The microbiology of acid mine drainage: genesis and biotreatment

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Waters draining abandoned mines, spoil heaps and mineral tailings are often acidic (sometimes extremely so) and rich in metals. A primary cause of acid mine drainage (AMD) formation is thought to be the oxidative dissolution of pyrite and other sulphidic minerals within mineral-rich strata and wastes, which is accelerated by chemolithotrophic acidophilic bacteria such as *Leptospirillum ferrooxidans* and *Acidithiobacillus ferrooxidans.* However, microbial life in AMD waters is not restricted to iron-oxidizing bacteria, but includes sulphur-oxidizing bacteria and archaea, heterotrophic microorganisms and some lower eukaryotic life-forms. Among the acidophilic heterotrophic bacteria that have been isolated from AMD are facultatively anaerobic *Acidiphilium* species which catalyse the dissimilatory reduction of ferric iron, and obligate anaerobes that catalyse the dissimilatory reduction of sulphate to sulphide. In a survey of AMD from seven mine sites in the U.K. and mainland Europe, iron-oxidizing bacteria were found in varying numbers (10^2 to 5.7×10^6 ml^{-1}) in waters draining metal mine sites. Smaller numbers (<10^2 ml^{-1}) were found in AMD at two abandoned coal mine sites, though iron-oxidizing bacteria could be readily isolated from enrichment cultures of these waters. Acid-tolerant/acidophilic sulphate-reducing bacteria were also isolated successfully from AMD stream sediment, using acidic overlay media. The importance of microbial oxidation of ferrous iron in AMD was demonstrated by comparing rates of oxidation in native and filter-sterilized mine water samples. Packed-bed bioreactor columns using porous glass beads as a support matrix for microbial biofilm development were set up, and different populations of acidophilic microorganisms isolated from AMD were introduced, as follows: (i) an iron-oxidation bioreactor, using a mixed population of iron-oxidizing bacteria, obtained by enrichment of coal mine AMD; (ii) iron-reduction bioreactors, using *Acidiphilium* species and (iii) sulphate-reduction bioreactors, using a mixed population of acidophilic and acid-tolerant sulphate-reducing bacteria isolated from mine waters and related environments. The performance of the bioreactors was assessed over several months in the laboratory. The results showed that indigenous acidophilic bacteria can be used successfully in packed-bed bioreactors to promote targeted changes in AMD water chemistry, and thereby facilitate bioremediation of these polluting waste waters.

Introduction

Abandoned coal and metal mines are often focal points of environmental pollution. Compared to non-polluted surface waters, those draining deep and opencast mines, spoil heaps and mineral tailings are characteristically enriched with metals, particularly iron. These waters are frequently acidic owing to the presence of hydrogen ions ('proton acidity') or dissolved metals ('mineral acidity'), usually iron and aluminium. Oxidation (in the case of ferrous iron) and hydrolysis of the metals lead to the genesis of additional hydrogen ions, as follows:

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$$Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+. \quad (1)$$

Other metals, such as manganese, can also contribute to the mineral acidity of mine effluents. Hedin *et al.*[1] proposed that the total acidity of coal mine drainage could be calculated using the equation

$$\text{Acidity calculated} = 50\left[\frac{2Fe^{2+}}{56} + \frac{3Fe^{3+}}{56} + \frac{3Al^{3+}}{27} + \frac{2Mn^{2+}}{55} + 1000(10^{-pH})\right], \quad (2)$$

where acidity is evaluated as mg l^{-1} calcium carbonate equivalent.

