 2 min and hourly averages logged into the computer with the atmospheric temperature data from the weather station (Wates, Meiring and Barnard, 1995a,b; Wates and Rykaart, 1999)

C.2.2.2. Rainfall

Rainfall was measured weekly in two rain-gauges on the pilot scale dumps and the mean calculated (differences between the duplicate readings were rare and slight). The gauges had a capacity of 110 mm. The rainfall of weeks where the precipitation exceeded this capacity was noted as more than 110 mm.

C.2.2.3. Oxygen and carbon dioxide in the coal waste

Two gas probes (37-mm-diameter x 100-mm-long sintered steel cylinders with 20-μm pores) were buried in the upper 15 cm of coal waste in each cell. Each probe was connected to the surface by means of an 8-mm-diameter stainless steel tube which was plugged at the top by a rubber stopper.

Oxygen and carbon dioxide were measured by means of a Gastechtor Model 3252-OX portable carbon dioxide/oxygen monitor (Gas Tech Inc., Newark, California) which was connected for the determinations to the unstoppered probe tube. By means of its pump the instrument extracted a sample of the atmosphere around the probe. The oxygen and carbon dioxide were measured weekly from October, 1993 (cells 1-8) or January, 1994 (cells 9 and 10) by Messrs Yoshan Nehro and Rishi Luckan of the Dundee office of the Department of Water Affairs and Forestry. Due to malfunctions in the portable carbon dioxide/oxygen monitor they could take no readings from March to May 1995 and February to May 1996.

C.2.2.4. Sampling and analysis of coal waste for moisture and pH

C.2.2.4.1. Sampling. Samples were taken from the completed cells on 27 and 28 September 1993 and every 6-8 weeks thereafter. Two samples were taken at each sampling from randomly selected sites about 0.5 m from the perimeter of each cell. At the first sampling coal waste was removed to a depth of 15-30 cm into the waste layer using a bucket corer (Thompson type) with internal diameter 4.4 cm from Johnson Soil Augers (Bergvlei, Gauteng). Thereafter a clay auger (Dutch or

Edelman type) with internal diameter 4.4 cm was used. On cells 4-10, the soil or soils of the cover had first to be removed by the same auger to permit extraction of the coal waste. After two samples were taken from the same cell, the auger was disinfected with 96% ethanol to prevent cross-contamination between the cells by the microbes under investigation. The holes created in the cells by the sampling procedure were subsequently filled with the same layers of material and compacted well using a broom handle.

Coal waste beneath soil covers was sampled to a depth of 15-30 cm beneath the cover. However, as the upper 15 cm of coal waste in the uncovered cells was considered too dry during much of the year for the maintenance of large numbers of viable iron-oxidizing bacteria, it was sampled at depths between 15 and 30 cm below the surface of the waste. The coal waste samples were placed in sterile bottles, transported to Stellenbosch by air on ice in a cooler box, then stored at 4°C until they were analysed. Analyses of moisture content, pH and populations of various groups of ferrous iron-oxidizing, thiosulphate- and sulphur-oxidizing bacteria (see sections **C.2.3.4 - C.2.3.6**) were undertaken within 3 days of sampling.

C.2.2.4.2. Moisture analyses. Moisture was determined by drying 50 g of coal waste overnight at 105 °C. The dried material was cooled in a desiccator and reweighed. The moisture content of a sample was the mean of two determinations expressed as g moisture/100 g dry coal waste.

C.2.2.4.3. pH analyses. For pH determinations, 10 g of coal waste were suspended in 25 ml of distilled water and stirred periodically for at least 1 hour. The pH of the water phase was measured using a Beckmann pHI^{TM}43 or A32 pH meter (Beckmann Instruments, Fullerton, California). The pH of a sample was the mean of two determinations.

C.2.3. Microbiological Studies

C.2.3.1. Experimental approach

As *T. ferrooxidans* has been generally regarded as the main acid-generating chemolithotrophic bacterial species in coal waste dumps (Evangelou and Zhang, 1995; Harrison, 1978; Kleinmann *et al.*, 1981), populations of this organism were monitored by most probable number (MPN) determinations in samples of the coal waste before construction of the mini-dumps and in 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, containing a high concentration (8900 mg/l) of ferrous iron as the energy source.

Acidophilic sulphur-oxidizing bacteria, with *T. thiooxidans* the major organism (also an oxidizer of thiosulphate), might also play a role in acid formation (Norris and Kelly, 1982), as might non-acidophilic sulphur (and thiosulphate)-oxidizing bacteria before the pH of the coal waste became too acid. Populations of these bacteria were also determined (MPN) in coal waste sampled before construction of the mini-dumps and in the early samples from the mini-dumps, using as selective MPN media Starkey's medium for the acidophilic group and Beijerinck's medium for the non-acidophilic group (Allen, 1957).

Later, populations of various other groups of iron-oxidizing bacteria were determined alongside the routine determinations of the high ferrous iron-oxidizing organisms believed to be *T. ferrooxidans*. These included presumed *Metallogenium* (Walsh and Mitchell, 1972), bacteria able to grow at higher temperatures (37 and 40°C) than usual for *T. ferrooxidans* in an acidified (pH 1.0-1.3) HJJ medium (designated L medium), and bacteria requiring the lower iron concentration (2800 mg/l) 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 low acid and very low ferrous iron conditions (*Metallogenium*; see Walsh and Mitchell, 1972), 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).

C.2.3.2. Media

C.2.3.2.1. 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 water. 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 $FeSO_4.7H_2O$ 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.

C.2.3.2.2. Starkey's medium. The medium as specified by Allen (1957) was used for MPN determinations and enrichment culturing of acidophilic chemolithotrophic thiosulphate-oxidizing bacteria, such as *T. thiooxidans* and *T. ferrooxidans*, and to test cultures growing in 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.

C.2.3.2.3. Beijerinck's medium. The medium was prepared according to Allen (1957), with the addition of bromothymol blue (Rupela and Tanro, 1973) as an indicator of acid production, for MPN determinations and enrichment culturing of non-acidophilic chemolithotrophic thiosulphate-oxidizing bacteria, such as *Thiobacillus thioparus*. Basal medium consisted of 0.10 g NH_4Cl, 0.20 g

Na_2HPO_4, 0.10 g $MgCl_2.6H_2O$, 5.00 ml of a 1% (m/v) solution of bromothymol blue in ethanol and 935 ml of distilled water. Aqueous solutions of $Na_2S_2O_3.5H_2O$ (5.00 g/20 ml), $NaHCO_3$ (1.00 g/20 ml) and $FeSO_4.7H_2O$ (0.01 g/20 ml) were also prepared. The basal medium and these solutions were sterilized separately at 121°C for 15 min., then mixed in the ratio 47:1:1:1.

C.2.3.2.4. ***Metallogenium*** **medium.** The medium of Walsh and Mitchell (1972) was used for MPN determinations of moderately acidophilic very low ferrous iron-oxidizing bacteria of the genus *Metallogenium*. The basal medium consisted of 1.00 g $(NH_4)_2SO_4$, 0.10 g $CaCO_3$, 0.20 g $MgSO_4.7H_2O$, 0.01 g K_2HPO_4 and 4.00 g KH-phthalate in 980 ml distilled water. The pH of the basal medium was adjusted to pH 4.0 with 0.2 M NaOH. After sterilization (121°C for 15 min), 20 ml acidified and filter-sterilized (as described for HJJ medium) $FeSO_4$ solution containing 0.25 g $FeSO_4.7H_2O$ was added.

C.2.3.2.5. L medium. This medium was used for MPN determinations of high ferrous iron-oxidizing bacteria able to grow at relatively high temperature (37°C) and extremely low pH. It was the same as HJJ medium but with the pH lowered to pH 1.0-1.3.

C.2.3.2.6. JLFe medium. This moderate ferrous iron medium developed by D.B. Johnson (personal communication), contained about one third of the ferrous iron of 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 solution (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 $ZnSO_4.7H_2O$, 1.0g $CuSO_4.5H_2O$, 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 salt 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).

C.2.3.2.7. 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.

C.2.3.3. Preliminary studies on the coal waste

Coal waste was obtained from the Kilbarchan Mine on 8 February 1993 (sample K/F1) and on 22 April 1993 (sample K/G1) for studies of populations of acidophilic iron-oxidizing, acidophilic and non-acidophilic thiosulphate-oxidizing bacteria. These samples were from coal waste which was used subsequently for construction of the pilot scale dumps. Within 1-2 weeks of sampling, the presence of acidophilic high ferrous iron-oxidizing, acidophilic and non-acidophilic thiosulphate-oxidizing bacteria was investigated in sample K/F1 by adding 10 g of the coal waste to HJJ, Starkey's and Beijerinck's medium, respectively, incubating at 26°C and examining after 33-35 days for ferrous iron or thiosulphate oxidation as specified later under MPN determinations (section **C.2.3.6.**). The same bacterial groups were enumerated in sample K/G1 within 1-2 weeks of sampling by MPN determinations using HJJ, Starkey's and Beijerink's medium, respectively. Sample K/G1 was also used subsequently for an incubation experiment to test the ability of the iron-oxidizing and acidophilic thiosulphate-oxidizing bacteria to multiply in the coal waste under favourable conditions. The coal waste was incubated in presterilized 250-ml glass beakers in three batches of 150 g wet mass that were treated initially with 30 ml sterile tap water, 30 ml of a 1 % or 30 ml of a 10 % suspension in sterile tap water of an 11-day old active enrichment culture of iron-oxidizing bacteria. This culture was the third serial enrichment subculture developed from an inoculum of 10 g coal waste K/F1 in HJJ medium. The beakers of treated coal waste were incubated at 26°C under covers of sterilized cotton wool below sterilized glass covers. Water loss through evaporation was replenished weekly. Samples were removed after 1, 23 and 44 days for MPN counts of acidophilic high ferrous iron-oxidizing bacteria using HJJ medium and (44 day sample only) acidophilic thiosulphate-oxidizing bacteria using Starkey's medium.

C.2.3.4. Coal waste samples from pilot scale dumps

Samples for MPN determinations of bacteria in the coal waste in the pilot scale dumps were portions of those described previously under section **C.2.2.4.1.**

C.2.3.5. MPN determinations: General procedures

For MPN determinations of specific chemolithotrophic bacterial groups, flasks of the appropriate medium (see section **C.3.2.3.6**) 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 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°C (most studies) or 37°C as specified in section **C.2.3.6.** After appropriate incubation times, flasks were recorded as positive or negative for bacterial growth as specified in section **C.2.3.6.**

From the patterns of cultures showing growth of the specific organisms in the MPN series, the populations of the organisms were determined where possible from the MPN values in Table 1 of de Man (1983). The indicated populations were corrected to populations per g dry coal waste using the moisture content of 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. For patterns of culture growth not included in de Man's Table 1, the MPN values and 95% confidence limits were obtained from a table computed by Dr J.H. Randall (formerly Biometrician, Faculty of Agricultural Sciences, University of Stellenbosch, Stellenbosch) and presented in **Appendix 1** (section **G.1**).

C.2.3.6. MPN determinations: Specific procedures for different bacterial groups

C.2.3.6.1. Acidophilic high ferrous iron-oxidizing bacteria. These iron-oxidizing bacteria were believed to be *T. ferrooxidans*. They were investigated using HJJ medium in the preliminary studies and in all the coal waste samples from the pilot scale dumps throughout the study period. 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.

C.2.3.6.2. Thiosulphate-oxidizing bacteria. Thiosulphate-oxidizing bacteria were determined using Starkey's medium for acidophilic and Beijerinck's medium for non-acidophilic organisms. The corresponding basal media without thiosulphate, as well as without the $NaHCO_3$ and $FeSO_4$ additions to Beijerinck's medium, were used to prepare the tenfold dilutions of the coal waste. After incubation for 4 weeks at 26°C in the dark, 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). Where necessary, the colour of the bromothymol blue of Beijerinck's medium was first brought to yellow by acidification with 1 N HCl. The titration volumes were compared with those for incubated control flasks containing uninoculated medium to determine the percentage of thiosulphate metabolized.

C.2.3.6.3. Moderately acidophilic very low ferrous iron-oxidizing bacteria (presumptive *Metallogenium* count). The *Metallogenium* medium is selective for iron-oxidizing bacteria which are tolerant of only very low ferrous iron concentrations and moderate acidity (presumed *Metallogenium,* which may develop in the early stages of dump acidification). The basal medium without ferrous sulphate was used for preparing the dilutions. After incubation for 5 weeks at 26°C in the dark, the cultures were examined for residual ferrous iron by adding 10 ml of a 15% H_3PO_4-15% H_2SO_4 solution to 20 ml of each culture and titrating it with acidified dichromate with acidified barium diphenylamine sulphonate as indicator (Vogel, 1951) as described by Loos *et al.* (1990a), but with the dichromate solution diluted 1:50 to make it suitable for the low iron concentration. An uninoculated incubated control was also titrated to assess the spontaneous chemical oxidation of the ferrous iron.

C.2.3.6.4. 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 higher temperature.

C.2.3.6.5. Acidophilic moderate ferrous iron-oxidizing bacteria (count in JLFe medium). The JLFe 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 determinations using HJJ and L medium.

C.2.3.6.6. 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 D.B. Johnson, University of Wales, Bangor) was taken as positive. *Thiobacillus ferrooxidans* ATCC 23270 (American Type Culture Collection, Rockville, Maryland, U.S.A.) was used as a positive control.

C.2.3.6.7. 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 medium in 250-ml Erlenmeyer flasks) and incubated at 26°C in the dark for 4 weeks. The metabolism of thiosulphate was detected by iodometric titration as described for the thiosulphate-oxidizing bacteria (section **C.2.3.6.2**).

C.3. RESULTS

C.3.1. Abiotic Ecological Determinants in Pilot Scale Coal Waste Dumps

C.3.1.1. Temperature conditions

C.3.1.1.1. Atmospheric temperatures. The weather station on the experimental site operated continuously from February 1994 giving hourly readouts of average air temperature (Wates,

Meiring and Barnard, 1995a) from which weekly mean maximum and minimum temperatures for the 15 months shown in Fig. 8 were calculated. Extremely large fluctuations (as much as 30°C in July 1994) were sometimes noted over a 24-hour period. Temperatures as high as 30°C during the day in summer and as low as -7°C during the early morning in winter were noted. Weekly mean temperatures averaged 24°C in February (summer) and 10°C during July (winter).

C.3.1.1.2. Soil and coal waste temperatures. The soil and coal waste temperatures measured at depths of 0.30 and 0.72 m in cell 6 from March to December 1994 and at a depth of 1.65 m from March 1994 to May 1995 are also shown in Fig. 8 as weekly mean maximum and minimum temperatures. The interface at 0.3-m depth between the top and the lower soil layers showed considerable temperature fluctuations (up to 5°C) over a 24-hour period. Temperatures of 29°C in March 1994 and 9°C in July 1994 were the highest and lowest soil interface temperatures in cell 6. Long term seasonal variations were noted, but the soil temperatures never reached as low or as high levels as the atmospheric temperatures.

At the 0.72-m depth in the Estcourt soil layer, the differences between corresponding day and night temperatures were less pronounced than at the 0.3-m depth, being almost negligible. However, long-term seasonal temperature variation was observed at this depth.

Corresponding day and night temperatures were almost identical (differences of less than 0.5°C) in the coal waste at 1.65-m depth. The seasonal changes were also less pronounced than at shallower depths, but mean temperatures of 24°C during summer and 16°C during winter were observed.

C.3.1.2. Moisture conditions

C.3.1.2.1. Rainfall. The weekly rainfall on the pilot scale dumps is indicated in Fig. 9. The rainfall is highly seasonal. The rain falls mostly from spring through summer and autumn, with the rainy seasons starting in October and lasting until May. The winter-early spring periods were dry with very little or no rain falling from June to September. Exceptional falls of winter rain occurred in August 1994 (4.5 and 23 mm), June-August 1996 (5, 50 and 18 mm) and June-July 1997 (total 55 mm).

The 1994/1995 rainy season was rather dry (486 mm). 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 walls separating the individual cells.

The subsequent 1995/1996 rainy season was particularly wet (> 1583 mm). The cracks in both the covers and the separating walls closed by the middle of the rainy season.

The 1996/1997 rainy season was not as wet as the 1995/1996 rainy season, but the rainfall events were well distributed with higher precipitation than during the rather dry 1994/1995 rainy season.

C.3.1.2.2. Moisture content of coal waste. The mean moisture contents of the duplicate coal waste samples from each cell over the period September 1993 to September 1996 are shown in

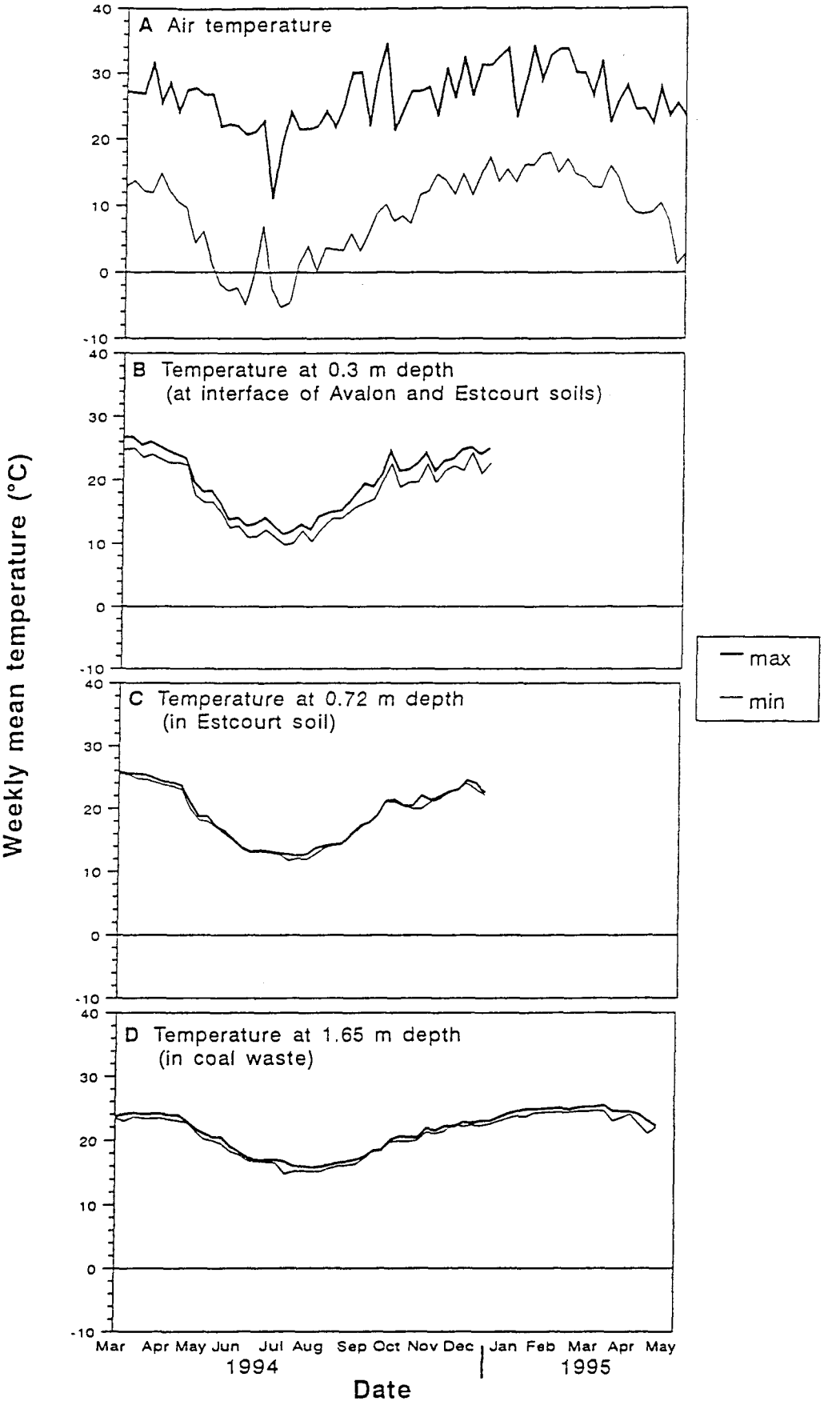

Fig. 8. Weekly mean maximum and minimum temperatures of atmosphere at weather station and at three depths in cell 6 from March 1994 to December 1994 or May 1995.