Discharge waters from coal and metal mines can also contain reduced inorganic sulphur compounds (RISCs), which arise during the oxidative dissolution of sulphide minerals (described below); these compounds can also give rise to acidity in mine waters, for example for trithionate:

$$S_3O_6^{2-} + 2O_2 + 2H_2O \rightarrow 3SO_4^{2-} + 4H^+. \quad (3)$$

The contribution of RISCs to the net acidity of mine effluents has generally been overlooked, owing to the sparseness of data on the abundance of RISCs in these waters. Pichtel and Dick,[2] however, found that trithionate (the dominant RISC in their experimental system) was present at 1.07 mmol kg^{-1} of incubated spoil material (a mixture of pyrite, coal and rock), compared with ferrous iron (at *c.* 25 mmol kg^{-1}) and total iron (at *c.* 90 mmol kg^{-1}) after the same period of incubation. In addition, Schippers *et al.*[3] noted that both tetrathionate and pentathionate were present in cultures of *L. ferrooxidans* during leaching of pyrite, though their combined concentration (*c.* 300 μM after 7 days' incubation) was again far lower than that of soluble iron (*c.* 20 mM).

Moreover, mine discharge waters that are moderately acidic to neutral (pH >4.5) do contain alkalinity, principally as bicarbonate arising from the dissolution of carbonaceous minerals. Mine effluents can therefore contain both bicarbonate alkalinity and mineral acidity, though the fact that the majority of these waters are net acidic has led to the widespread use of the generic term 'acid mine drainage' (AMD) to describe them.

There is consensus that a prime cause of AMD genesis is the oxidative dissolution of sulphidic minerals in mine strata, spoils and tailings. Coal typically contains up to 10% sulphur, about half of which is commonly pyrite (FeS_2, the dominant sulphide mineral in the lithosphere); the major ores of many metals are either sulphidic or else are associated with pyrite and other sulphides. Exposure of these minerals to combined water and oxygen will cause them to oxidize spontaneously, though the oxidative process is greatly accelerated in the presence of certain chemolithotrophic (literally 'rock eating') bacteria. As ferric iron is a more powerful oxidant of pyrite than is molecular oxygen,[4] bacteria and archaea that generate this ion by the dissimilatory oxidation of ferrous iron in low-pH liquors (where ferric iron is soluble and therefore a more effective oxidant) can accelerate the oxidation of pyrite and other sulphides by a factor of up to 10^6.

The process of sulphide mineral dissolution by acidophilic iron-oxidizing bacteria has been the subject of much research

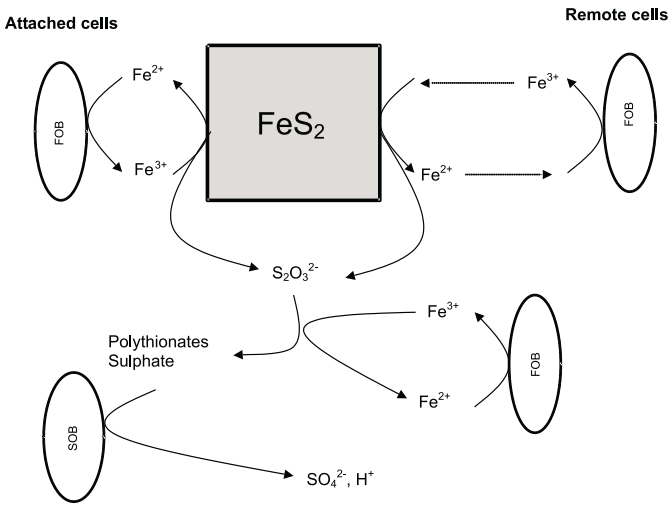

Fig. 1. Hypothetical scheme for the oxidative dissolution of pyrite by acidophilic bacteria.[3,7] FOB denotes iron-oxidizing bacteria and SOB sulphur-oxidizing bacteria.

and debate.[5–7] Figure 1 outlines the mechanism described by Schippers *et al.*[3] In this scheme, cycling of iron between its ferrous and ferric ionic states provides the driving force for mineral oxidative dissolution. Iron is reduced on reaction with pyrite, and oxidized by bacteria that use the energy to fuel their metabolic processes. The sulphide moiety of the mineral is initially only partly oxidized, accounting for the occurrence of RISCs in AMD. Subsequent oxidation of RISCs by ferric iron and by sulphur-oxidizing acidophiles produces sulphate, as well as additional proton acidity.