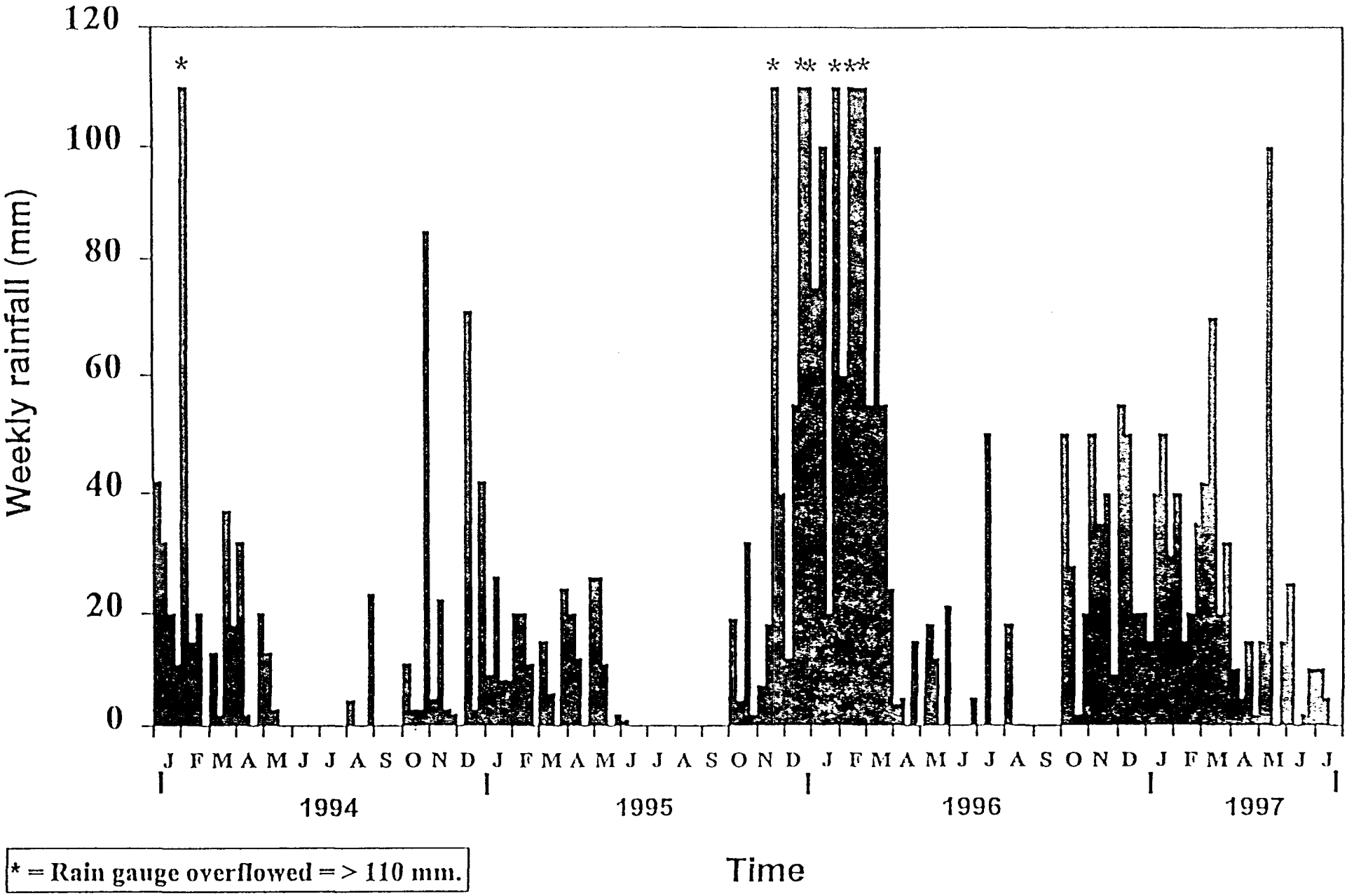

Fig. 9. Weekly rainfall on the pilot scale dumps from January 1994 to July 1997.

Fig. 10 and the values for individual samples in Appendix Tables 2.1-2.20a (except values for July 1995, for which only the means are available). Because the cells were watered during construction, the initial moisture content of the cells was generally above 8%.

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 and at their lowest levels in July or September 1995, following two dry winters and the dryish 1994/1995 summer.

The moisture content of the coal waste in the cells covered with Avalon soil only (cells 4, 5 and 7) rose slightly during the 1993/1994 rainy season, after which it returned to the initial levels of 8-10 %. The moisture content remained at approximately this level, although with a slight decline as dry conditions persisted, until the very wet 1995/1996 rainy season when it increased sharply, before returning to about 10% during the winter of 1996.

In the cells covered with both an Estcourt and an Avalon soil layer (cells 6, 8, 9 and 10), considerable scatter of measured moisture contents was evident during the first year and a half. This made the recognition of responses to rainfall during this period impossible. Part of the problem arose from samples from the bottom of the slope of cell 10, which were very wet from the accumulation of drainage water in the coal waste just above the plastic liner. Subsequently, less scatter was observed when the moisture content of the cells declined to 8-10%. During the 1995/1996 rainy season, the moisture content rose sharply before declining again to means of about 9-12% during the winter of 1996.

C.3.1.3. 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 (means of two determinations at different sites in each cell) are shown in Fig. 11 and 12. These concentrations are monthly means of the weekly measurements with the results grouped in the figures as the concentrations in the uncovered waste (cells 1, 2 and 3), the Avalon soil-covered waste (cells 4, 5 and 7) and the Estcourt and Avalon soil-covered waste (cells 6, 8, 9 and 10).

The oxygen and carbon dioxide concentrations 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. 12 could have been higher.

C.3.1.3.1. Uncovered cells (1, 2 and 3). All three uncovered cells remained aerobic during the course of the experiment, with oxygen concentrations generally above 15% and carbon dioxide concentrations below 2%. 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

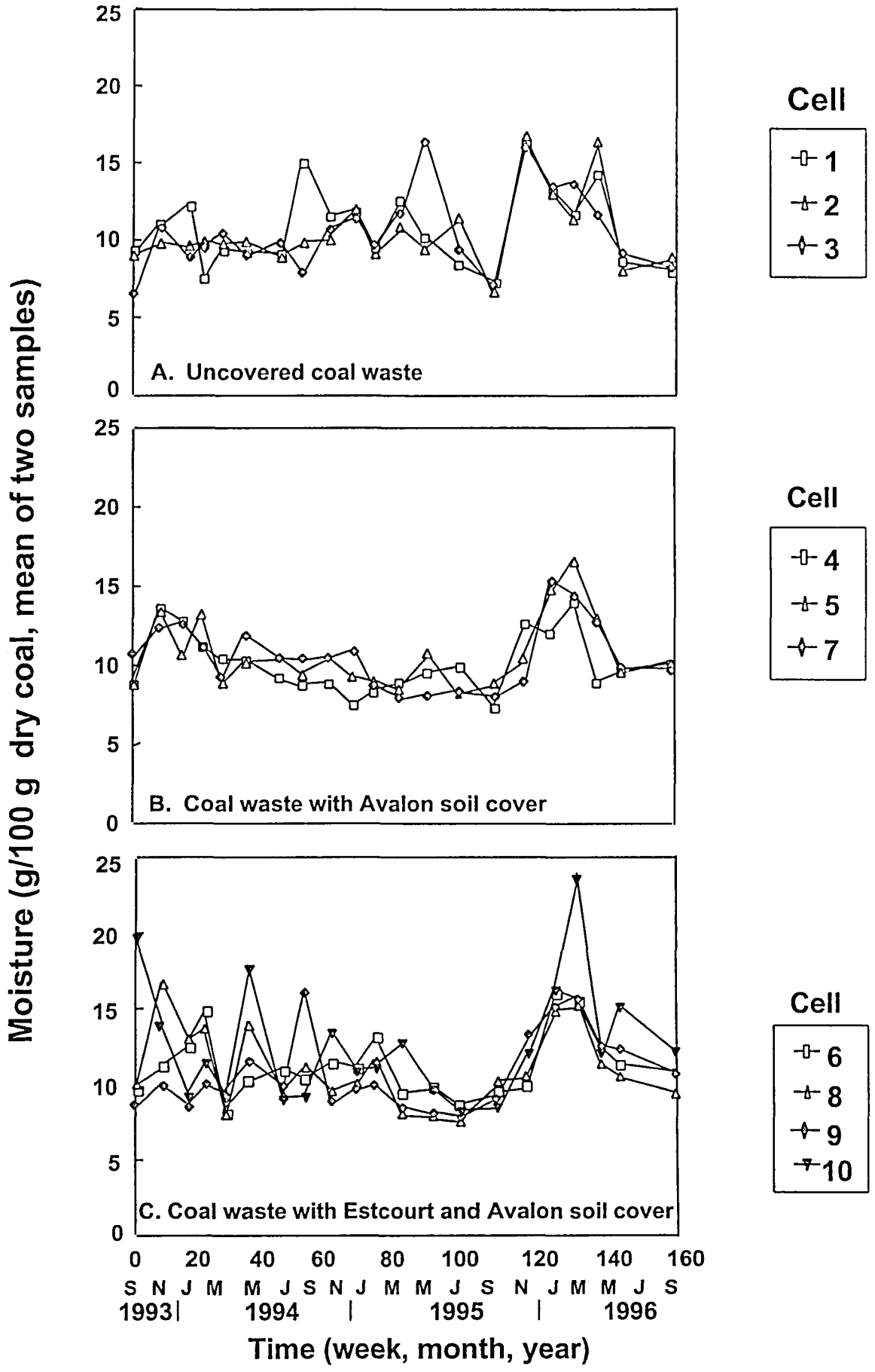

Fig. 10. Mean moisture content of duplicate samples from the upper 30 cm of coal waste in the cells of the pilot scale dump rehabilitation experiment in relation to the time of sampling.

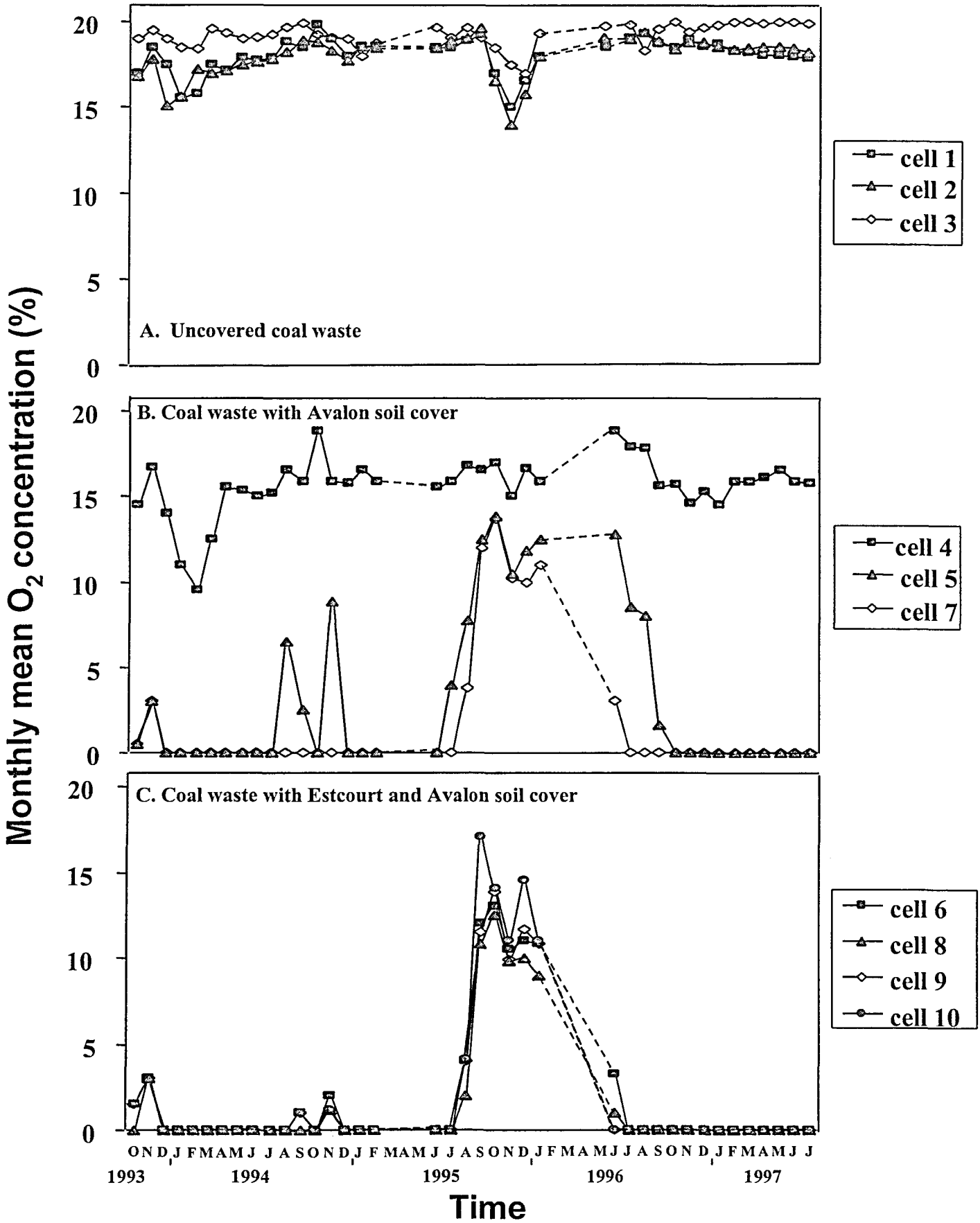

Fig. 11. Monthly mean oxygen concentrations (from weekly measurements) at depths of 5-15 cm below the upper surface of the coal waste in cells of the pilot scale dump rehabilitation experiment. Broken lines indicate periods when no determinations could be made.

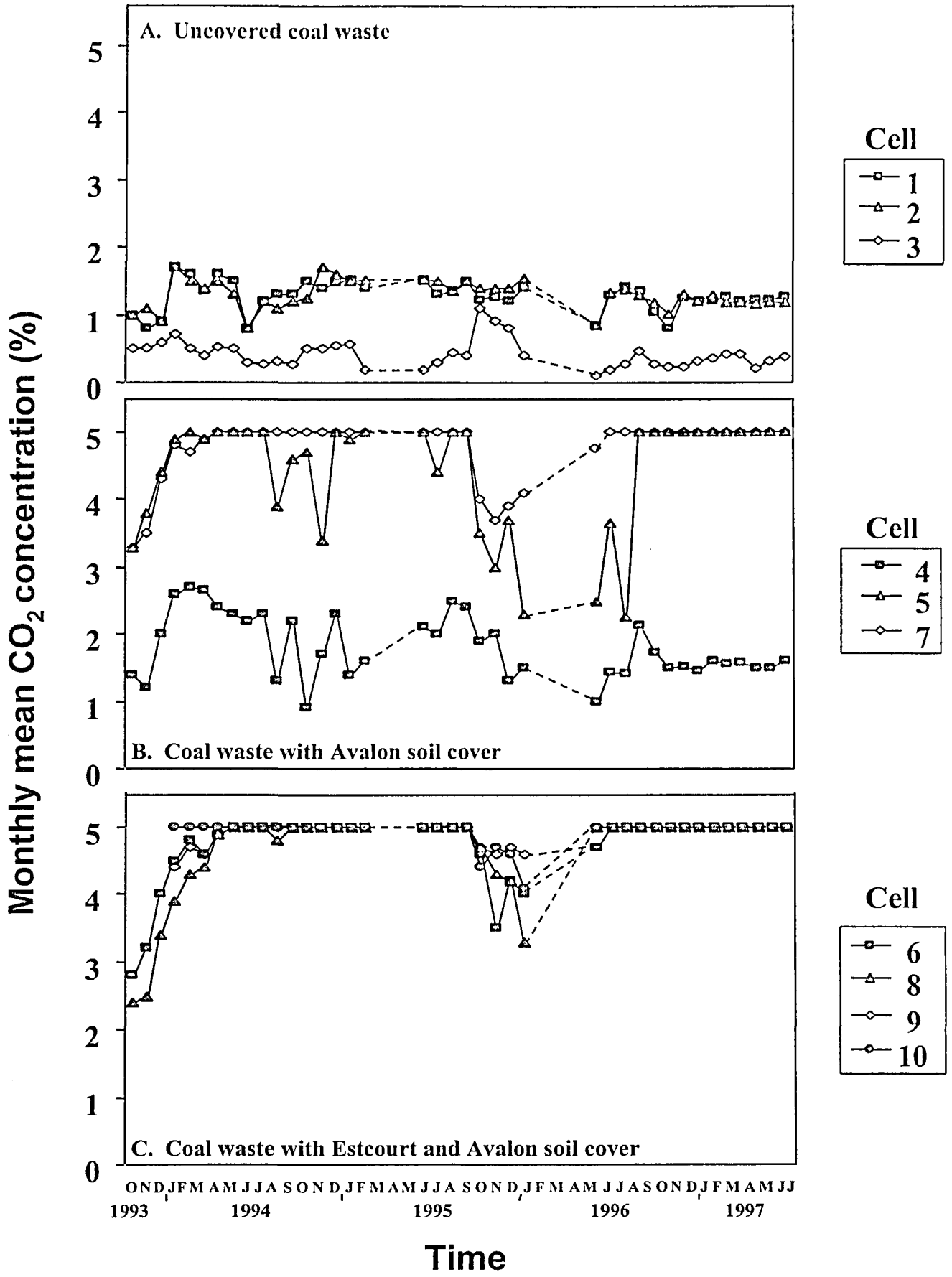

Fig. 12. Monthly mean carbon dioxide concentrations (from weekly measurements) at depths of 5-15 cm below the upper surface of the coal waste in cells of the pilot scale dump rehabilitation experiment. Broken lines indicate periods when no determinations could be made.

ascribed to compaction. During extended wet periods such as the summers of 1993/1994 and 1995/1996, all three cells showed temporary declines in oxygen concentration, but not always a corresponding rise in carbon dioxide concentration.

C.3.1.3.2. 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. It showed only one major decline in oxygen concentration, during the summer of 1993/1994.

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 declined sharply to become anaerobic in September 1996, then remained anaerobic until the end of the experimental period in July 1997.

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 also remained anaerobic until July 1997.

C.3.1.3.3. Estcourt and Avalon soil-covered cells (6, 8, 9 and 10). These cells became anaerobic soon after construction. During the spring of 1994, following the dry winter, small amounts of oxygen penetrated the cells, but otherwise they remained anaerobic until July 1995.

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 again became anaerobic, remaining in this state until July 1997.

C.3.1.4. pH of coal waste

The mean pH of the two samples from each cell at each sampling is shown in Fig. 13 and the values for individual samples in Appendix Tables 2.1-2.21 (except values for July 1995, for which only the means are available). Large differences were often observed between duplicate samples from the same cell, indicating that acidification of the coal waste was initiated in pockets and not uniformly throughout a cell. This pattern of variation among samples caused the substantial scatter of the mean pH values in Fig. 13 around the acidification trends for the individual cells. Nonetheless, different trends among the cells are evident.

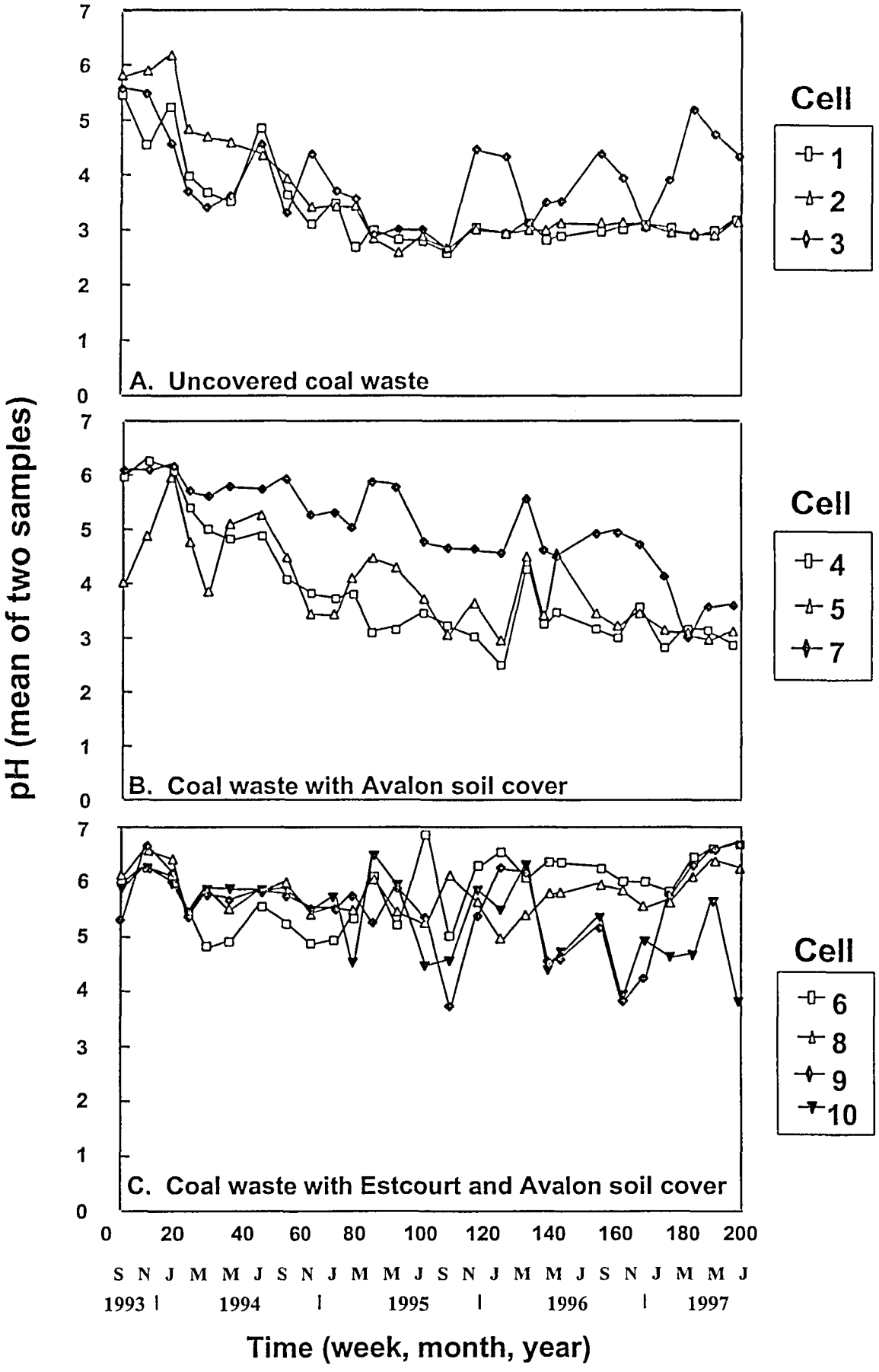

Fig. 13. Mean pH of duplicate samples from the upper 30 cm of coal waste in the cells of the pilot scale dump rehabilitation experiment in relation to the time of sampling.

C.3.1.4.1. 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, after which the pH of the coal waste 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, and thereafter showed considerable fluctuation in the range approximately pH 3-6, probably as a result of uneven treatment of the coal waste with the lime.

C.3.1.4.2. Avalon soil-covered cells (4, 5 and 7). As these cells differed considerably in construction and aerobiasis, their acidification trends also differed substantially.

Cell 4, covered with 30 cm of Avalon soil, remained aerobic during the experimental period (Fig. 11). Its acidification proceeded similarly to that of the uncovered cells (Fig. 13). After 80 weeks it had acidified to approximately pH 3. The samples of January 1996 were highly acidic at pH 2.53 and 2.50. The subsequent short-lived rise in the mean pH during March 1996 resulted from the low aciditiy of a single sample.

Cell 5, covered with 50 cm of compacted Avalon soil, fluctuated between periods of being aerobic and anaerobic with seasonal fluctuations of moisture content (Fig. 10, 11). It already contained pockets of acidification at the onset of the experiment causing considerable scatter of pH values around the general pH trend. However, from Fig. 13 it appears that the pH declined to about pH 3 by September 1995, then remained (most samples) in the range pH 3-3.5 for the rest 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 (Fig. 11). Slight and slow acidification occurred over the experimental period (Fig. 13). The mean pH values during the last 6 months of observation had dropped to pH 3.0-3.5 from those of pH 5.7-6.1 recorded during the first year.

C.3.1.4.3. 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 (Fig. 11). Until January 1995 very little change in pH of these cells occurred, with pH values remaining mostly in a narrow range between pH 5 and 6 (Fig. 13). However, from February 1995 pockets of acidification were detected, particularly in cells 9 and 10 with the sloped surfaces, suggesting that acidification may have started in the cells during the 1994/1995 drought; however, there was no substantial consistent downward trend of the pH in any of the four cells during the 4-year duration of the experiment. Cells 6 and 8 resisted acidification particularly well.

C.3.2. Microbial Populations in Coal Waste of the Pilot Scale Dumps

C.3.2.1. Preliminary studies on coal waste used for dump construction

The pH and moisture contents of coal waste samples K/F1 and K/G1 from the Kilbarchan Mine, representing waste that was later used for the construction of the pilot scale dumps are shown in Table 7. With a pH of 5.85 and 5.75, respectively, neither K/F1 nor K/G1 showed acidification. When K/F1 was inoculated into HJJ, Starkey's or Beijerinck's medium at a rate of 10 g/100 ml medium to detect and produce enrichment cultures of ferrous iron-oxidizing, acidophilic and non-acidophilic thiosulphate-oxidizing organisms, respectively, all cultures showed the appropriate complete metabolism of substrate after incubation at 26°C for 33-35 days.

In a further investigation of these populations in sample K/G1 by MPN determinations (Table 8) , moderate populations of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing organisms were found, but the population of non-acidophilic thiosulphate-oxidizing organisms was very low. The responses of the ferrous iron- and acidophilic thiosulphate-oxidizing microorganisms in coal discard K/G1 to moistening and incubation of the waste at 26°C were investigated (Table 9). The responses without inoculation and following inoculation with two levels of an enrichment culture of iron-oxidizing bacteria from sample K/F1 were compared. Initially (after 1 day) uninoculated coal waste showed a moderate population of iron-oxidizing organisms and that with the highest inoculum level (3 ml culture/180 g wet coal waste) a very high population, but that which had received the lowest level of inoculum (0.3 ml/180 g coal) unexpectedly showed a low population. With this treatment the population of iron-oxidizing organisms had not changed by 23 days, but the populations of the other two had declined to a similar low level. Subsequently, all treatments showed modest increases of these populations by 44 days. The populations of the thiosulphate-oxidizing organisms after 44 days of incubation were higher than those of the iron-oxidizing organisms, being very high in the inoculated coal waste (at both levels of inoculum).

C.3.2.2. Acidophilic high ferrous iron-oxidizing bacteria

Population sizes of acidophilic iron-oxidizing bacteria capable of growth at 26°C in HJJ-medium from various cells of the pilot scale experiment from September 1993 to September 1996 are shown in Table 10 and Appendix Tables 2.1-2.20. The mean log populations of the duplicate samples from each cell at the various samplings are plotted in Fig. 14. 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:

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

(2) The Avalon soil-covered cells 4 and 5, that were aerobic for all or part of the time, respectively, showed similar populations to those of the uncovered cells. However, samples from cell 7, that was covered by 1 m of Avalon soil and was anaerobic for most of the time, had

Table 7. pH and moisture content of coal waste samples, K/F1 and K/G1, as received from the Kilbarchan Mine in northern Kwazulu-Natal and moisture content of incubated sample K/G1

Sample	pH[a]	Moisture in sample as received (g/100 g dry mass)	Moisture in incubated sample (g/100 g dry mass)
K/F1	5,85[b]	9,30[b]	
K/G1	5,75[b]	10,67[c]	29,75[c]

[a] pH of suspension of 10 g sample in 25 ml distilled water.

[b] Mean of triplicate determinations.

[c] Mean of duplicate determinations.

Table 8. Acidophilic high ferrous iron-oxidizing, acidophilic and non-acidophilic thiosulphate-oxidizing microbial populations (MPN estimates) in coal waste sample K/G1 from the Kilbarchan mine

Microbial population[a]	MPN/g dry mass
(1) Ferrous iron-oxidizing	1 029 (1)[b]
(2) Acidophilic thiosulphate-oxidizing	1 660 (1)
(3) Non-acidophilic thiosulphate-oxidizing	4.8 (1)

[a] Determined 14 days after sampling in the following MPN media for the respective groups: (1) HJJ, (2) Starkey's and (3) Beijerinck's medium.

[b] Figure in parenthesis indicates the likelihood category of the test result according to de Man (1983).

Table 9. Acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations (MPN estimates) in moistened coal waste from the Kilbarchan mine (sample K/G1, without and with inoculation with HJJ enrichment culture from sample K/F1) incubated at 26°C

Micro-organisms counted[a]	Coal waste incubation time (days)	MPN/g dry coal waste		
		No initial inoculation	Inoculated initially with K/F1 enrichment culture 0.3 ml	3.0 ml
Ferrous iron oxidizers	1	1 207 (1)[b]	195 (1)	120 670 (1)
	23	56 (1)	182 (2)	93 (2)
	44	195 (1)	506(1)	597 (1)
Thiosulphate oxidizers	44	1 207 (1)	311 405 (1)	557 934 (1)

[a] HJJ medium was used for the ferrous iron oxidizers and Starkey's medium for the thiosulphate oxidizers (acidophilic).

[b] Figure in parenthesis indicates the likelihood category of the test result according to de Man (1983).

Table 10. Populations (MPN estimates) 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

Cell and (sample)	MPN/g dry coal waste in samples of						
	28/09/93	22/11/93	18/01/94	28/02/94	11/04/94	28/05/94	01/08/94
1 (1)	4 683	5	4 913	100 747	164	2 280	41
1 (2)	102	16 496	83	245 962	47 064	102	47
2 (1)	160 965	47	47	25 445	412	2 535	1 305
2 (2)	4 202	231	47	4 703	25 567	474	16 392
3 (1)	100	2 559	82	1 013	4 761	1 017	220
3 (2)	253	1 027	10 060	23 157	2 532	228 354	102
4 (1)	101	4 280	49	258	102	101	161
4 (2)	102	49	26	2 317	3 779	220	166
5 (1)	46 603	24 026	123	13 940	46 401	152	25
5 (2)	16 414	48	823	25 463	16 512	48	165
6 (1)	47	103	47	27	130	102	25
6 (2)	48	84	26	26	15	103	26
7 (1)	26 311	105	231	254	101	486	254
7 (2)	479	26	496	258	474	256	25
8 (1)	3 080	11	262	2 448	251	17	25
8 (2)	47	47	48	48	14 979	258	25
9 (1)	809	1 659	3	164	1 000	259	26
9 (2)	2 512	102	130	255	3 114	829	25
10 (1)	1 802	272	415	248	250	1 099	10
10 (2)	5 135	164	821	4 959	252 747	5 045	25

Cell and (sample)	MPN/g dry coal waste in samples of					
	26/09/94	21/11/94	16/01/95	27/02/95	10/04/95	06/06/95
1 (1)	108 940	104 039	10 438	41 667	490	103 155
1 (2)	260 429	3 896	479	164 340	4 785	3 109
2 (1)	16 455	1 654	10 671	16 391	10 374	1 022 814
2 (2)	0.3	473	425	25 123	33	16 304
3 (1)	1 000	2 546	424	22 831	803	2 671
3 (2)	46 573	ND[a]	2 561	47 571	424	17 523
4 (1)	47 046	812	12 917	46	25	230 706
4 (2)	8 116	3 944	24 668	46 926	4 775	468 184
5 (1)	3 190	1 659	25 233	164	47	12 229
5 (2)	2 615	ND[a]	46 853	21 790	4 653	13 214
6 (1)	26	49	48	26	25	103
6 (2)	25	25	48	26	470	31 775
7 (1)	254	1 029	230	4 685	25	102
7 (2)	121	166	105	101	100	2 144
8 (1)	479 020	102	475	4 802	1 004	47 257
8 (2)	25	4 7	47	84	41	15 929
9 (1)	1 121	251	3 143	9 957	1 605	162
9 (2)	104	1 007	255	10 479	102 123	16 271
10 (1)	47	131	8 405	165 510	46	47
10 (2)	10 140	89	703	8 401	444	394

Table 10 (continued)

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)	382 598	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	428	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

[a]ND = not determined.

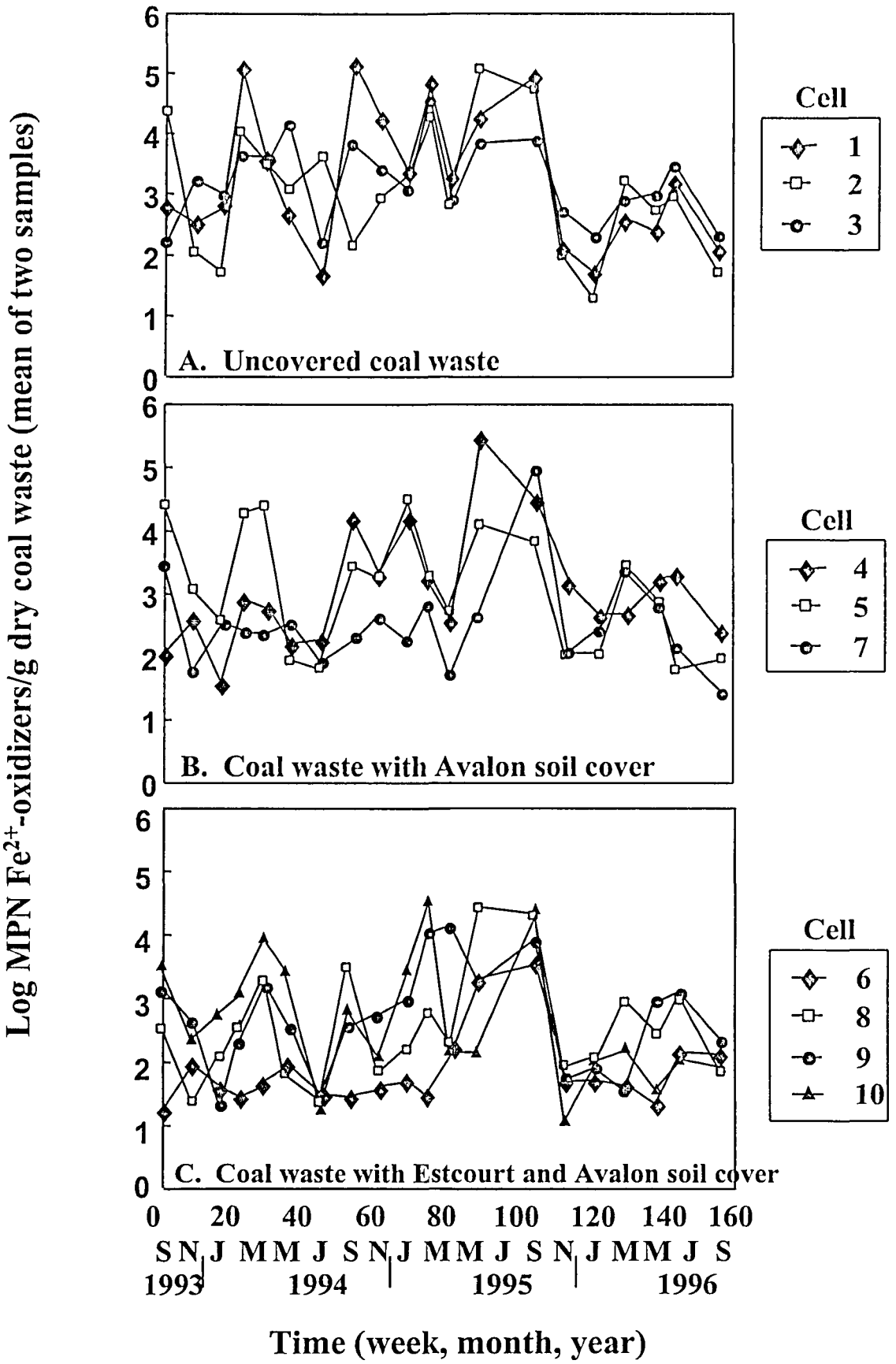

Fig. 14. Mean log MPN estimates of acidophilic ferrous iron-oxidizing bacteria in duplicate samples from the upper 30 cm of coal waste in the cells of the pilot scale dump rehabilitation experiment in relation to the time of sampling.

populations mainly between 100 and 1000/g dry coal waste, hence comparable with the lowest counts for cells 4 and 5.

(3) 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/g dry coal waste.

(4) The populations in all cells were comparable in the low range of about 10 to 1000/g dry coal waste from November 1995 to September 1996, i.e. they seemed to stabilize at a relatively low level during the third year.

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

In Table 11 the counts of acidophilic high ferrous iron-oxidizing bacteria capable of growth in HJJ-medium are cross-tabulated according to population size frequency and pH. From this cross-tabulation it is obvious that the higher populations of these bacteria tended to be associated with more acidic (lower pH) samples, and the lower populations with the higher pH samples, where acidification had not occurred.

C.3.2.3. Acidophilic and non-acidophilic thiosulphate-oxidizing bacteria.

Populations of acidophilic thiosulphate-oxidizing organisms in the coal waste from six samplings and of non-acidophilic thiosulphate-oxidizing organisms in the waste from three other samplings are shown in Tables 12 and 13 and Appendix Tables 2.1-2.8 and 2.10. The populations of both groups of organisms were generally very low (often smaller than 10/g dry coal waste), with only 18 out of 120 individual samples showing > 100 acidophilic thiosulphate oxidizers/g dry waste and 6 out of 60 individual samples showing > 100 non-acidophilic thiosulphate oxidizers/g dry waste.

C.3.2.4. Moderately acidophilic very low ferrous iron-oxidizing bacteria (presumptive *Metallogenium*)

The populations of moderately acidophilic very low ferrous iron-oxidizing bacteria counted by the MPN procedure using *Metallogenium* medium are shown in Table 14 and Appendix Tables 2.9, 2.11 and 2.12. The counts were very low, mainly less than 100/g dry coal waste and mostly between 1 and 10/g. These counts were much smaller than the counts of acidophilic high ferrous iron-oxidizing bacteria for the same samples.

C.3.2.5. Highly 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 15 and

Table 11. 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

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)**

Table 12. Populations (MPN estimates) of acidophilic thiosulphate-oxidizing micro-organisms in coal waste samples from cells of the pilot scale dump rehabilitation experiment

Cell and (sample)	MPN of acidophilic thiosulphate-oxidizing organisms per gram dry mass of coal waste sample on:					
	28/09/93	22/11/93	18/01/94	11/04/94	01/08/94	16/01/95
1 (1)	4	3	4 913	5	10	4 826
1 (2)	82	5	0.5	102	25	3
2 (1)	23	1 019	2	10	3	5
2 (2)	22	42	25	8	47	48
3 (1)	8	7	47	476	8	3
3 (2)	2 425	13	10	165	10	3
4 (1)	47	3	1	22	2	2
4 (2)	25	24	104	4	8	2
5 (1)	2	49	0.3	25	155	3
5 (2)	470	5	0.3	10	3	8
6 (1)	25	3	1	2	5	10
6 (2)	25	2	0.3	2	26	5
7 (1)	1 019	17	47	5	47	4 704
7 (2)	16	8	0.3	474	10	11
8 (1)	102	5	2	4	8	25
8 (2)	47	5	0.8	5	47	3
9 (1)	2	5	0.3	8	42	3
9 (2)	4	3	0.8	8	25	103
10 (1)	11 177	5	25	23	5	26
10 (2)	513	1	0.8	231	25	13

Table 13. Populations (MPN estimates) of non-acidophilic thiosulphate-oxidizing micro-organisms in coal waste samples from the pilot scale dump rehabilitation experiment

Cell and (sample)	MPN of non-acidophilic thiosulphate-oxidizing organisms/g dry mass of coal waste sampled on:		
	28/02/94	28/05/94	26/09/94
1 (1)	2	8	1
1 (2)	2	3	0.5
2 (1)	3	4	10
2 (2)	3	4	0.8
3 (1)	0.2	3	0.1
3 (2)	3	3	0.04
4 (1)	5	2	5
4 (2)	3	5	3
5 (1)	3	250	1 023
5 (2)	5	5	16
6 (1)	5	5	3
6 (2)	3	8	3
7 (1)	ND[a]	486	166
7 (2)	8	22	5
8 (1)	11	16	256
8 (2)	3	104	7
9 (1)	3	3	11
9 (2)	3	4	3
10 (1)	2	3	2
10 (2)	3	3	5

[a]ND = not determined.

Table 14. Populations (MPN estimates) of moderately acidophilic very low ferrous iron-oxidizing bacteria (presumptive *Metallogenium*) in coal waste samples from the pilot scale dump rehabilitation experiment

Cell and (sample)	MPN/g dry coal waste		
	21/11/94	27/02/95	10/04/95
1 (1)	5	32	9
1 (2)	0.2	102	48
2 (1)	1	23	0.4
2 (2)	< 0.03	16	17
3 (1)	1	0.3	10
3 (2)	ND[a]	10	1
4 (1)	1	4	2
4 (2)	3	0.8	3
5 (1)	8	10	0.5
5 (2)	ND[a]	10	1
6 (1)	17	8	0.3
6 (2)	0.3	106	5
7 (1)	51	5	2
7 (2)	10	250	3
8 (1)	2	104	16
8 (2)	1	10	2
9 (1)	3	2	2
9 (2)	10	3	5
10 (1)	3	25	2
10 (2)	51	7	11

[a]ND = not determined.

Table 15. Populations (MPN estimates) 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).

Appendix Tables 2.11a, 2.12a, 2.14 and 2.16. Figure 15 compares the 95% confidence intervals of their log MPN estimates with those of the bacteria which grew at 26°C in HJJ medium (section **C.3.2.2.**) and were present in the same samples. Paired log MPN estimates with overlapping confidence intervals are considered similar and those without overlap different. The paired log MPN estimates were often similar, but when different, the MPN obtained at 37°C using L medium was usually lower than that obtained at 26°C using HJJ medium. These results indicate the possibility that the MPN determinations at 37°C using L medium might often have estimated the same populations of organisms as the determinations at 26°C using HJJ medium. However, with some samples, not all organisms which grew at 26°C in HJJ medium of pH 2.0 could grow under the more extreme conditions of incubation at 37°C in L medium of pH 1.0-1.3. Only rare exmples of the reverse situation, i.e. organisms tolerant of the more extreme conditions being more numerous, were observed.

Table 15 also indicates that a large proportion of the cultures growing at 37°C in L medium could grow in this medium at 40°C.

C.3.2.6. 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 16 and Appendix Tables 2.18–2.20. The 95% confidence intervals of their log MPN estimates are compared in Fig. 16 with those for the organisms that grew in HJJ medium (section **C3.2.2.**) Among the 59 MPN estimates of iron-oxidizing organisms in JLFe medium, 25 were higher, 32 not significantly different and 2 lower than the corresponding MPN estimates of iron-oxidizers that grew in HJJ medium according to overlap or non-overlap of the 95% confidence intervals. It appeared, therefore, that in almost half of the samples more groups of organisms were counted using JLFe medium than HJJ medium. As the JLFe medium is suitable for *T. ferrooxidans*, the differences in the pairs of MPN estimates in these samples indicate the organisms that can grow in this moderate ferrous iron medium but not in the high iron HJJ medium, i.e. organisms other than *T. ferrooxidans*. The samples where these moderate iron-oxidizing organisms exceeded the high iron-oxidizing organisms were mainly from the acidified aerobic cells 1-4 and periodically aerobic cells 5 and 7. Conditions in these cells could have favoured various groups of the acidophilic mesophilic iron-oxidizing bacteria discussed in section **B.2.1.**, including *L. ferrooxidans*.