While mines are active, control of water tables within them effectively minimizes the amount of AMD that is produced. When mines are closed, however, and water pumps are switched off, water tables rebound, causing the products of sulphide mineral dissolution on wall and roof surfaces to come into solution. Rising water levels can impose considerable pressure on plugs and other devices meant to seal the mines, and catastrophic breakouts, such as occurred at the Wheal Jane tin mine in Cornwall in 1992, are not uncommon. Flooding of mines tends to reduce the rate of sulphide mineral oxidation due to decreased accessibility to oxygen, though fluctuating water tables can accelerate mineral oxidation. However, since ferric iron oxidation of sulphides does not require molecular oxygen, this process can continue even in anoxic areas of mines. The same is true of mine spoils, where ferric iron produced in the aerobic outer layer of the mounds can leach sulphide minerals within the anaerobic core. In both scenarios, the dominant form of iron at the point of discharge is mostly ferrous, even in extremely acidic waters.

Knowledge of the microbiology of AMD advanced considerably during the latter part of the 20th century. Once thought to be limited to a few specialized iron- (*Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*) and sulphur-oxidizing (*Acidithiobacillus thiooxidans*) acidophiles, the biodiversity of acidic, metal-rich mine waters is known to be extensive, though it is generally restricted to prokaryotic and simple eukaryotic life-forms.[8] A variety of indigenous microorganisms can catalyse the dissimilatory oxidation and reduction of iron and sulphur. Interactions between acidophilic microorganisms are also many and varied, and can accelerate or retard mineral dissolution. This suggests that selection and control of certain microorganisms that thrive in these 'extreme' environments could conceivably be used in systems aimed at remediating mine water pollution. Previously, immobilized iron-oxidizing bacteria (predominantly *At. ferrooxidans*) have been used in laboratory and pilot scale operations to remove iron (as insoluble ferric iron) from AMD.[9–11]

Reductive processes, on the other hand, can generate alkalinity, for example (in the case of iron):

$$Fe(OH)_3 + e^- \rightarrow Fe^{2+} + 3OH^-. \quad (4)$$

In addition, biogenic reduction of sulphate can lead to the effective removal of many toxic metals as highly insoluble sulphides, for example:

$$Cu^{2+} + H_2S \rightarrow CuS + 2H^+, \quad (5)$$

where the solubility product (log K_{sp}) of CuS is –35.9 at 20°C.

In this paper, we describe areas of research in which we have sought to characterize the microbiology of acid mine drainage waters from various locations in the U.K. and beyond, and of the development of novel bioremediation prototypes based on the use of microorganisms which are indigenous to AMD and similar acidic environments.

Materials and methods

Mine sites

Mine water samples from a variety of sites were analysed. Mine sites in the United Kingdom were: Cae Coch (pyrite; Gwynedd), Parys (copper; Gwynedd), Wheal Jane (tin; Cornwall), Bullhouse (coal; Yorkshire) and Ynysarwed (coal; mid-Glamorganshire). Samples were also taken from the Rio Tinto (Spain) just downstream from the Rio Tinto copper mine, and of water seeping from a spoil dump at the site of the former King's copper mine, Roeros, Norway (by Eigil Iversen of the Norwegian Institute for Water Research, NIVA, Oslo). Sterilized (filtered through 0.2 μm membrane filters), acidified, and non-modified (in sterile plastic vessels) samples were taken to facilitate a full range of microbiological and physico-chemical analyses.