C.3.2.7. 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 sulphur as well as ferrous iron as energy sources (Kelly and Harrison, 1989) whereas other mesophilic iron-oxidizing bacteria described in section **B.2.1.** do not; this test could therefore possibly be used to indicate in which JLFe cultures *T. ferrooxidans* was the organism responsible for iron oxidation (Harrison, 1978) and to obtain MPN estimates 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

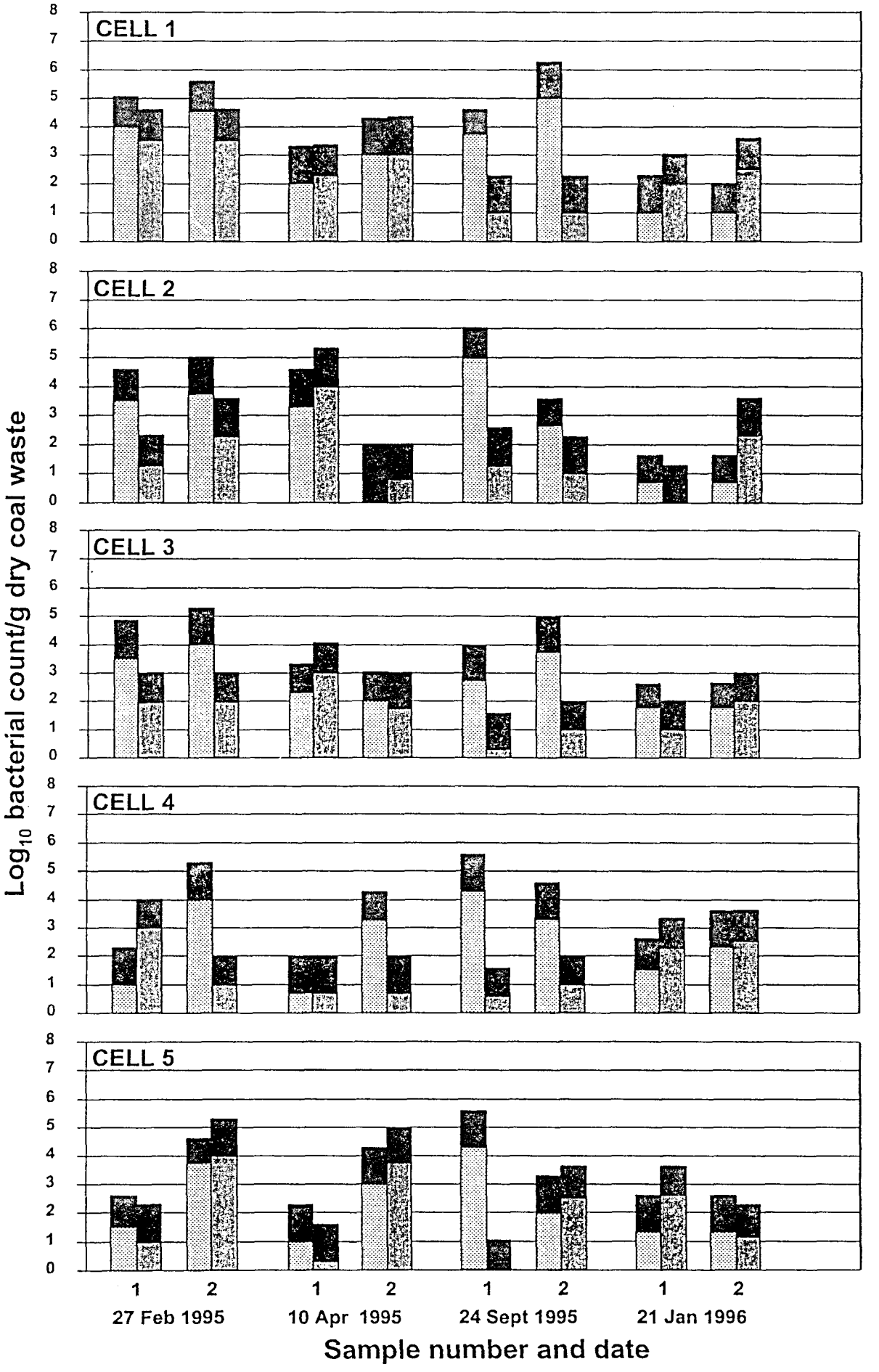

Fig. 15. Ninety-five percent confidence intervals ( ) of $\log_{10}$ MPN estimates of high ferrous iron-oxidizing bacteria in coal waste samples from the pilot scale dump rehabilitation experiment on four dates as determined at 26°C using HJJ medium ( ) or at 37°C using L medium ( ).

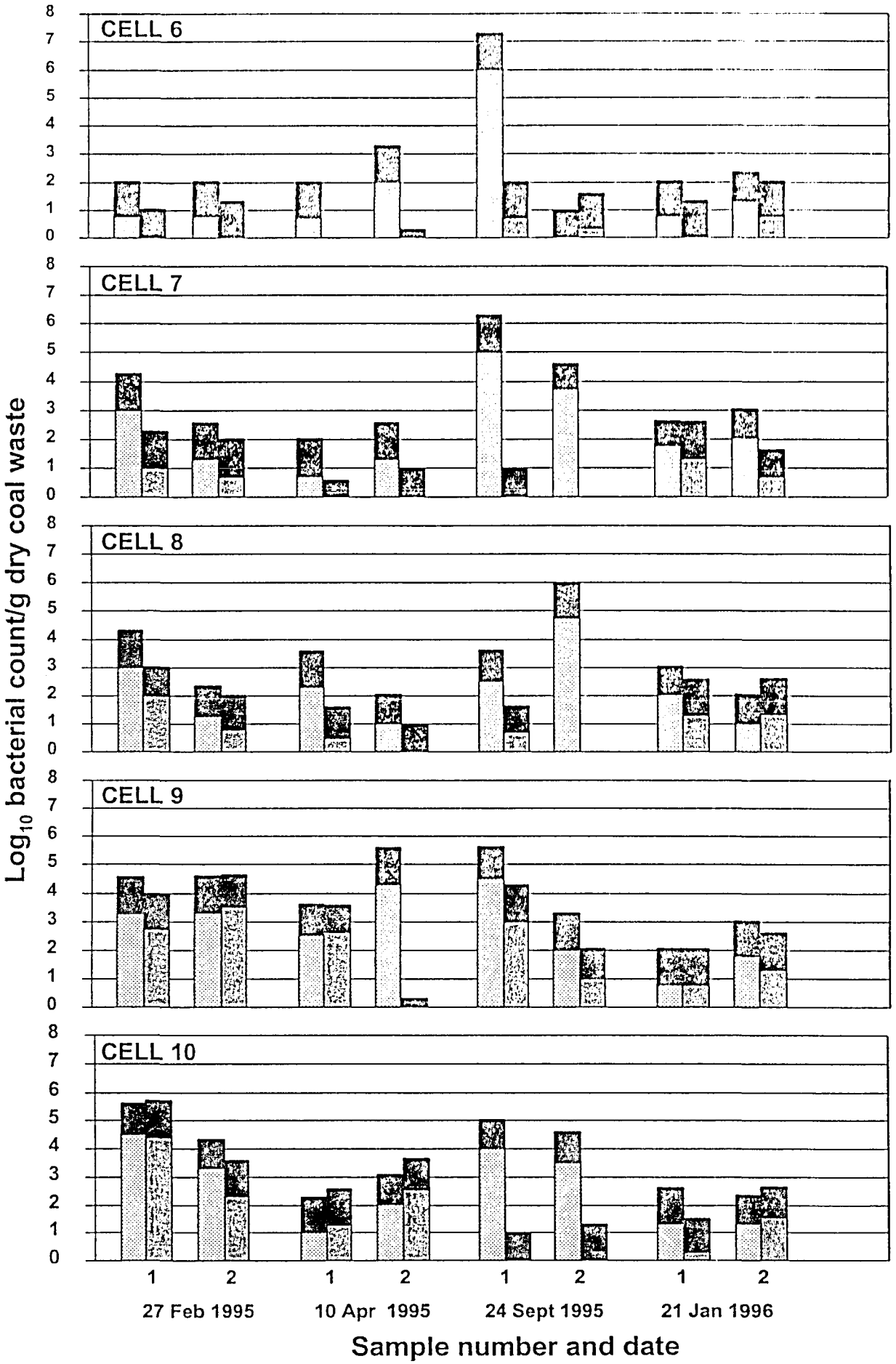

Fig. 15 (continued).

Table 16. Populations (MPN estimates) of acidophilic moderate ferrous iron-oxidizing bacteria capable of growth in JLFe medium, as well as the populations (MPN estimates) 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	251 264	120 170	<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

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 estimated making the assumption that negative tubes in the lower dilutions would have been positive in the absence of this repression. The MPN estimates of the sulphur-oxidizing organisms are shown with those of the iron-oxidizers in JLFe medium in Table 16 and Appendix Tables 2.18a, 2.19a and 2.20a, while the 95% confidence intervals for the log MPN estimates are included in Fig. 16. These confidence intervals show that populations of these organisms were similar to those for the moderate ferrous iron-oxidizing bacteria in 16 samples and lower in 28 samples, while 15 samples yielded no sulphur-oxidizing cultures (<0.3 sulphur oxidizers/g dry coal waste). These results suggest that *T. ferrooxidans* usually formed only a portion of the total iron-oxidizing populations in the coal waste samples. The non-sulphur-oxidizing organisms may have been *L. ferrooxidans* or other mesophilic iron-oxidizers described in section **B.2.1.**, such as heterotrophic iron-oxidizing bacteria. There appeared to be a tendency for their numbers to be higher (often about 10^4/g dry coal waste or higher) in the acid aerobic cells 1-4 and cell 5, than in the low acid mainly anaerobic cells 6 and 8-10, as well as cell 7, where their MPN estimates were often below 10^4/g dry coal waste.

In 25 samples the populations of sulphur oxidizers were similar to those of iron oxidizers estimated using HJJ medium; these populations probably represented *T. ferrooxidans*. However, many samples showed lower populations of the former than of the latter organisms, suggesting that the MPN determinations of acidophilic high ferrous iron-oxidizing bacteria in HJJ medium sometimes estimated not only *T. ferrooxidans* populations, but populations of other species as well. Possibly these could include high ferrous iron-oxidizing strains of *L. ferrooxidans*. In three samples of 2 September 1996 the counts of sulphur oxidizers exceeded those of iron oxidizers able to grow in HJJ medium; in these cases mesophilic *Thiobacillus* species other than *T. ferrooxidans* (section **B.2.2.**) may have grown in association with the iron oxidizers that developed in JLFe medium.

C.3.2.8. 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 sampling of 3 June 1996 growing in the S^0 medium were subcultured into flasks containing Starkey's medium to test their ability to utilize thiosulphate. However, many of the 27 cultures utilizing sulphur failed to utilize thiosulphate (Table 16, Fig. 16 and Appendix Table 2.20a). 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.

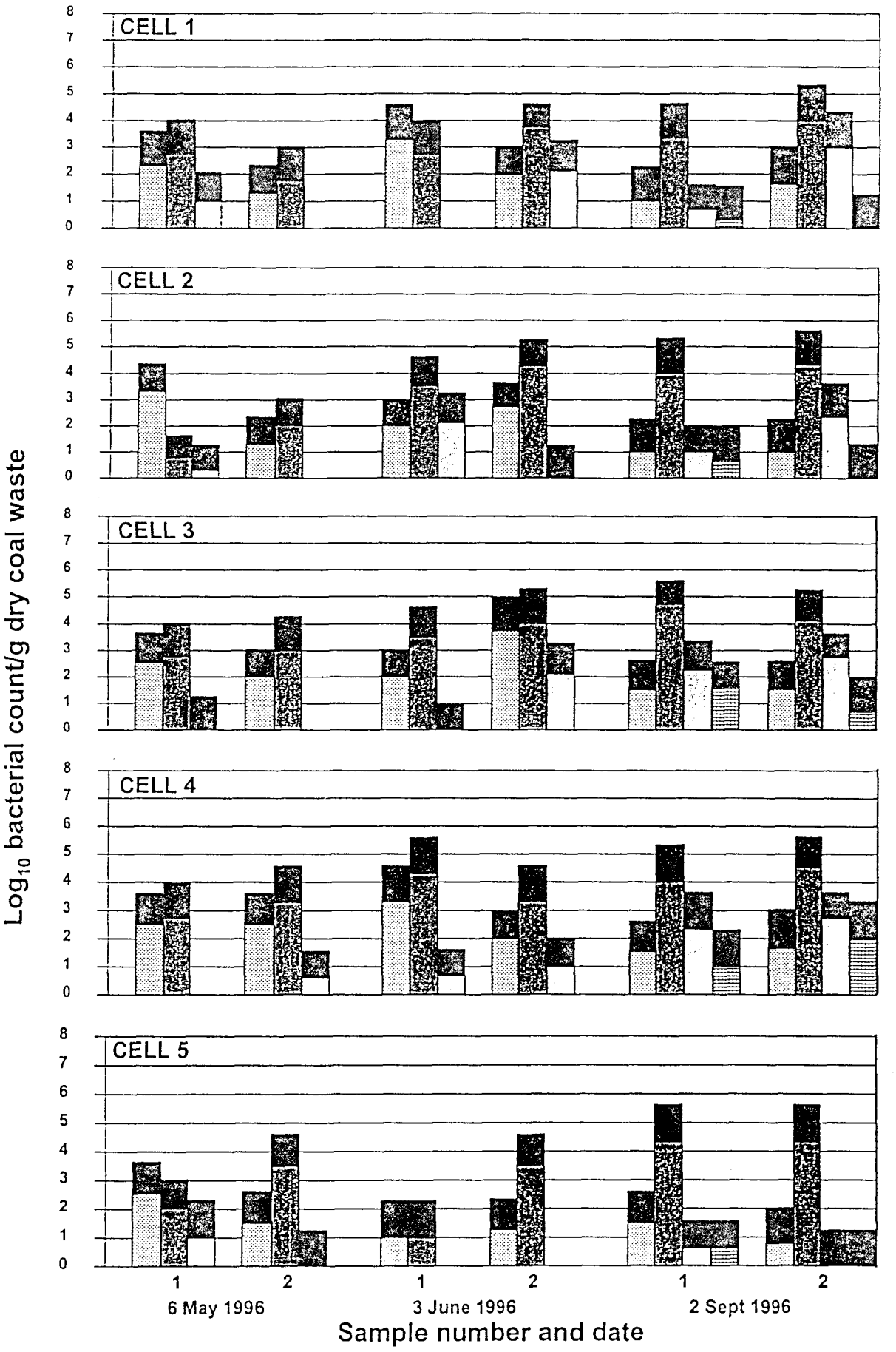

Fig. 16. Ninety-five percent confidence intervals ( ) of $\log_{10}$ MPN estimates of high ferrous iron-oxidizing organisms growing in HJJ medium ( ), moderate ferrous iron-oxidizing organisms growing in JLFe medium ( ), sulphur-oxidizing organisms growing in S^0 medium ( ) and thiosulphate-utilizing organisms growing in Starkey's medium ( ), in coal waste samples from the pilot scale dump rehabilitation experiment on three dates.

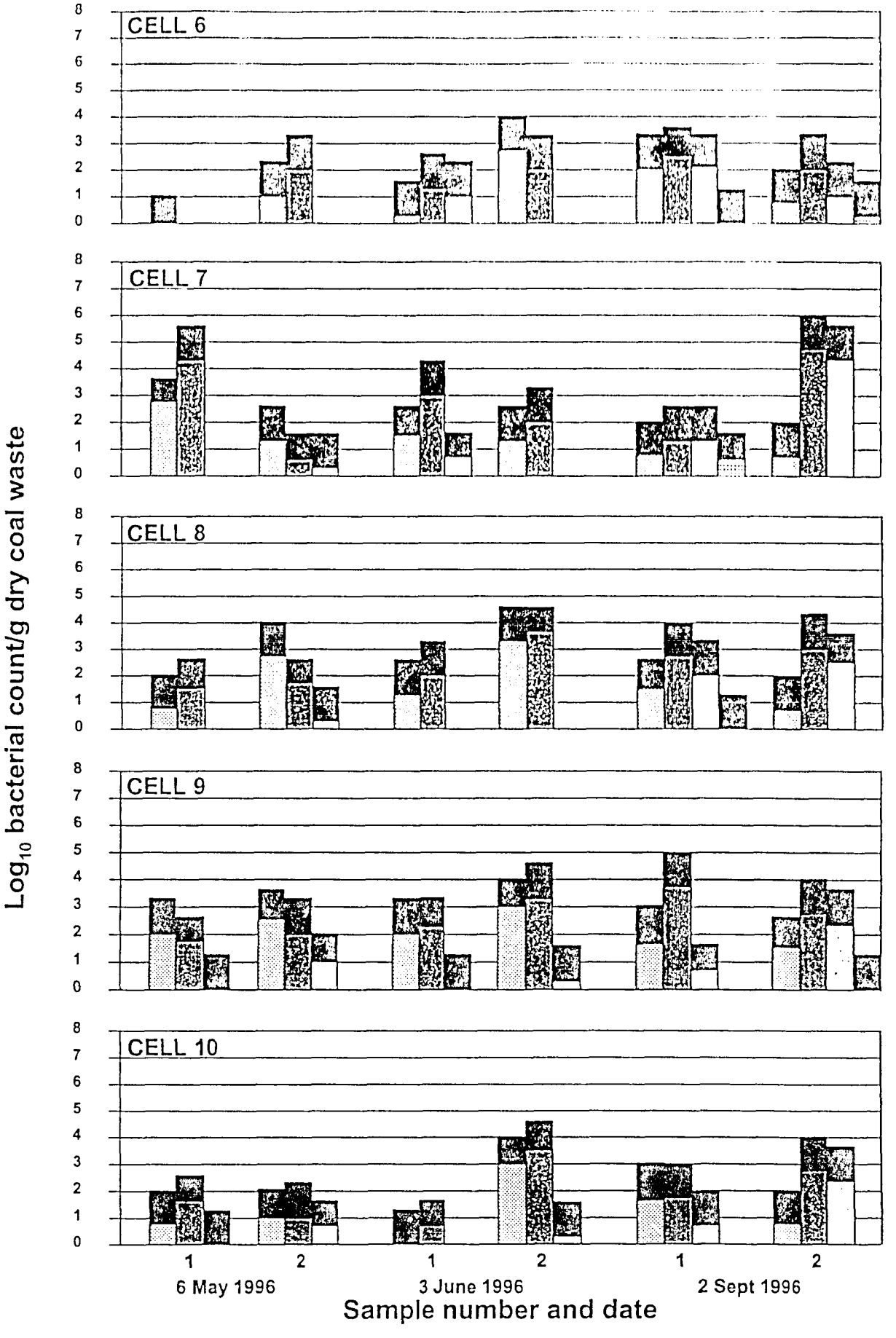

Fig. 16 (continued).

C.4. DISCUSSION

C.4.1. Characteristics of Coal Waste Used in Construction of Pilot Scale Dumps

C.4.1.1. Physicochemical characteristics

The acid potential of the coal waste used to construct the mini-dumps, from analysis of oxidized waste by Prof. F.D.I. Hodgson and colleagues, was neutralized by 24 500 mg $CaCO_3$/kg, which far exceeded the total base potential (equivalent to 780 mg $CaCO_3$/kg) of the waste. This acid potential was equivalent to 0.77% unoxidized sulphur in the coal waste, corresponding well with one of the two values (0.81 and 4.15% sulphur) obtained by Mrs. L. Strydom, as well as one of the two values (0.91 and 1.54% sulphur) supplied by Wates, Meiring and Barnard (1995b). These sulphur concentrations indicated that the chemical and bacterial reactions responsible for the oxidation of sulphur would yield significant acidity and hence acid mine drainage.

Texture analysis performed by Lorentz *et al.* (Wates, Meiring and Barnard, 1995a; Wates and Rykaart, 1999) on the coal waste used in the construction of the mini-dumps indicated that more than 90% of the coal waste was at least of sand particle size (> 0.05 mm cross section) with small amounts of silt- and clay-sized particles. According to this texture analysis, the coal waste resembled a sandy soil (Soil Classification Working Group, 1991). The compacted and uncompacted coal samples revealed little difference in their particle size distribution and density. The compacted and the uncompacted, treated and vegetated coal waste used in cells 2 and 3, respectively, had the lowest porosity and permeability of the uncovered cells. A higher bulk density and lower porosity in cells 2 and 3 indicated that the infiltration of water and gases from the atmosphere into these cells could be reduced compared to that of the uncompacted coal waste in cell 1. These results corresponded well with the initial decline in the outflow from cell 1 to 3 reported by Wates, Meiring and Barnard (1995a,b), although the differences were not large. However, these differences were not sustained, and among the three cells over the entire experimental period cell 2 showed the lowest percentage outflow of precipitation water and cell 3 the highest (Wates and Rykaart, 1999). Lorentz *et al.* (Wates, Meiring and Barnard, 1995a,b) found that the coal waste was generally able to retain 10-15% of its water at a matric pressure head of 100 cm, indicating that in this respect the coal waste was probably comparable to a sandy loam soil (Gray and Williams, 1971), implying good drainage of water and diffusion of gases.