Microbiological analyses

Aerobic acidophilic microorganisms were isolated from AMD samples using serial dilution of samples and plating onto selective solid media.[12] Several media formulations were used, mostly based on the 'overlay' technique in which an acidophilic heterotrophic bacterium (*Acidiphilium* strain SJH) is incorporated into the lower layer of the gel in order to remove organic compounds that are inhibitory or toxic to many of the more fastidious acidophiles, such as *L. ferrooxidans.* In addition to the media quoted by Johnson,[12] two additional overlay solid media were used: (i) a variant of the FeSo medium, in which potassium tetrathionate was replaced by sodium thiosulphate (5 mM, final concentration), the ferrous sulphate concentration lowered to 5 mM, and the final medium pH adjusted to pH 3.5 (as opposed to pH 2.5 in FeSo medium); (ii) yeast extract (0.02%, w/v) overlay medium, in which *Acidiphilium* SJH was replaced by *Acidocella* WJB3, an acidophilic heterotroph that does not metabolize yeast extract.[13] Classification and identification of aerobic isolates was carried out using the techniques described by Johnson and Roberto.[14] Sulphate-reducing bacteria in sediment samples collected at the Parys mine site were enumerated using two media: (i) a glycerol (5 mM)/tryptone soya broth (0.025%)/ ferrous sulphate (10 mM) overlay medium, pH ~3.5; (ii) the same medium prepared without glycerol. Plates were incubated anaerobically ('Anaerogen' system; Oxoid, U.K.) for up to four weeks. Colonies of sulphate-reducing bacteria were identified from their black colouration.

Table 1. Physico-chemical and microbiological data from a range of acid mine drainage waters. For details of site locations, see text.

	Cae Coch	Parys	Wheal Jane	Bullhouse	Ynysarwed	Rio Tinto	Roeros
pH	2.5	2.5	3.6	5.9	6.2	2.5	3.7
Eh (mV)	+705	+685	+500	+257	+214	nd	nd
Fe (total)	1350	650	130	61	160	2500	6.7
Fe^{2+}	150	650	130	45	140	nd	nd
Al^{3+}	60	70	nd	1.2	20	nd	4.3
Mn^{2+}	15	10	nd	15	nd	nd	0.25
Cu^{2+}	<1	40	nd	<1	nd	50	3.76
Zn^{2+}	<1	60	200	<1	nd	150	11
Fe^{2+}-oxidizing bacteria	1.1×10^4	2.0×10^3	1.0×10^2	40	<10	5.7×10^6	6.4×10^3
Sulphur-oxidizing bacteria*	$<10^3$	$<10^2$	<10	<10	<10	$<10^4$	1.0×10^3
Heterotrophic acidophilic bacteria	9.0×10^3	2.0×10^3	3.0×10^2	<10	<10	1.0×10^6	2.1×10^5

*Not including S-oxidizing bacteria that also oxidize ferrous iron.
Metal concentrations are in mg l^{-1}, and bacterial counts in no. ml^{-1}; nd, not determined.

Iron oxidation in mine water samples

Rates of ferrous iron oxidation in filter-sterilized and non-sterilized AMD samples were compared by incubating water samples (50 ml) in sterilized shake flasks (100 ml); the samples were incubated, shaken, at 20°C and were analysed for ferrous iron using the ferrozine reagent.[15] The effects of nutrient addition on iron oxidation in native water samples were tested by addition of a concentrated basal salts mix, used routinely for culturing iron-oxidizing acidophiles.[16] Replicate samples were set up and analysed in each case.