The coal waste used for the construction of the pilot scale dumps had more than 95% of its particles smaller than 3 mm in cross-section. This fine material was suitable for mini-dump construction as it facilitated sampling and handling of the material. As the surface area of the coal waste was larger than it would have been if the particles were larger, more pyrite should theoretically have been exposed to oxygen and pyrite-oxidizing microbial populations than in the case of material with larger particles. The pyrite-oxidizing bacteria should have utilized the smaller particles more effectively than they would have utilized larger particles (Caruccio, 1970; Good *et al.*, 1970), thereby readily oxidizing the pyrite and acidifying this fine waste material.

C.4.1.2. Microbiological characteristics

In preliminary microbiological studies, two samples of coal waste (K/F1 and K/G1), with pH 5.85 and 5.75 respectively, were analysed to determine the suitability of this waste as a material for sustaining large chemolithotrophic bacterial populations with a potential to cause acidification. After inoculation of K/F1 into HJJ, Starkey's or Beijerinck's medium at a rate of 10 g/100 ml medium and incubation at 26°C for 33-35 days, all cultures showed a complete metabolism of substrate. Further investigation of these organisms (MPN determinations) in sample K/G1 showed moderate populations of acidophilic ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing bacteria, but a very low population of non-acidophilic thiosulphate-oxidizing organisms. The most important representatives of the first two groups are *T. ferrooxidans* and *T. thiooxidans*, respectively, which play a role in the production of acid mine drainage from pyrite (Lizama and Suzuki, 1991b; Norris and Kelly, 1982), although *T. ferrooxidans* is regarded as the major organism involved. The presence of these populations in the coal waste at the presumed unfavourable measured pH of 5.75 (Buchanan and Gibbons, 1974) suggests that they may initiate the process of microbially catalysed acid formation in pyrite-containing micro-environments, with limited production of acidity, before the measured pH of a 10-g sample shows a decrease.

The responses of ferrous iron-oxidizing and thiosulphate-oxidizing microorganisms in coal waste K/G1, without and following inoculation with two levels of an enrichment culture of iron-oxidizing bacteria from sample K/F1, were compared after incubation at 26°C. An initial decrease in the ferrous iron-oxidizing bacteria in the uninoculated sample K/G1 with a possible slight rise later, as well as the possible slight increases over time with both of the K/F1-inoculated treatments seemed to indicate that the coal waste was able to sustain a moderate population of ferrous iron-oxidizing bacteria. However, these populations were far smaller than the large thiosulphate-oxidizing populations ($> 10^5$/g coal waste) in the inoculated samples at the end of the incubation period. These MPN estimates showed that most of the organisms oxidizing thiosulphate must have been acidophilic organisms other than *T. ferrooxidans*; a likely possibility is *T. thiooxidans* for which Starkey's medium is an enrichment medium (Allen, 1957).

The results of the studies discussed in this section suggested that the Kilbarchan coal waste would support populations of acidophilic iron-oxidizing bacteria and thiosulphate-oxidizing bacteria which could catalyse acid mine drainage formation from pyrite. Microbiologically the coal waste therefore seemed suitable for use in the pilot scale dump rehabilitation experiment.

C.4.2. Characteristics of Soil Cover Materials

The Avalon soil consisted of 37-44% sand, 26-29% silt and 30-34% clay and the Estcourt soil of 47% sand, 20% silt and 33% clay. According to the texture chart of the Soil Classification Working Group (1991) the Avalon soil was a clay loam and the Estcourt soil a sandy clay loam. However, the higher bulk density of the Estcourt soil (compacted) than that of the Avalon soil (compacted or uncompacted) indicated a closer packing of the particles of the Estcourt soil. No conclusion could be drawn from the particle density values as they were almost all the same. The porosity determinations confirmed that the Avalon soil (compacted or uncompacted) was more porous than the compacted

Estcourt soil. The permeability (saturated hydraulic conductivity) of the uncompacted and compacted Avalon soil was also greater than that of the compacted Estcourt soil by at least an order of magnitude, while the permeability of the uncompacted Avalon soil was one order of magnitude greater than that of the compacted Avalon soil when measured using the small ring infiltrometer or Guelph permeameter, indicating that the larger pore sizes were significantly reduced by compaction of the material. The Estcourt soil also retained more water than the Avalon soil at comparable matric pressures (Wates, Meiring and Barnard, 1995a, b; Wates and Rykaart, 1999).

The porosity, permeability and water retention characteristics of the Estcourt and Avalon soils provided the reasons for placing the Estcourt soil as the "clay" layer below the Avalon "soil" layer in the pilot scale dumps. However, as the Estcourt and Avalon soils had similar clay contents and as the Avalon soil exhibited "clayey" water retention characteristics, it seemed possible that a sufficient amount of compacted Avalon soil might prove almost as effective as the Estcourt soil in limiting the diffusion of gases and infiltration of water to the underlying coal waste. Compaction of the Avalon soil as in cells 5 and 7 would reduce the large void spaces in the macrostructure of the soil, thereby reducing the porosity and hence permeability. However, when used as a topsoil layer for the establishment of vegetation, as in cells 4 and 6-10, the Avalon soil was not compacted.

Clays have a tendency to crack under extreme conditions of desiccation (Daniel and Wu, 1993), as happened during the extremely dry winter of 1995. Large cracks were noted on the Estcourt soil side walls separating the cells from each other. At the same time smaller cracks were noted on the surface of the Avalon soil cover on most of the cells. This was not suprising as the Avalon soil had a high clay content. The cracks allowed much oxygen, which is the electron acceptor in 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 and Herman, 1991; Moses *et al.*, 1987). The cracking of the covers of the mini-dumps and oxidation of ferrous to ferric iron by oxygen as a biotic or abiotic reaction could therefore have far-reaching negative consequences for the coal waste underlying the covers. Oxygen infiltrating the underlying material would 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 of the pH (Atlas and Bartha, 1993; Kleinmann *et al.*, 1981).

C.4.3. Abiotic Ecological Determinants in Pilot Scale Dumps

C.4.3.1. Temperature conditions

C.4.3.1.1. Atmospheric temperatures. The large fluctuations of the air temperature at the site (Fig. 8) had a significant effect on vegetation growth and the state of the covers on the mini-dumps. High temperatures with periods of summer rain provided suitable conditions for the vegetation growth from the beginning of the 1994-1995 rainy season, although the relatively low rainfall of this summer may not have been particularly favourable, but very low night temperatures (-7°C) in winter caused frosting of the vegetation. On the other hand, the warm day temperatures combined with extended periods of drought or low rainfall caused the Avalon soil covers and particularly the

Estcourt soil walls that separated the cells from one another to dry out to such an extent that large cracks (20 mm and wider) had formed at the surface by the end of the 1995 dry season.

C.4.3.1.2. Soil and coal waste temperatures. Continuous fluctuations of daily and weekly mean maximum and minimum temperatures were expected as the soil covers or coal waste were exposed to the daily maximum and minimum air temperature variations. However, there was very little fluctuation from week to week of the mean maximum and minimum temperatures at 0.30 m depth in cell 6 at the interface between the Avalon and Estcourt soil layers (Fig. 8), and almost none at greater depths in the Estcourt soil (0.72 m) and coal waste (1.65 m). Longer term variations (over several weeks or seasons) were found to be associated with the patterns of variation in the air temperature over the same periods.

The lower Estcourt soil layer and even more so, the coal waste, seemed to take longer than the upper Avalon soil layer to show seasonal changes of temperature following change in the air temperature, either downwards or upwards. The little variation in the temperature of the layer of coal waste at 1.65-m depth can be attributed to its separation by the cover materials from fluctuations of the air temperature. During the summer months the constant temperature of about 24°C in the coal waste layer would have been favourable for sustaining large microbial populations. *Thiobacillus ferrooxidans* and related species would proliferate readily at this temperature in the mesophilic temperature range (Harrison, 1984; Kleinmann *et al.*, 1981). A drop in temperature to about 15°C in the winter might cause the bacterial population to decline as biological processes slow down by about 50% for every 10°C drop in temperature (Atlas and Bartha, 1993).

C.4.3.2. Moisture conditions

C.4.3.2.1. Rainfall. The rainfall occurred almost entirely during the summer and other warm months (October to May), with drought occurring through the winter months (June to September). The frequency and intensity of the rainfall on the soil covers significantly influences the infiltration rate. A high volume of rain over a short period on the covers will result in a highly water-saturated zone close to the surface of the cover material, thereby reducing rapid water infiltration into the cover and underlying material. Wates, Meiring and Barnard (1995a,b) reported that the rainfall occurred mainly as events lasting for 1 to 2 hours. Continuous rain over long periods (> 1 day) rarely occurred.

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 years of the experiment (Fig. 9). The most extreme dry and wet seasons were the winter of 1995 and the summer of 1995/1996, respectively. Both of these extreme seasons had profound effects on the soil covers used in the experiment 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 section **C.4.3.3**).

C.4.3.2.2. Moisture content of coal waste. The moisture content of the coal waste samples taken over a 3-year period of the experiment generally ranged between 8 and 16% (Fig. 10). 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 the 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 a general drying trend through the winters of 1994 and 1995 as well as the intervening rather dry 1994/1995 summer, then a sharp increase during the 1995/1996 rainy season. Cells 6, 8, 9, and 10 covered with both an Estcourt and an Avalon soil layer, showed a rather similar trend but with more fluctuation, particularly when the drainage water that collected at the bottom of cell 10 was 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 of relevance 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.

C.4.3.3. 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 generally above 15% and carbon dioxide concentrations below 2% (Fig. 11, 12). 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 and 1995/1996, 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 and would 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. As the coal waste was covered with different depths of soil cover material, 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 soil cover slowed 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 section **C.4.3.4**). Cell 7 covered with 70 cm compacted and 30 cm uncompacted Avalon soil, also became anaerobic soon after construction. It remained anaerobic through 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, acidification of the cell through the chemical and bacterial oxidation of pyrite was slow (see section **C.4.3.4**). However, the acidification indicated that the cover of cell 7 was not a completely satisfactory barrier to oxygen diffusion into the cell.

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, which would result in acidification of the cells, were completely or almost completely inhibited (see section **C.4.3.4**).

This study has clearly shown that all cells without a soil cover or covered with less than 0.5 m of 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 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 long-term acidification of the coal waste.

C.4.3.4. 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, to a mean pH of approximately 3, but with pockets of higher pH in the coal waste of cell 3 following the lime application of November 1995 (Fig. 13). 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 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, the 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 by 120 days. The coal waste in the Witbank mini-dumps was not a small particle fairly homogeneous 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 effectively covered cells where oxygen penetrated only occasionally and less uniformly.

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 contained pockets of acidity from the start 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 113 weeks with little fluctuation subsequently. The cover was therefore not effective in preventing acidification of the coal waste.

Cell 7 with the 1-m 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 appears that it 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. These cells 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 the 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 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. Cells 6 and 8 showed no evidence of general acidification but whether the observed pockets of acidity in cells 9 and 10 increased following the return of anaerobic conditions, with the acidity becoming more general in the coal waste, remains to be determined.

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, suggested that bacteria were involved in the oxidation of pyrite, although the leachate pH was 6.7-7.4 (Wates,

Meiring and Barnard, 1995a,b). However, neutralization of acid by carbonate present in the coal waste can be expected to have occurred initially when pyrite oxidation still occurred in localized acidic regions ("pockets"). Thus, the overall high pH of the leachate from the cells could fail to reflect localized pyrite oxidation and acid production.

C.4.4. Microbial Populations in Coal Waste of Pilot Scale Dumps

C.4.4.1. 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) (Fig. 14). 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 possibly have included other high ferrous iron-oxidizing bacteria, such as strains of *L. ferrooxidans* or mesophilic heterotrophic iron-oxidizing bacteria (Johnson and Roberts, 1997), 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 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 11 clearly shows a tendency for the larger populations of these bacteria to be associated with low pH, reflecting both the favourable effect of acidity on the bacteria and the generation of acid as a result of their metabolism.

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 from 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 high ferrous iron-oxidizing bacteria most of the time. The pH also remained high except in isolated pockets and generally in cell 7 during 1997; it was therefore mainly unfavourable for acidophilic iron-oxidizers such as *T. ferrooxidans, L. ferrooxidans* and mesophilic heterotrophs (section **B.2.1**).

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* (Norris, 1990; Norris and Kelly,

1982; Rawlings, 1997). The only possible limiting factor for this organism in the MPN determinations would be the high ferrous iron concentration (8900 mg/l) of the HJJ medium, but *L. ferrooxidans* can grow in medium with 30 g/l $FeSO_4.7H_2O$ (6043 mg/l ferrous iron) (Hallmann *et al.*, 1992). Mesophilic acidophilic heterotrophic iron-oxidizing bacteria can tolerate 300->400 mM (16800->22400 mg/l) ferrous iron, although strain CCH7 had a tolerance of only 50 mM (2800 mg/l) ferric iron (Johnson and Roberto, 1997), but would require suitable organic compounds to be supplied by the inoculum or associated chemoautotrophic organisms for growth in HJJ medium. This specificity issue will be considered further in later sections.

C.4.4.2. Acidophilic and non-acidophilic thiosulphate-oxidizing bacteria

Starkey's and Beijerinck's media used for the MPN determinations of acidophilic and non-acidophilic thiosulphate-oxidizing bacteria were developed for bacteria such as *T. thiooxidans* and *Thiobacillus thioparus*, respectively (Allen, 1957). The former species has received attention as an organism possibly contributing to pyritic mineral dissolution and the production of acid during bioleaching (Norris, 1990; Hallmann *et al.*, 1992). Bacteria of the latter group have been found in uranium mine waste dumps (Sand *et al.*, 1995), but their role in acid generation from pyrite remains to be demonstrated. However, they could perhaps have a role in the initiation thereof before the pH has dropped to a level suitable for the iron- and sulphur-oxidizing acidophiles. The generally low MPN estimates of acidophilic thiosulphate-oxidizing microorganisms in the coal waste of the pilot scale dumps compared to the generally higher MPN estimates of acidophilic high ferrous iron oxidizers suggest that the same organisms were not responsible for the two oxidations, or if they were, the populations with this dual ability formed a minority in most of the sampled iron-oxidizing microbial populations. Actually, the populations where they were a minority could have been less abundant than the MPN estimates appear to suggest, as 54 of the 120 samples tested for both groups showed overlapping 95% confidence intervals for the two estimates (Appendix Tables 2.1-2.3, 2.5, 2.7, 2.10), indicating that they could have been estimates of the same bacterial population. The consistently low populations of both the acidophilic and the non-acidophilic thiosulphate-oxidizing microorganisms in the coal waste suggest that they had little effect on acidification of the pilot scale dumps, even by iron oxidation where they may also have had this ability. There were also no obvious indications that the acidophilic or non-acidophilic thiosulphate-oxidizing populations were influenced by the absence or presence of the different dump covers.

C.4.4.3. Moderately acidophilic very low ferrous iron-oxidizing bacteria (presumptive *Metallogenium*)

Moderately acidophilic *Metallogenium*-type iron-oxidizers showed very low counts in the coal waste samples from all of the cells during November 1994 to April 1995 (much lower than many of the corresponding counts of acidophilic high ferrous iron-oxidizing bacteria). It can be concluded from these results that *Metallogenium* probably played no role in acidification of the pilot scale dumps, in agreement with Harrison (1978) and Kleinmann and Crerar (1979).

It is not clear how the pH of the waste was reduced from the initial pH of approximately pH 6 to lower levels favourable for the growth of acidophilic iron-oxidizing bacteria. Walsh and Mitchell (1972) postulated a role of *Metallogenium* in initiating acidification of coal at about pH 4.5 or 5.0 (Walsh, 1978), which they supposed to be too high for growth of *T. ferrooxidans*. Nonetheless, our results and those of Harrison (1978) and Kleinmann and Crerar (1979) in laboratory simulations of coal waste dump conditions indicate that a mechanism other than ferrous iron oxidation by *Metallogenium* or sulphide or thiosulphate oxidation by sulphur-oxidizing bacteria must be responsible for the initial reduction in pH. Perhaps the oxidation of pyrite by the initial chemical reactions of Kleinmann *et al.* (1981) can create enough acidity in micro-environments (but undetectable by pH measurements on macro-samples) to give acidophilic bacteria suitable conditions for growth.

C.4.4.4. Highly acidophilic relatively high temperature high ferrous iron-oxidizing bacteria

Most MPN estimates of these organisms obtained using L medium of pH 1.0-1.3 at 37°C were either similar to or lower than those of the acidophilic iron-oxidizers which grew in HJJ medium of pH 2.0 at 26°C. The two different MPN determinations may have estimated the same or mainly the same group(s) of organisms, especially *T. ferrooxidans*, but with less tolerant strains in some samples being eliminated by the more extreme conditions (higher temperature and lower pH) of the former MPN procedure. This explanation seems likely, as many strains of *T. ferrooxidans* can grow at 40°C (Norris, 1990), as could most of the 37°C cultures subsequently incubated at 40°C in our study, while pH 1.0 can also be suitable for the growth of strains of this species (Razzell and Trussell, 1963). Strains of *L. ferrooxidans* that might tolerate the ferrous iron concentration of L and HJJ medium or heterotrophic iron-oxidizers that might find adequate organic nutrients in the L or HJJ medium during MPN determinations (see section **C.4.4.1**), might also be estimated by one or both procedures.

C.4.4.5. 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 often higher than, but mainly similar to those in HJJ medium containing 44 g/l $FeSO_4.7H_2O$. A higher count in JLFe than in HJJ medium suggests that more groups of organisms may have been counted in the particular sample using the former 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 if adequate organic nutrients were available from the inoculum or growth of autotrophs (Johnson and Roberto, 1997). 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, 1997) 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.

C.4.4.6. Acidophilic moderate ferrous iron- and sulphur-oxidizing bacteria

Tubes showing iron oxidation in JLFe medium in MPN determinations were tested for sulphur utilization in S^0-medium, as *T. ferrooxidans* utilizes ferrous iron and sulphur as energy sources (Kelly and Harrison, 1989). The pattern of tubes positive for sulphur utilization was therefore used to determine the MPN of presumptive *T. ferrooxidans* (Harrison, 1978) in a given sample. The estimates of presumptive *T. ferrooxidans* determined in this way were lower than those for the moderate ferrous iron-oxidizing bacteria in 47% of the samples and the high ferrous iron-oxidizing bacteria in 27% of the samples, while 27% of the 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 in many of the samples *T. ferrooxidans* may form a smaller percentage of the total iron-oxidizing population and even of the high ferrous iron-oxidizing population, than originally suspected. In the 25 samples (42% of the total) where the MPN estimates of high ferrous iron oxidizers and sulphur oxidizers were similar, it can be suggested that both were probably estimates of a *T. ferrooxidans* population.

C.4.4.7. Acidophlic moderate iron-, sulphur- and thiosulphate-oxidizing bacteria

The thiosulplate utilization 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 very few utilized thiosulphate. However, the identity of the others 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). The result may partly explain the lower counts of acidophilic thiosulphate-oxidizing than of acidophilic high ferrous iron-oxidizing bacteria in the coal waste samples, suggesting that the populations of *T. ferrooxidans* may have been greater than would be anticipated from the count of acidophilic thiosulphate oxidizers.

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

D.1. 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 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*.

D.2. MATERIALS AND METHODS

D.2.1. Microbiological Media

D.2.1.1. HJJ, 9K, L, and S^0 media

The composition and use of the HJJ, L and S^0 media have been described in section **C.2.3.2.** 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).

D.2.1.2. H medium

This medium was devised for the enrichment of heterotrophic 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 $MgSO_4 \cdot 7H_2O$, 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 min. To this basal medium, a filter-sterilized solution of 14 g $FeSO_4 \cdot 7H_2O$ 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.

D.2.1.3. *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 $MgSO_4 \cdot 7H_2O$ 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 min 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, U.S.A., cat. no. 50071) as the modified component in 250 ml distilled water, which was autoclaved at 121°C for 15 min and cooled to 55°C. The solutions were mixed gently prior to pouring the plates.

D.2.1.4. *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, Baningstoke, Hampshire, England) in 500 ml non-acidified distilled water. Both solutions were autoclaved at 121°C for 15 min and cooled to 45-50°C. After cooling, the solutions were mixed gently and poured into sterile Petri dishes aseptically.

D.2.1.5. FeSo medium

This solid medium developed by Johnson (1995b) and his colleagues (Johnson *et al.*, 1987; Johnson and McGinness, 1991a) was tested for the isolation of iron-oxidizing bacteria from enrichment cultures. It consisted of four solutions that were sterilized separately and mixed after cooling to 45°C in a water bath. The basal salts stock solution (BSS) and trace element stock solution (TES) were as described in Part 1 under JLFe medium (section **C.2.3.2.6**).

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, Maine, U.S.A.) 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. The 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 mixing thoroughly, 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.

D.2.2. Enrichment Cultures

D.2.2.1. Cultures from coal waste

Mines at which coal waste was sampled are shown in Fig. 17. The Corby Rock sample (CD1) was duff sampled on 13 July 1992 and the Kilbarchan sample K/E1 was fine soft waste from a duff-washing process, which was sampled on 13 October 1992. The moisture content of the samples after transport from KwaZulu-Natal was 6.19 and 10.46 g/100 g dry duff or waste and the pH 2.10 and 3.50, respectively. Sample K/G1 from the Kilbarchan Mine has been described in Part 1 (section **C.2.3.3**); it was sampled on 22 April 1993 and had a moisture content of 10.67 g/100 g dry waste and a pH of 5.75. The WC1 enrichment culture line was from an iron-oxidizing MPN culture from the Kilbarchan pilot scale dump rehabilitation experiment. Enrichment cultures were started from these and other Corby Rock and Kilbarchan samples by inoculating 10 g of sample into 100 ml 9K or HJJ medium in a 250 ml Erlenmeyer flask. The first incubation was stationary at 26°C.

The active ferrous iron-oxidizing 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). Samples were removed periodically for determination of residual ferrous iron by titration with acidic dichromate (Loos *et al.*, 1990a). Cultures were inoculated (10% v/v inoculum) into fresh medium when most of the ferrous iron had been metabolized. From early 1993, the use of 9K medium for the development or subculturing of enrichment cultures of iron-oxidizing bacteria was replaced by the use of HJJ medium.

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 sections **C.2.3.2** and **D.2.1.2**. The moisture content and pH of these samples are indicated later under section **D.3.4.1**. 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 and the cultures transplanted into fresh

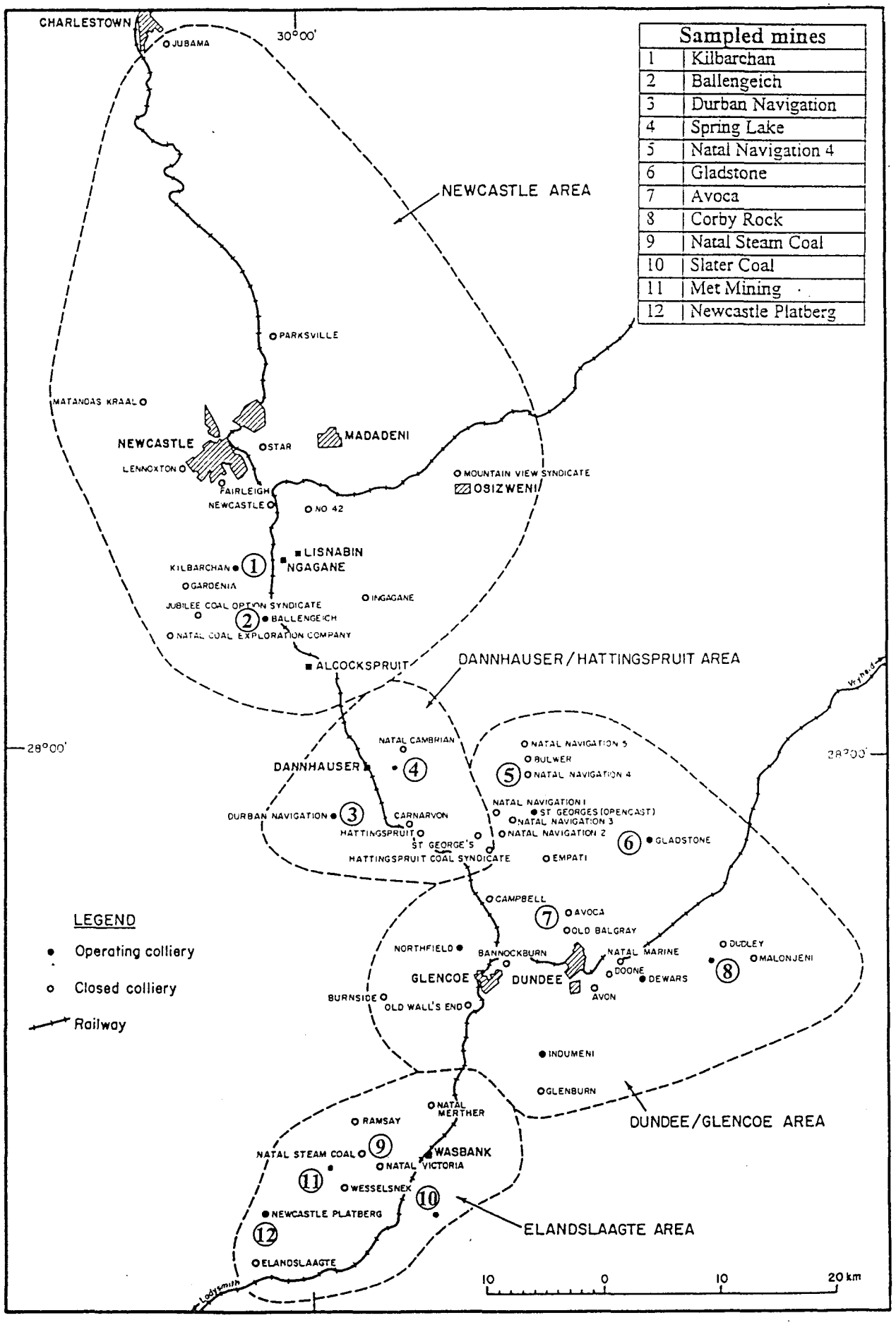

Fig. 17. Mines of the Klip River Coalfield in northern KwaZulu-Natal (Bell and Spurr, 1986), with sampled mines indicated by numbers on the map.

medium. Subsequently these enrichment cultures were transplanted into fresh medium every 4-6 weeks.

D.2.2.2. Cultures from mine dump drainage water

Enrichment cultures of iron-oxidizing bacteria were started in May 1994 by inoculating 10 ml samples of mine drainage water collected at the mines listed on Fig. 17 into 100 ml HJJ medium. The pH of the samples from which active enrichment cultures were derived, is reported under section **D.3.4.2**. The cultures were incubated at 26°C with shaking at 80 r.p.m. on a Gerhardt RO 20 rotary shaker. 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.

D.2.2.3. Cultures for microbial isolations

The isolations of organisms from the cultures described under sections **D.2.2.1** and **D.2.2.2** 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.

D.2.3. Isolation of Iron-oxidizing Bacteria

D.2.3.1. 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.

D.2.3.2. 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.

D.2.3.3. 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 (10% v/v inoculum) and incubating the cultures with shaking (80 r.p.m.) at 26°C.

D.2.4. 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.

D.2.4.1. 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 to obtain 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.

D.2.4.2. 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.

D.2.5. 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 0.01 N 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.

D.2.6. Characterization of Heterotrophic Organisms

D.2.6.1. 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 (Oxoid Ltd., Basingstoke, Hampshire, England) as directed by the manufacturer.

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 *T. 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.

D.2.6.2. 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, Detroit, Michigan, U.S.A.) supplemented with 50 mM test carbon source. The basal medium was sterilized at 121°C for 15 min 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, Detroit, Michigan, U.S.A.) 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 (Difco Laboratories, Detroit, Michigan, U.S.A.) to which 5 mM test nitrogen source and 1 g/l glucose were added (Barnett *et al.*, 1990).

D.2.6.3. 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.

D.2.7 Studies of Interactions between *T. ferrooxidans* and Fungi from Enrichment Cultures

Five sets of media were prepared, consisting of JLFe medium (section **C.2.3.2.6**) 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:

1. *T. ferrooxidans* ATCC 23270 alone.
2. *T. ferrooxidans* ATCC 23270 and *Acidiphilium organovorum*, a known bacterial associate of *T. ferrooxidans.*
3. *T. ferrooxidans* ATCC 23270 and a *Penicillium* sp.
4. *T. ferrooxidans* ATCC 23270 and a fungus of the unidentified Type 2.
5. *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 relative to the mean concentration of unoxidized ferrous iron in duplicate uninoculated 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.

D.3. RESULTS

D.3.1. Enrichment Cultures

D.3.1.1. Cultures from coal waste

Enrichment cultures in HJJ medium started from 1992 to 1994 from samples CD1, K/E1, K/G1 and WC1 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 heterotrophic acidophilic high temperature high ferrous iron-oxidizing bacteria), and cultures in H-medium, which were incubated at 26 and 40°C (selection for heterotrophic 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.

D.3.1.2. 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.

D.3.2. Isolation and Identification of Iron-oxidizing Bacteria from Enrichment Cultures

D.3.2.1. 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*.

D.3.2.2. 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.

D.3.3. Isolation and Identification of Heterotrophic Organisms from Enrichment Cultures

D.3.3.1. 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.

D.3.3.2. Identification

D.3.3.2.1. 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 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.

D.3.3.2.2. 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*.

D.3.3.2.3. 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 positively identified with certainty, these fungi closely resembled the drawings and photographs of Braun and Feiler (1995) and can be described as *Cladophialophora*-like.

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.

D.3.4. Distribution of Isolated Organisms in Enrichment Cultures from Coal Waste or Mine Drainage Water

D.3.4.1. 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 a Kilbarchan mini-dump 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.

D.3.4.2. 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 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 asolates, 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.

D.3.5. 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. 18. 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:

(1) 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. 18).

(2) 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.

(3) 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.

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	*Acidi-philium*	Type 1 *Penici-llium*	Type 2 uniden-tified	Type 3 uniden-tified
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	12.12	2.10	1A@NNC4	+	+	-	+
Navigation	9.75	2.50	1B@NNC4	+	-	-	+
4							
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	12.51	2.13	1A@SLC	-	+	-	+
Coal	14.73	2.13	1B@SLC	-	+	-	+
Met Mining	9.47	2.60	1A@MM	-	+	-	+
Newcastle	9.87	2.88	1A@PB	+	-	-	-
Platberg	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.

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 dump 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	2.38	PB#1	3	+	Y
Platberg	2.72	PB#2	0	+	-

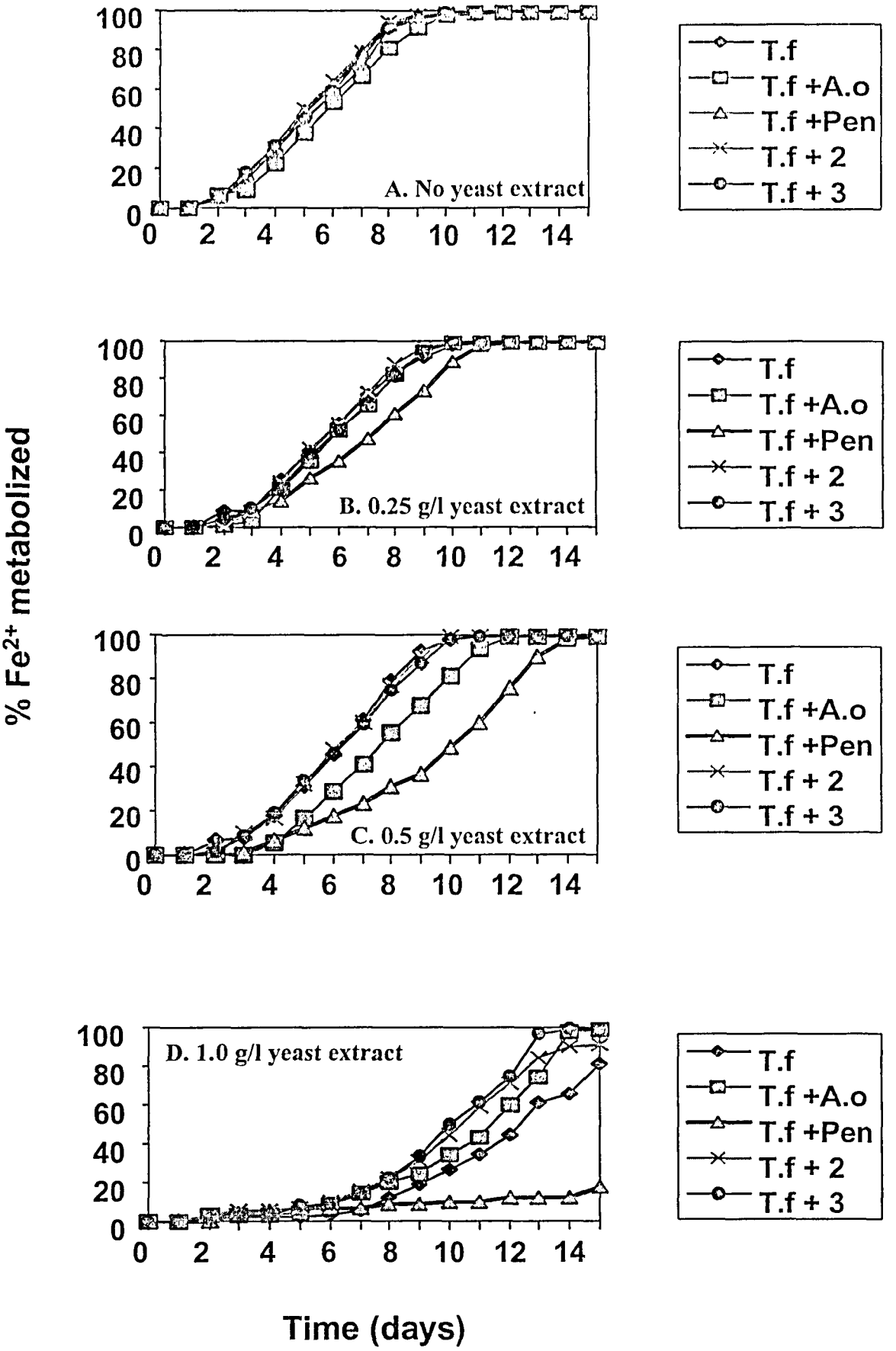

Fig. 18. Metabolism of ferrous iron by *T. ferrooxidans* in JLFe medium without organic supplementation (A) or supplemented with 0.25, 0.50 or 1.00 g/l yeast extract (B, C and D, respectively), alone (T.f) or in association (T.f +) with *A. organovorum* (A.o), *Penicillium* sp. (Pen) or unidentified fungus of Type 2 or 3 (2 and 3, respectively).

(4) 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 the best capable of overcoming the limited inhibition of 1.0 g/l yeast extract for *T. ferrooxidans*.

D.4. DISCUSSION

D.4.1. 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.

D.4.1.1. Cultures from coal waste

Many of the iron-oxidizing enrichment cultures in HJJ medium remained active and stable through numerous successive subcultures, indicating the presence of iron-oxidizing bacteria and the possibility of an association between the iron-oxidizing lithotrophs in these cultures and the heterotrophs that remained associated with them throughout the enrichment procedure.

The enrichment cultures CD1, K/E1, K/G1 and WC1, started from 1992 to 1994, were subjected to the most transfers to fresh media. The heterotrophic components of these cultures included both fungi (unidentified) and bacteria. The bacterial associates were identified as belonging to the genus *Acidiphilium*, in agreement with much research elsewhere showing bacteria of this genus to be consistent associates of *T. ferrooxidans* (Hallmann *et al.*, 1992; Harrison, 1984; Johnson, 1995a).

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.

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.

D.4.1.2. 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, benefiting 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 often gave higher MPN estimates of iron-oxidizing organisms than HJJ medium in the pilot scale dump study of Part 1, might yield a wider range of iron-oxidizing bacterial species (D.B. Johnson, 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.

D.4.2. Interaction Studies between *T. ferrooxidans* and Fungi from Enrichment Cultures

Interactions between the iron-oxidizing bacteria and the fungi that occur in acid mine drainage-generating environments may form an integral and important part of the ecology of acid mine drainage generation. For example, Bosch (1990) and Loos *et al.* (1990b) reported the degradation of sodium lauryl sulphate (an artificial inhibitor of *T. ferrooxidans*) by yeasts isolated from a pilot scale coal waste dump rehabilitation experiment in the Witbank area of Mpumalanga. The interaction studies between *T. ferrooxidans*, that was subjected to organic compound stress, and fungi isolated from enrichment cultures derived from coal waste in northern Kwazulu-Natal suggest that yeasts and fungi may stimulate the growth of *T. ferrooxidans* in a similar way to that of heterotrophic bacteria (see Johnson, 1995a,b; Harrison, 1984), i.e. 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. 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 clearly warrants further investigation.

E. DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

E.1. 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 over 4 years with their cycles of seasons supplied valuable information 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 coal waste 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, indicate 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 cells 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 0.3 m clay layer covered by topsoil which need perhaps not be 0.7 m thick, could be investigated as a possible cheaper cover. The effectiveness of the double soil covers of Escourt and Avalon soil in limiting the diffusion of oxygen into the coal waste and the generation of acid drainage was, however, not paralleled by reduction of the drainage outflow from the waste in the hydrological study of Wates, Meiring and Barnard (Wates and Rykaart, 1999); the greatest reduction of outflow was observed with the double layer of Avalon soil in cell 7, while the 0.5-m single layer of compacted Avalon

soil of cell 5 was as effective in reducing outflow as the double soil covers. This superiority of the Avalon soil alone in reducing outflow was not expected from the literature surveyed by Wates and Rykaart (1999), but was ascribed to the high water retention properties of the Avalon soil coupled with less susceptibility to desiccation cracking than the Estcourt soil.

The presence of a vegetation cover should prove valuable, by preventing the erosion of soil covers on dumps and reducing the movement of water and oxygen to the coal waste, but the advantages were not evaluated in the present study.

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 extracted for analysis in a portable oxygen/carbon dioxide meter. Samples of coal waste were removed 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.

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 indicated the dominance of acidophilic iron-oxidizing bacteria rather than thiosulphate- and/or sulphur-oxidizing bacteria. However, the high ferrous iron-oxidizing *T. ferrooxidans* may not have been the dominant iron-oxidizer, as population estimates using media with a lower ferrous iron concentration indicated that large numbers of other iron oxidizers with a lower ferrous iron tolerance were often 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 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 our 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. Our 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.

E.2. RECOMMENDATIONS

E.2.1 Recommendations Concerning Coal Waste Dump Rehabilitation Procedures

From the results presented in this report, the following recommendations can be made in connection with procedures for the rehabilitation of coal waste dumps involving the construction of clay and/or soil covers to prevent acid mine drainage formation and other undesirable occurrences, such as spontaneous combustion:

(1) The use of covers consisting of two layers, namely, an underlying less permeable clay or clayey soil layer and an upper topsoil layer, both of a suitable thickness, is recommended. In the present investigation, an underlying layer of 30 or 70 cm compacted Estcourt soil covered by 70 or 30 cm, respectively, of uncompacted Avalon soil (total cover thickness 1 m) was effective in preventing acidificaton of the underlying coal waste over a 4-year period.

(2) Use of a cover of a single soil type, exemplified by Avalon soil in this investigation, is not recommended, even though a 1-m cover of 70 cm compacted and 30 cm uncompacted Avalon soil greatly slowed acidification of the coal waste in the present study and showed the greatest reduction of water outflow. Thirty- and 50-cm-thick covers of Avalon soil had no or little delaying effect on acidification of the coal waste, although the latter was comparable with the double soil covers in limiting water outflow.

(3) Coal waste should be compacted during construction of a dump to counteract spontaneous combustion and soil or clay/soil covers should be vegetated to prevent erosion.

(4) The inhibition of acidification of the coal waste below a cover depends on the effectiveness of the cover as a barrier to oxygen diffusion into the dump. It is recommended that oxygen in the coal waste below covers be monitored routinely as in the present study, using permanently buried probes that extend through the covers into the coal waste. Using an appropriate meter, the oxygen concentration in the atmosphere of the coal waste below each probe can be measured in turn. An immediate result is obtained. Anaerobic conditions (no oxygen) indicate that the cover should be effective in blocking acidification; the presence of oxygen indicates that it is not.

(5) Acidification below covers should also be monitored from time to time by sampling coal waste below the cover by auger and measuring its pH.

(6) 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 the usefulness of the studies will depend on whether the bacteria investigated are the dominant group(s) involved in acid generation or not. Also they do not give a reliable indication of whether acidification is taking place extensively or not, as the numbers of iron- and sulphur-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 under (4) and (5).

E.2.2. Recommendations for Further Research

The following further research is recommended:

(1) Continued monitoring of the oxygen and carbon dioxide concentrations in the Kilbarchan mini-dumps and correlation of the results with rainfall, to check the maintenance of anaerobic conditions beneath the effective covers and in particular the effect of future drought conditions on the entrance of oxygen through dried or cracked covers.

(2) Monitoring of the pH in the coal waste beneath the apparently effective covers (for example, three times per year) to detect acidification if it should occur.

(3) A study of the acidophilic moderate ferrous iron-oxidizing bacteria in uncovered and effectively covered mini-dumps to ascertain whether their populations are better correlated with acidification than those of the acidophilic high ferrous iron-oxidizing bacteria which were the group monitored routinely in the present study. The moderate ferrous iron-oxidizers often showed greater populations than the high ferrous iron oxidizers in the present study (in a very limited investigation) but their significance is unknown. It may be greater than that of the high ferrous iron-oxidizers, exemplified by *T. ferrooxidans*.

(4) Interactions of heterotrophic microorganisms, particularly fungi, with *T. ferrooxidans* and other iron-oxidizing bacteria that appear significant in acidification, with a view to identifying organisms that stimulate or inhibit iron oxidation in the presence of organic matter. The latter group, in particular, may be of value in controlling acid drainage production if the conditions under which they can achieve inhibition in the field are established.

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G. APPENDICES

G.1. APPENDIX 1. TABLE OF MPN ESTIMATES WITH 95% CONFIDENCE LIMITS FOR ALL POSSIBLE COMBINATIONS OF FERTILE TUBES INOCULATED IN TRIPLICATE WITH 1, 0.1 AND 0.01 G OR ML OF INOCULUM

Certain combinations of fertile tubes observed in the MPN studies of coal waste microorganisms are not included in Table 1 of de Man (1983), having been excluded as highly unlikely results. However, MPN values, but not their 95% confidence intervals, are available for all possible combinations of fertile tubes where three successive tenfold dilutions are inoculated in triplicate into the test medium, for example, in Table 21c of the American Public Health Association *et al.* (1955). To overcome the problem of 95% confidence limits being unavailable for certain MPN estimates, Dr J.H. Randall, formerly Biometrician of the Faculty of Agricultural Sciences, University of Stellenbosch, computed the MPN values with their standard errors and 95% confidence limits shown in Appendix Table 1.1 (Randall, 1997). All values are rounded to the third decimal digit, but with further rounding, the MPN estimates in Appendix Table 1.1 agree with those of the American Public Health Association *et al.* (1955) and de Man (1983), while the 95% confidence limits agree with or are very close to those of de Man (1983). The MPN estimates and 95% confidence limits shown in Appendix Table 1.1, suitably rounded, were therefore used for those combinations of fertile tubes not included in de Man's Table 1. Each MPN estimate was rounded to the same number of significant digits as the estimate in Table 21c of the American Public Health Association *et al.* (1955) and each confidence limit to the same decimal digit as its MPN estimate. These rounded values were then multiplied by appropriate factors to correct them for the dilutions used for estimating the MPN and the moisture content of the coal waste sample.

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Appendix Table 1.1. Computed MPN estimates (d) of microorganisms/g or ml, with standard errors and 95% confidence limits, for all possible combinations of fertile responses ($f_1 f_2 f_3$) in media inoculated in triplicate with 1, 0.1 and 0.01 g or ml of inoculum (Randall, 1997)

$f_1 f_2 f_3$	d	stderr (d)	95% Confidence limits	
			Lower	Upper
000	0.000	-	0.000	0.942
001	0.301	0.301	0.015	0.953
002	0.602	0.305	0.125	1.763
003	0.905	0.537	0.145	2.026
010	0.305	0.426	0.016	1.062
011	0.611	0.432	0.125	1.764
012	0.918	0.622	0.145	2.026
013	1.226	0.517	0.363	3.806
020	0.619	0.530	0.126	1.767
021	0.931	0.545	0.356	3.739
022	1.243	0.645	0.363	3.806
023	1.556	0.787	0.452	3.809
030	0.944	0.631	0.356	3.739
031	1.261	0.758	0.363	3.806
032	1.579	1.000	0.452	3.810
033	1.899	0.803	0.452	3.810
100	0.357	0.522	0.017	1.763
101	0.723	0.613	0.126	1.791
102	1.099	0.707	0.357	3.739
103	1.484	0.772	0.452	3.808
110	0.736	0.438	0.127	2.026
111	1.118	0.776	0.357	3.751
112	1.511	0.879	0.452	3.808
113	1.914	0.916	0.452	3.810
120	1.138	0.359	0.358	3.806
121	1.539	0.669	0.452	3.809
122	1.950	1.021	0.452	3.810
123	2.372	0.898	0.874	9.419
130	1.568	0.897	0.452	3.810
131	1.989	1.122	0.452	3.810
132	2.420	1.107	0.874	9.419
133	2.861	1.591	0.877	9.424
200	0.918	0.696	0.145	3.739
201	1.433	0.526	0.363	3.808
202	1.992	0.672	0.452	3.811
203	2.599	1.318	0.874	9.419

Appendix Table 1.1 (continued)

$f_1 f_2 f_3$	d	stderr (d)	95% Confidence limits	
			Lower	Upper
210	1.469	0.657	0.369	3.808
211	2.047	0.873	0.452	3.870
212	2.678	1.529	0.874	9.419
213	3.361	1.808	0.877	9.424
220	2.106	1.072	0.454	4.098
221	2.763	1.144	0.874	9.421
222	3.477	1.421	0.877	9.425
223	4.242	2.108	0.903	10.440
230	2.855	1.367	0.875	9.424
231	3.604	1.660	0.877	9.425
232	4.408	2.731	1.686	17.649
233	5.257	4.275	1.686	17.649
300	2.312	1.272	0.459	9.419
301	3.850	1.891	0.877	10.440
302	6.356	5.491	1.686	17.682
303	9.538	6.768	3.710	31.528
310	4.273	1.652	0.903	17.649
311	7.489	3.211	1.691	19.965
312	11.521	8.138	3.711	31.657
313	15.880	9.150	3.806	38.080
320	9.328	5.232	1.763	31.528
321	14.936	6.795	3.739	38.072
322	21.466	11.687	3.808	40.400
323	29.172	17.408	9.003	99.945
330	23.979	14.572	3.808	99.922
331	46.218	36.254	9.021	199.989
332	109.895	81.540	17.649	407.734
333	∞	-	31.528	∞

G.2. APPENDIX 2. DETAILS OF pH, MOISTURE AND MPN DETERMINATIONS OF MICROBIAL POPULATIONS OF COAL WASTE SAMPLES FROM THE TEN PILOT SCALE DUMPS CONSTRUCTED NEAR THE KILBARCHAN MINE[a]

Appendix Table	Sampling date	Microbial population investigated besides acidophilic high ferrous iron-oxidizing microorganisms (26°)[b]
2.1	27-28/9/93	Acidophilic thiosulphate-oxidizing (26°)
2.2	22/11/93	Acidophilic thiosulphate-oxidizing (26°)
2.3	18/1/94	Acidophilic thiosulphate-oxidizing (26°)
2.4	28/2/94	Non-acidophilic thiosulphate-oxidizing (26°)
2.5	11/4/94	Acidophilic thiosulphate-oxidizing (26°)
2.6	28/5/94	Non-acidophilic thiosulphate-oxidizing (26°)
2.7	1/8/94	Acidophilic thiosulphate-oxidizing (26°)
2.8	26/9/94	Non-acidophilic thiosulphate-oxidizing (26°)
2.9	21/11/94	Presumed *Metallogenium* (26°)
2.10	16/1/95	Acidophilic thiosulphate-oxidizing (26°)
2.11	27/2/95	Presumed *Metallogenium* (26°)
2.11a	27/2/95	Highly acidophilic high ferrous iron-oxidizing (37°)
2.12	10/4/95	Presumed *Metallogenium* (26°)
2.12a	10/4/95	Highly acidophilic high ferrous iron-oxidizing (37°)
2.13	6/6/95	-
2.14	24/9/95	Highly acidophilic high ferrous iron-oxidizing (37°)
2.15	20/11/95	-
2.16	21/1/96	Highly acidophilic high ferrous iron-oxidizing (37°)
2.17	18/3/96	-
2.18	6/5/96	Acidophilic moderate ferrous iron-oxidizing (26°)
2.18a	6/5/96	Acidophilic moderate ferrous iron- and sulphur-oxidizing (26°)
2.19	3/6/96	Acidophilic moderate ferrous iron-oxidizing (26°)
2.19a	3/6/96	Acidophilic moderate ferrous iron- and sulphur-oxidizing (26°)

Appendix Table 2.1. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Starkey's medium, respectively, of coal waste samples obtained on 27-28 September 1993 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/S7	5.07	8.91	4683	1	982 - 19700	4	1	1-20
K1/W8	5.85	9.39	102	1	20 - 386	82	1	19 - 218
K2/E3	5.64	7.31	160965	1	32200 - 410000	23	2	3 - 43
K2/W2	5.87	10.59	4202	1	995 - 11500	22	0	6 - 42
K3/E7	5.73	7.63	100	1	19 - 387	8	1	2 - 21
K3/E9	5.38	5.45	253	1	42 - 1040	2425	1	527 - 9200
K4/S4	5.89	8.51	101	1	20 - 391	47	1	10 - 196
K4/W1	6.06	9.21	102	1	20 - 393	25	1	5 - 103
K5/S2	2.83	8.38	46603	1	9750 - 196000	2	1	0.5 - 10
K5/S6	5.17	9.43	16414	1	3280 - 41600	470	1	98 - 1980
K6/N7	5.91	8.95	47	1	10 - 197	25	1	5 - 102
K6/W8	6.17	10.63	48	1	10 - 200	25	1	6 - 104
K7/E4	6.10	9.63	26311	1	4360 - 109000	1019	1	197 - 3950
K7/W9	6.14	11.58	479	1	100 - 2020	16	1	3 - 42
K8/E6	6.00	10.02	3080	3	990 - 10300	102	1	20 - 396
K8/E9	6.21	10.13	47	1	10 - 199	47	1	10 - 199
K9/E3	6.17	7.92	809	1	183 - 2150	2	1	0.5 - 10
K9/E5	4.45	9.23	2512	1	546 - 10300	4	1	1 - 10
K10/N10	5.78	20.19	1802	1	361 - 4570	11177	1	2160 - 43300
K10/W7	5.92	19.43	5135	1	1070 - 21600	513	1	107 - 2160

[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); /g = /g dry coal waste.

Appendix Table 2.2. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Starkey's medium, respectively, of coal waste samples obtained on 22 November 1993 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/W5	5.32	12.06	5	1	1 - 20	3	1	0.6 - 11
K1/N2	3.29	9.97	16496	1	3300 - 41700	5	1	1 - 20
K2/S4	5.77	9.56	47	1	10 - 198	1019	1	197 - 3940
K2/S8	6.08	10.21	231	2	33 - 441	42	1	10 - 115
K3/S10	5.69	11.25	2559	1	556 - 10500	7	3	2 - 20
K3/E8	5.39	10.46	1027	1	199 - 3980	13	3	3 - 40
K4/W5	6.23	12.64	4280	1	1010 - 11700	3	1	0.6 - 11
K4/S5	6.32	14.82	49	1	10 - 208	24	2	3 - 46
K5/N8	3.69	14.41	24026	2	3430 - 45800	49	1	10 - 207
K5/W8	6.12	12.49	48	1	10 - 204	5	1	1 - 20
K6/N4	6.23	10.80	103	1	20 - 399	3	1	0.6 - 10
K6/W4	6.34	11.54	84	1	19 - 222	2	1	0.3 - 4
K7/E7	5.84	13.29	105	1	20 - 408	17	1	3 - 43
K7/W5	6.37	11.58	26	1	6 - 105	8	1	2 - 22
K8/S3	6.69	23.49	11	1	2 - 44	5	1	1 - 22
K8/S7	6.54	10.00	47	1	10 - 199	5	1	1 - 20
K9/E9	6.72	10.62	1659	1	332 - 4200	5	1	1 - 20
K9/E8	6.66	9.30	102	1	20 - 393	3	1	0.5 - 10
K10/W4	6.56	18.38	272	1	59 - 1110	5	1	1 - 21
K10/S3	5.98	9.45	164	1	33 - 416	1	1	0.2 - 4

[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); /g = /g dry coal waste.

Appendix Table 2.3. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Starkey's medium, respectively, of coal waste samples obtained on 18 January 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/W8	4.71	14.26	4913	1	1030-20600	4913	1	1030 - 20600
K1/S4	5.94	10.35	83	1	19 - 220	0.5	1	0.1 - 2
K2/N2	6.26	8.99	47	1	10 - 197	2	1	0.3 - 4
K2/S8	6.09	10.29	47	1	10 - 200	25	1	6 - 104
K3/S10	4.89	9.70	82	1	19 - 218	47	1	10 - 199
K3/W2	4.29	8.17	10060	1	1940 - 38900	10	1	2 - 39
K4/W9	6.31	13.69	49	1	10 - 206	1	1	0.2 - 4
K4/W3	5.87	12.07	26	1	6 - 105	104	1	20 - 403
K5/E6	5.87	11.75	123	3	45 - 391	0.3	1	0.06 - 1
K5/S6	6.15	9.68	823	1	186 - 2180	0.3	1	0.05 - 1
K6/S9	6.00	10.33	47	1	10 - 200	1	1	0.2 - 4
K6/E5	6.22	14.94	26	1	6 - 108	0.3	1	0.06 - 1
K7/N1	6.32	10.07	231	2	33 - 440	47	1	10 - 199
K7/S3	5.99	15.29	496	1	104 - 2090	0.3	1	0.06 - 1
K8/E8	6.56	14.06	262	1	57 - 1070	2	2	0.3 - 5
K8/S2	6.31	12.13	48	1	10 - 203	0.8	1	0.2 - 2
K9/E3	6.15	8.60	3	1	0.5 - 10	0.3	1	0.05 - 1
K9/E1	6.26	8.68	130	3	33 - 391	0.8	1	0.2 - 2
K10/E6	6.00	9.19	415	1	98 - 1130	25	1	5 - 103
K10/S7	5.99	9.40	821	1	186 - 2180	0.8	1	0.2 - 2

[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); /g = /g dry coal waste.

Appendix Table 2.4. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and non-acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Beijerinck's medium, respectively, of coal waste samples obtained on 28 February 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Beijerinck's medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/W8	4.15	8.33	100747	1	19500 - 390000	2	1	0.5 - 10
K1/S1	3.92	6.94	245962	1	53500 - 1010000	2	1	0.5 - 10
K2/S2	3.84	10.63	25445	1	5350 - 104000	3	1	0.6 - 10
K2/N1	5.71	9.37	4703	1	984 - 19800	3	1	0.5 - 10
K3/S8	4.69	8.90	1013	1	196 - 3920	0.2	3	0.05 - 0.4
K3/N8	2.68	10.27	23157	2	3308 - 44100	3	1	0.6 - 10
K4/E9	5.37	12.23	258	1	56 - 1055	5	1	1 - 20
K4/N6	5.44	10.33	2317	2	331 - 4413	3	1	0.6 - 10
K5/S9	4.63	16.17	13940	3	3485 - 41800	3	1	0.6 - 11
K5/W7	4.96	10.71	25463	1	5540 - 104000	5	1	1 - 20
K6/E4	5.41	16.08	27	1	6 - 109	5	1	1 - 21
K6/E7	5.65	13.92	26	1	6 - 107	3	1	0.6 - 11
K7/E2	5.64	10.24	254	1	55 - 1036	ND[e]	-	-
K7/N2	5.65	12.38	258	1	56 - 1056	8	1	2 - 22
K8/W1	5.69	16.57	2448	2	350 - 4663	11	1	2 - 42
K8/W4	5.42	10.54	48	1	10 - 200	3	1	0.6 - 10
K9/E5	5.27	9.44	164	1	33 - 416	3	1	0.5 - 10
K9/S5	5.52	10.75	255	1	55 - 1040	3	1	0.6 - 10
K10/S2	5.46	7.68	248	1	54 - 1010	2	1	0.5 - 10
K10/S7	5.50	15.32	4959	1	1040 - 20900	3	1	0.6 - 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); /g = /g dry coal waste.

[e] ND : Not determined.

Appendix Table 2.5. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Starkey's medium, respectively, of coal waste samples obtained on 11 April 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/N9	4.38	9.37	164	1	33 - 416	102	1	20 - 394
K1/E3	2.94	9.45	47064	1	9851 - 198000	5	1	1 - 20
K2/W3	4.92	8.29	412	1	97 - 1126	10	1	2 - 39
K2/S5	4.63	11.16	25567	1	5558 - 104000	8	1	2 - 22
K3/N5	3.31	10.72	4761	1	996 - 20180	476	1	100 - 2004
K3/E4	3.48	10.10	2532	1	551 - 10300	165	1	33 - 418
K4/N10	4.70	9.58	102	3	20 - 394	22	2	5 - 42
K4/S9	5.21	11.16	3779[e]	-	1000 - 10449	4	1	1 - 12
K5/S3	5.34	7.91	46401	1	9712 - 195000	25	1	5 - 101
K5/W1	2.31	10.08	16512[e]	-	5504 - 41830	10	1	2 - 40
K6/S6	4.34	8.45	130	3	33 - 390	2	1	0.5 - 10
K6/S2	5.29	7.91	15	2	4 - 38	2	1	0.5 - 10
K7/N6	5.52	8.44	101	1	20 - 390	5	1	1 - 20
K7/E10	5.62	10.23	474	1	99 - 1995	474	1	99 - 1995
K8/E2	5.81	9.30	251	1	55 - 1027	5	1	1 - 20
K8/W10	5.89	6.99	14979	2	4280 - 37400	5	1	1 - 20
K9/N1	5.80	7.58	1000	1	194 - 3873	8	1	2 - 21
K9/W2	5.81	11.20	3114	3	1001 - 10453	8	1	2 - 21
K10/N4	5.88	8.48	250	1	54 - 1020	23	2	3 - 43
K10/E9	5.87	9.89	252747	1	54900 - 1034000	231	2	33 - 440

[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) ; /g = /g dry coal waste.

[e] MPN and 95 % confidence limits according to J. H. Randall (see Appendix 1); no category.

Appendix Table 2.6. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and non-acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Beijerinck's medium, respectively, of coal waste samples obtained on 28 May 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Beijerinck's medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/S7	3.44	8.57	2280	2	326 - 4343	8	1	2 - 22
K1/S9	3.45	9.93	102	1	20 - 396	3	1	0.5 - 10
K2/S10	4.42	10.20	2535	1	551 - 10359	4	1	1 - 11
K2/N10	4.73	10.34	474	1	99 - 1997	4	1	1 - 11
K3/E4	3.78	9.35	1017	1	197 - 3937	3	1	0.5 - 10
K3/N2	3.36	8.74	228354	2	32600 - 435000	3	1	0.5 - 10
K4/N6	4.38	8.19	101	1	32 - 389	2	1	0.5 - 10
K4/W4	5.20	9.97	220	2	55 - 418	5	1	1 - 20
K5/S10	5.08	8.69	152	2	43 - 380	250	1	54 - 1022
K5/W1	5.18	11.93	48	1	10 - 203	5	1	1 - 20
K6/S4	5.45	9.73	102	1	20 - 395	5	1	1 - 20
K6/E6	4.42	10.67	103	1	20 - 398	8	1	2 - 22
K7/E7	5.49	12.94	486	1	102 - 2044	486	1	102 - 2044
K7/W9	6.04	11.15	256	1	56 - 1045	22	2	6 - 42
K8/W3	6.10	16.60	17	1	3 - 11	16	2	5 - 41
K8/N3	4.81	12.31	258	1	56 - 1056	104	1	20 - 404
K9/E2	5.50	12.69	259	1	56 - 1059	3	1	0.6 - 11
K9/N7	5.81	10.51	829	1	188 - 2199	3	1	0.6 - 11
K10/E8	5.90	18.17	1099	1	213 - 4254	3	1	0.6 - 11
K10/W2	5.86	17.33	5045	1	1056 - 21200	3	1	0.6 - 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); /g = /g dry coal waste.

Appendix Table 2.7. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Starkey's medium, respectively, of coal waste samples obtained on 1 August 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/W9	4.97	8.89	41	1	10 - 113	10	1	2 - 39
K1/W7	4.81	9.48	47	1	10 - 198	25	1	5 - 103
K2/N2	4.47	8.75	1305	3	326 - 3915	3	1	0.5 - 10
K2/W10	4.26	9.28	16392	1	3278 - 41500	47	1	10 - 198
K3/N1	4.65	10.18	220	0	55 - 419	8	1	2 - 22
K3/E2	4.54	9.61	102	1	20 - 395	10	1	2 - 39
K4/E5	4.77	7.64	161	1	32 - 409	2	1	0.5 - 10
K4/W6	5.06	10.56	166	1	33 - 420	8	1	2 - 22
K5/S6	5.20	10.70	25	1	6 - 104	155	2	44 - 387
K5/E8	5.37	10.14	165	1	33 - 419	3	1	0.6 - 10
K6/N8	5.56	10.76	25	1	6 - 104	5	1	1 - 20
K6/S8	5.61	11.37	26	1	6 - 105	26	1	6 - 105
K7/N10	5.70	10.28	254	1	55 - 1037	47	1	10 - 200
K7/E1	5.76	10.58	25	1	6 - 104	10	1	2 - 40
K8/W1	5.82	9.69	25	1	5 - 103	8	1	2 - 22
K8/W4	5.87	9.12	25	1	5 - 103	47	1	10 - 198
K9/N4	5.84	11.33	26	1	6 - 105	42	1	10 - 116
K9/N3	5.86	8.65	25	1	5 - 102	25	1	5 - 102
K10/E9	5.86	9.17	10	1	2 - 39	5	1	1 - 20
K10/E7	5.85	9.01	25	1	5 - 102	25	1	5 - 102

[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); /g = /g dry coal waste.

Appendix Table 2.8. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and non-acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Beijerinck's medium, respectively, of coal waste samples obtained on 26 September 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Beijerinck's medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/S10	3.75	17.14	108940	1	21100 - 422000	1	1	0.2 - 4
K1/E8	3.58	13.23	260429	1	56600 - 1070000	0.5	1	0.1 - 2
K2/N3	3.91	9.70	16455	1	3291 - 41700	10	1	2 - 39
K2/W5	4.04	10.08	0.3	1	0.05 - 1	0.8	1	0.2 - 2
K3/N8	3.13	7.49	1000	1	193 - 3869	0.1	1	0.02 - 0.4
K3/E6	3.42	8.31	46573	1	9748 - 196000	0.04	1	0.002 - 0.2
K4/N1	3.88	9.41	47046	1	9847 - 198000	5	1	1 - 20
K4/N9	4.35	8.21	8116	1	1840 - 21500	3	1	0.5 - 10
K5/S9	4.33	9.99	3190	3	990 - 10340	1023	1	198 - 3960
K5/W10	4.69	8.96	2615[e]	-	980 - 10242	16	1	3 - 41
K6/E3	5.18	10.74	26	1	6 - 104	3	1	0.5 - 10
K6/W1	5.35	10.03	25	1	6 - 103	3	1	0.5 - 10
K7/E1	5.97	10.51	254	1	55 - 1039	166	1	33 - 420
K7/S4	5.92	10.23	121	3	44 - 386	5	1	1 - 20
K8/W4	5.96	11.40	479020	1	101000 - 2020000	256	1	56 - 1047
K8/S2	6.01	8.75	25	1	5 - 102	7	3	2 - 20
K9/S5	6.00	20.54	1121	1	217 - 4339	11	1	2 - 43
K9/W3	5.58	11.82	104	1	20 - 403	3	1	0.6 - 11
K10/N6	5.98	9.50	47	1	10 - 198	2	2	0.3 - 4
K10/E4	5.78	9.03	10140	1	1963 - 39300	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); /g = /g dry coal waste.

[e] MPN and 95 % confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.9. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and presumptive *Metallogenium* populations able to grow at 26°C in HJJ and *Metallogenium* medium, respectively, of coal waste samples obtained on 21 November 1994 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in *Metallogenium* medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/N3	3.04	11.87	104039	1	20137 - 402732	5[e]	-	1 - 12
K1/W4	3.12	11.30	3896	0	1002 - 10462	0.2	1	0.06 - 0.4
K2/W1	3.19	10.27	1654	1	331 - 4190	1	1	0.2 - 4
K2/E1	3.63	9.90	473	1	99 - 1989	<0.03	-	-
K3/W8	4.42	10.69	2546	1	553 - 10405	1	1	0.2 - 4
K3/E4	ND[f]	ND	ND	-	-	ND	-	-
K4/E5	3.71	8.28	812	1	184 - 2155	1	1	0.2 - 4
K4/S4	3.94	9.55	3944	0	986 - 10298	3	1	0.5 - 10
K5/N7	3.44	10.57	1659	1	332 - 4202	8	1	2 - 22
K5/S8	ND	ND	ND	-	-	ND	-	-
K6/S10	4.66	12.85	49	1	10 - 204	17	1	3 - 43
K6/N4	5.08	10.26	25	1	6 - 104	0.3	3	0.1 - 1
K7/N9	5.19	10.60	1029	1	199 - 3982	51	1	10 - 219
K7/N5	5.32	10.43	166	1	33 - 420	10	1	2 - 40
K8/E7	5.41	10.07	102	1	20 - 396	2	2	0.3 - 4
K8/S2	5.50	9.28	47	1	10 - 198	1	1	0.2 - 4
K9/S5	5.54	9.17	251	1	55 - 1026	3	1	0.5 - 10
K9/N1	5.56	9.28	1007	1	195 - 3898	10	1	2 - 39
K10/S1	5.48	8.90	131	3	33 - 392	3	1	0.5 - 10
K10/N10	5.50	18.06	89	1	20 - 235	51	1	11 - 214

[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); /g = /g dry coal waste.

[e] MPN and 95 % confidence limits according to J.H. Randall (see Appendix 1); no category.

[f] ND: Not determined.

Appendix Table 2.10. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and acidophilic thiosulphate-oxidizing microbial populations able to grow at 26°C in HJJ and Starkey's medium, respectively, of coal waste samples obtained on 16 January 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/E8	3.83	12.24	10438	1	2020 - 40046	4826	1	1010 - 20315
K1/N2	3.32	11.51	479	1	100 - 2018	3	1	0.6 - 10
K2/S7	2.88	12.33	10447[e]	-	4044 - 42011	5	1	1 - 20
K2/E9	3.96	11.87	425	1	101 - 1163	48	1	10 - 202
K3/S9	3.93	11.65	424	1	100 - 1161	3	1	0.6 - 10
K3/S6	3.51	11.38	2561	1	557 - 10470	3	1	0.6 - 10
K4/E1	3.27	7.64	12917	3	3229 - 38750	2	1	0.5 - 10
K4/W2	4.15	7.25	24668	1	5363 - 100815	2	1	0.5 - 10
K5/S3	2.86	9.71	25233	1	5486 - 103127	3	1	0.5 - 10
K5/N6	3.95	8.96	46853	1	9806 - 197218	8	1	2 - 22
K6/W5	4.77	11.57	48	1	10 - 202	10	1	2 - 40
K6/W8	5.09	10.93	48	1	10 - 201	5	1	1 - 20
K7/W7	5.29	9.39	230	2	33 - 438	4704	1	985 - 19800
K7/E4	5.35	12.63	105	1	20 - 405	11	1	2 - 41
K8/E6	5.41	10.43	475	1	99 - 1999	25	1	6 - 104
K8/W1	5.68	9.90	47	1	10 - 199	3	1	0.5 - 10
K9/S1	5.46	8.35	3143	3	975 - 10727	3	1	0.5 - 10
K9/N10	5.62	10.91	255	1	55 - 1043	103	1	20 - 399
K10/E5	5.71	12.06	8405	1	1905 - 22300	26	1	6 - 105
K10/S5	5.72	9.88	703	3	176 - 1989	13	3	3 - 40

[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); /g = /g dry coal waste.

[e] MPN and 95 % confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.11. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and presumptive *Metallogenium* populations able to grow at 26°C in HJJ and *Metallogenium* medium, respectively, of coal waste samples obtained on 27 February 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in *Metallogenium* medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/W5	2.53	9.65	41667	1	9869 - 114036	32	3	10 - 109
K1/N7	2.68	9.56	164340	1	32868 - 416328	102	1	20 - 394
K2/N9	3.18	9.27	16391	1	3278 - 41523	23	2	3 - 143
K2/E6	3.68	9.23	25123	1	5462 - 102676	16	1	3 - 42
K3/W10	4.24	8.72	22831	2	3262 - 74880	0.3	1	0.05 - 1
K3/E1	2.87	10.63	47571	1	9957 - 200240	10	1	2 - 40
K4/N2	2.96	7.64	46	1	10 - 195	4	1	1 - 11
K4/W8	4.71	9.13	46926	1	9822 - 197525	0.8	1	0.2 - 2
K5/N1	5.16	9.28	164	1	33 - 415	10	1	2 - 39
K5/W7	3.04	8.95	21790	1	5448 - 41401	10	1	2 - 39
K6/N6	5.47	12.42	26	1	6 - 106	8	1	2 - 22
K6/W4	5.26	14.14	26	1	6 - 107	106	1	21 - 411
K7/S8	5.12	8.95	4685	1	981 - 19720	5	1	1 - 20
K7/W3	4.94	8.74	101	1	20 - 391	250	1	54 - 1022
K8/S1	5.39	11.67	4802	1	1005 - 20212	104	1	20 - 402
K8/E9	5.53	11.53	84	1	19 - 222	10	1	2 - 40
K9/S2	5.67	7.06	9957	1	1927 - 38542	2	1	0.5 - 10
K9/W4	5.88	12.68	10479	1	2028 - 40565	3	1	0.6 - 11
K10/S6	4.79	10.34	165510	1	33102 - 419292	25	1	6 - 10
K10/N5	4.22	12.01	8401	1	1904 - 22290	7	3	2 - 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); /g = /g dry coal waste.

Appendix Table 2.11a. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing microbial populations able to grow at 37° C in L medium, of coal waste samples obtained on 27 February 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[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/W10	4.24	8.72	360	1	90 -1080
K3/E1	2.87	10.63	266[e]	-	100-1040
K4/N2	2.96	7.64	3767	0	969 - 10118
K4/W8	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/W4	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/E9	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); /g = /g dry coal waste.

[e] MPN and 95% confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.12. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing and presumptive *Metallogenium* populations able to grow at 26°C in HJJ and *Metallogenium* medium, respectively, of coal waste samples obtained on 10 April 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in *Metallogenium* medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/E6	3.25	13.94	490	1	103 - 2062	9	1	2 - 23
K1/N9	2.76	11.27	4785	1	1001 - 20140	48	1	10 - 201
K2/E1	2.86	11.55	10374	1	2008 - 40158	0.4	0	0.1 - 1
K2/W5	2.83	10.26	33	3	1 - 105	17	1	3 - 42
K3/E4	3.08	12.04	803	3	201-2028	10	1	2 - 40
K3/E10	2.67	11.53	424	1	100 - 1160	1	1	0.2 - 4
K4/E5	2.74	9.43	25	3	5 - 103	2	2	0.3 - 4
K4/W7	3.52	8.53	4775[e]	-	1845 - 19101	3	3	1 - 10
K5/N6	6.01	8.71	47	1	10 - 197	0.5	1	0.2 - 2
K5/W3	2.92	8.22	4653	1	974 - 19588	1	3	0.3 - 4
K6/S8	6.05	9.29	25	1	5 - 103	0.3	3	0.1 - 1
K6/N5	6.23	9.26	470	1	98 - 1978	5	1	1 - 20
K7/E2	6.16	8.57	25	2	5 - 102	2	1	0.3 - 4
K7/W2	5.66	7.54	100	1	19 - 387	3	3	1 - 10
K8/E3	6.03	7.92	1004	1	194 - 3885	16	1	3 - 41
K8/N10	6.14	8.24	41	1	10 - 113	2	1	0.5 - 2
K9/S2	4.46	7.00	1605	3	321 - 4066	2	1	0.3 - 4
K9/W4	6.03	9.81	102123	1	19766 - 395316	5	1	1 - 20
K10/N1	6.45	7.87	46	1	10 - 195	2	1	0.4 - 4
K10/S9	6.57	16.79	444	3	105 - 1215	11	3	2 - 42

[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); /g = /g dry coal waste.

[e] MPN and 95 % confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.12a. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing microbial populations able to grow at 37° C in L medium, of coal waste samples obtained on 10 April 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[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/W7	3.52	8.53	25	1	5- 102
K5/N6	6.01	8.71	10	1	2- 39
K5/W3	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/W4	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); /g = /g dry coal waste.

Appendix Table 2.13. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing populations able to grow at 26°C in HJJ medium of coal waste samples obtained on 6 June 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/N3	2.98	10.84	103155	1	19966 - 399312
K1/E3	2.66	10.15	3109	3	984 - 10273
K2/E8	2.59	8.73	1022814	1	197964 - 3959280
K2/S10	2.57	8.69	16304	1	3261 - 41302
K3/N6	3.20	16.09	2671	1	581 - 10916
K3/E4	2.82	16.82	17523	1	3505 - 44392
K4/N1	3.05	9.86	230706	1	54930 - 439440
K4/S9	3.38	8.57	468184	1	97992 - 1970728
K5/W7	5.01	11.71	12229	3	4468 - 39095
K5/E10	3.59	10.12	13214	3	3304 - 39643
K6/S4	4.44	10.27	103	1	20 - 397
K6/S8	5.96	9.57	31775	3	9861 - 108474
K7/N2	6.28	8.89	102	1	20 - 392
K7/W9	5.33	7.18	2144	0	536 - 4073
K8/N4	5.98	9.90	47257	1	9891 - 198919
K8/E1	4.96	6.20	15929	1	5186 - 40358
K9/W3	6.08	8.01	162[e]	-	54 - 410
K9/E9	5.76	8.47	16271	1	3254 - 41219
K10/S6	5.56	9.89	47	1	10 - 199
K10/E6	5.92	9.35	394	0	98 - 1028

[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); /g = /g dry coal waste.

[e] MPN and 95 % confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.14. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ medium or at 37°C in L medium, of coal waste samples obtained on 24 September 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[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 - 4126
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	<0.03	-	-
K8/S1	6.10	9.84	1648	1	330 - 4174	22	2	5 - 42
K8/W2	6.27	10.51	254166	1	55253 - 1005613	<0.03	-	-
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); /g = /g dry coal waste.

[e] MPN and 95% confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.15. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ medium, of coal waste samples obtained on 20 November 1995 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[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/W5	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/Nl0	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	626	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); /g = /g dry coal waste.

Appendix Table 2.16. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ medium or at 37°C in L medium, of coal waste samples obtained on 21 January 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in L medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[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]	-	411-4271
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]	-	6-44
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); /g = /g dry coal waste.

[e] MPN and 95% confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.17. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ medium, of coal waste samples obtained on 18 March 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ-medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/W4	3,10	12,60	105	1	20 - 405
K1/E10	3,18	10,88	1663	1	333 - 4213
K2/S2	3,02	11,15	2557	1	556 - 10448
K2/W6	2,99	11,48	1037	1	200 - 5013
K3/N1	3,09	13,89	239	2	34 - 456
K3/S7	3,17	13,41	2608	1	567 - 10661
K4/N6	5,74	15,06	26	1	6 - 108
K4/W10	2,84	13,00	8475	1	1921 - 22448
K5/N2	4,09	15,72	4976	1	1042 - 20946
K5/E7	4,96	17,61	1764	1	353 - 4469
K6/W7	6,04	16,52	50	1	10 - 211
K6/W8	6,19	14,80	26	1	6 - 108
K7/N7	5,65	12,42	4834	1	1012 - 20348
K7/S3	5,52	16,56	1748	1	350 - 4429
K8/E5	5,99	15,86	50	1	10 - 210
K8/W2	4,89	14,81	17221	1	3444 - 43627
K9/N4	6,14	12,02	10	1	2 - 40
K9/S9	6,25	19,50	111	1	22 - 430
K10/S10	6,39	26,91	55	1	11 - 230
K10/W3	6,29	20,74	519	1	109 - 2185

[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); /g = /g dry coal waste.

Appendix Table 2.18. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high and moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ and JLFe medium, respectively, of coal waste samples obtained on 6 May 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in JLFe medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[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	1986 - 23253	18	3	6-44
K2/N6	3.08	16.25	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	48	1	10 - 202	480	1	100 - 2020
K7/S10	3.29	14.53	2291	2	573 - 4352	106512	1	20 615 - 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	555 - 4215
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); /g = /g dry coal waste.

Appendix Table 2.18a. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in JLFe medium (see Appendix Table 2.18) and subsequently in S^0 medium, of coal waste samples obtained on 6 May 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Sulphur oxidizers in S^0-medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/S7	2.98	13.57	34	2	10-114
K1/N7	2.79	15.20	<0.03	-	-
K2/S3	3.15	16.85	7	2	2-19
K2/N6	3.08	16.25	<0.03	-	-
K3/E5	3.58	13.45	4	1	0.2-19
K3/N10	3.43	10.01	<0.03	-	-
K4/W3	3.51	8.81	<0.03	-	-
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	<0.03	-	-
K6/N5	6.42	11.59	<0.03	-	-
K7/S10	3.29	14.53	<0.03	-	-
K7/E6	6.10	11.11	10	1	2-39
K8/E7	6.19	11.81	<0.03	-	-
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); /g = /g dry coal waste.

Appendix Table 2.19. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high and moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ and JLFe medium, respectively, of coal waste samples obtained on 3 June 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in JLFe medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]	MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/N2	3.06	8.56	10096	1	1954-39082	2497	1	543-10205
K1/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	47674[e]	-	18420-190696
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); /g = /g dry coal waste.

[e] MPN and 95% confidence limits according to J.H. Randall (see Appendix 1); no category.

Appendix Table 2.19a. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in JLFe medium (see Appendix Table 2.19) and subsequently in S^0 medium, of coal waste samples obtained on 3 June 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Sulphur oxidizers in S^0 medium		
			MPN/g[d]	Category[d]	95% Confidence limits/g[d]
K1/N2	3.06	8.56	<0.03	-	-
K1/W6	3.10	8.88	675	3	131-1851
K2/W2	3.38	7.85	669	3	129-1833
K2/W4	3.04	8.35	8	2	1-18
K3/W9	3.56	8.80	3	3	0.1-10
K3/S8	3.66	9.76	670	3	132-1866
K4/N1	3.22	9.68	22	0	5-42
K4/N5	3.75	9.80	33	3	10-104
K5/N4	5.76	10.15	<0.03	-	-
K5/E10	3.45	9.07	<0.03	-	-
K6/S10	6.15	12.49	48	1	10-204
K6/E9	6.37	10.23	<0.03	-	-
K7/W1	4.34	9.65	22	0	5-42
K7/E9	4.67	10.33	<0.03	-	-
K8/N3	6.04	10.65	<0.03	-	-
K8/E4	5.33	10.39	<0.03	-	-
K9/W8	6.36	12.74	4	1	0.2-19
K9/E3	3.19	11.85	10	1	2-39
K10/S9	6.45	20.31	<0.03	-	-
K10/S1	3.12	10.13	10	1	2-39

[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); /g = /g dry coal waste.

Appendix Table 2.20. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic high and moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in HJJ and JLFe medium, respectively, of coal waste samples obtained on 2 September 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Ferrous iron oxidizers in HJJ medium			Ferrous iron oxidizers in JLFe medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/W3	2.96	8.23	47	1	10-196	11905	1	2165-43290
K1/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	21742-434 470
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	332-4205
K6/N1	6.61	11.12	26	1	6-104	511	1	100-2200
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	44-1087	25248	1	5489-103189
K9/W4	5.98	11.82	168	1	34-425	2572	1	559-10511
Kl0/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); /g = /g dry coal waste.

Appendix Table 2.20a. Details of pH, moisture and MPN determinations (including category and 95% confidence limits) of acidophilic moderate ferrous iron-oxidizing microbial populations able to grow at 26°C in JLFe medium (see Appendix Table 2.20), subsequently also in S^0 medium and then in Starkey's medium, of coal waste samples obtained on 2 September 1996 from the 10 pilot scale dumps constructed near the Kilbarchan Mine

Mini-dump/ sample[a]	pH[b]	Moisture in sample (g/100 g dry mass)[c]	Sulphur oxidizers in S^0 medium			Thiosulphate oxidizers in Starkey's medium		
			MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]	MPN/g[d]	Cate-gory[d]	95% Confidence limits/g[d]
K1/W3	2.96	8.23	17	3	5-41	10	1	2-38
K1/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	<0.03	-	-
K8/N4	5.88	9.94	506	1	99-2177	8	1	1-20
K8/N7	6.05	9.03	1635	1	327-4143	<0.03	-	-
K9/E7	4.42	9.78	23	1	5-44	<0.03	-	-
K9/W4	5.98	11.82	1230	1	224-4473	4	1	0.2-19
K10/E6	4.83	9.42	25	1	5-103	<0.03	-	-
K10/S8	5.93	15.16	1267	1	230-4606	<0.03	-	-

[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); /g = /g dry coal waste.

Appendix Table 2.21. Details of pH of samples taken from the 10 pilot scale dumps constructed near the Kilbarchan mine, during the period 21 October 1996 to 7 August 1997

Mine-dump/ sample	Sample date, position and pH										
	21/10/96[a]	09/12/96		02/02/97		24/03/97		12/05/97		07/07/97	
	pH[b]	Position[c]	pH[b]	Position[c]	pH[b]	Position[c]	pH[b]	Position[c]	pH[b]	Position[c]	pH[b]
K1/1	3.07	E7	3.12	E2	2.99	E4	2.82	E2	3.00	W5	3.23
K1/2	3.03	E8	3.14	S2	3.06	S4	2.97	W4	2.91	E5	3.21
K2/1	3.12	N3	3.06	N7	2.99	W8	2.95	S6	2.88	S7	3.15
K2/2	3.08	W8	3.16	S7	2.90	N3	2.94	N7	2.91	N3	3.19
K3/1	3.62	W6	2.98	S9	3.98	E8	5.35	S9	3.54	W4	4.32
K3/2	4.32	W4	3.16	S5	3.90	S6	5.11	S7	6.01	S3	4.41
K4/1	2.85	S3	3.51	S4	2.84	N2	3.50	S8	2.92	E9	2.99
K4/2	3.14	S6	3.69	W3	2.76	E7	2.88	W3	3.34	E6	2.76
K5/1	3.36	W2	3.55	E5	3.26	N5	2.90	N2	3.02	E3	3.23
K5/2	3.04	S9	3.37	E9	3.00	W3	3.25	S8	2.89	N6	2.98
K6/1	5.90	E3	5.75	E7	5.75	W7	6.28	E8	6.57	W2	6.65
K6/2	6.13	S7	6.25	N2	5.86	W2	6.60	N3	6.67	N5	6.79
K7/1	3.92	S5	3.96	S6	4.40	W9	3.16	W4	3.87	N4	3.65
K7/2	6.03	E2	5.54	S8	3.93	E5	2.73	S3	3.27	N2	3.56
K8/1	6.03	N2	6.03	E8	5.60	E9	6.02	E9	6.47	W6	6.29
K8/2	5.70	N7	5.07	N3	5.73	S3	6.18	E6	6.38	S5	6.21
K9/1	3.44	E9	3.02	W8	5.74	W6	6.26	W5	6.67	W7	6.66
K9/2	4.20	S8	5.45	W6	5.81	W4	6.33	E5	6.57	N8	6.73
K10/1	4.49	N2	4.11	W4	4.43	N8	4.05	N9	5.26	W3	4.05
K10/2	3.40	N8	5.80	S3	4.85	S2	5.31	W7	6.17	W9	3.53

[a] Position not available.

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

[c] 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).