Bioremediation of AMD using oxidative processes

Fixed-bed bioreactors were set up using immobilized iron-oxidizing bacteria, and monitored for their abilities to remove iron from synthetic AMD. Porous glass beads (8–16 mm diameter), made from recycled glass by Dennert Poraver GmbH (Germany), provided the support matrix on which bacterial biofilms developed. The beads were acid-washed with H_2SO_4 to remove any soda-glass present, rinsed repeatedly with distilled water and put into 10-l flat-bottomed flasks, together with 5 mM ferrous sulphate and basal salts and trace elements. The flasks were inoculated with AMD from the Ynysarwed mines, and incubated at room temperature (unshaken, but aerated at 1 l air min^{-1}). When ferrous iron was oxidized (which took about two weeks in the first instance) the greater part of the spent medium was removed and replaced with fresh medium. This pattern was continued for at least five cycles, by which time the rate of ferrous iron oxidation in added medium had increased considerably, indicating that biofilms had established successfully on the beads. At this point the beads were put into cylindrical perspex columns (height 20 cm, diameter 9.5 cm), covered with a layer (2 cm) of sterile gravel (5–10 mm diameter) and percolated with ferrous sulphate medium at about 500 ml hr^{-1}. Column aeration was maintained at about 1 l min^{-1}. Following an initial period during which the medium was recirculated, a flow-through system was established. This further encouraged the development of the biofilm on the beads, and was continued for 2–3 weeks ahead of initial monitoring of bioreactor performance. The flow-through system entailed percolating the column at different flow rates (50–1250 ml hr^{-1}), with synthetic AMD (basal salts at 10% of growth medium concentration) containing 1–10 mM ferrous sulphate, at varying pH (2.5–4.0). Experimental variants also included eliminating column aeration and removing basal salts from the influent water. Differences in influent pH, redox potential and ferrous iron concentrations were used to assess the efficiencies of the bioreactors at oxidizing and removing iron.

Bioremediation of AMD using reductive processes

Column bioreactors were also prepared in which the inocula were either acidophilic iron-reducing bacteria (*Acidiphilium* spp.[17]) or acid-tolerant/acidophilic sulphate-reducing bacteria.[18] Poraver glass beads were again used as the support matrix. A similar protocol to that described above was used to establish microbial biofilms and commission the bioreactors to that described above, except that microaerobic conditions were used for iron-reducing *Acidiphilium* spp., and anaerobic conditions to encourage the development of sulphate-reducing bacteria biofilms. As both populations are heterotrophic, it was necessary to provide organic compound(s) as carbon and energy sources. These were glucose (1 mM) for iron reduction, and ethanol, lactic acid and glycerol (in varying combinations and concentrations) for sulphate reduction. Bioreactor performance was monitored by measuring differences in influent and effluent liquors (pH, ferrous and ferric iron, sulphate and organic substrates).

Results

Physico-chemical and microbiological data of AMD waters from the U.K. and two mine sites in mainland Europe are shown in Table 1. While the pH of the AMD samples at the point of discharge was as high as 6.2, all were net acidic waters and developed pH values of below 4 following oxidation and hydrolysis of dissolved metals. The data for Wheal Jane relate to AMD sampled in October 1999. During peak discharge shortly after the catastrophic release of AMD from this mine (1992), water pH was 2.8, and concentrations of iron (at >5 g l^{-1}) and zinc (at >2 g l^{-1}) were far in excess of those in more recent discharge waters. This pattern of gradual improvement of AMD quality with time is typical of mine effluents, though the time frame over which the improvement occurs is highly variable.

There were significant differences in numbers of indigenous acidophilic iron- and sulphur-metabolizing bacteria, and of acidophilic heterotrophic bacteria, in these waters. The highest numbers of iron-oxidizing bacteria were found in the more acidic AMD samples from the metal mines, with this group of bacteria being below detectable limits (10 bacteria ml^{-1}) in Ynysarwed AMD and present in low number (40 ml^{-1}) in AMD from the Bullhouse coal mine site. This distribution was paralleled by heterotrophic acidophilic bacteria (*Acidiphilium* spp. etc). Concentrations of dissolved organic carbon tended to be low (<10 mg l^{-1}) in AMD waters. Numbers of sulphur-oxidizing bacteria (other than *T. ferrooxidans*) were small in all samples, except in the Norwegian (Roeros) AMD. The fact that iron-oxidizing bacteria did occur in the coal mine effluent was confirmed by incubation of AMD samples and plating following comple-

Table 2. Numbers (bacteria per gram sediment) of sulphate-reducing bacteria isolated from two sediment core samples taken from 4–5 cm depth below the sediment surface at the Afon Goch, Parys copper mine, Wales. Core samples were diluted in basal salts (pH 3.5) and plated onto overlay media, with or without 10 mM glycerol.

Sample	Medium formulation	
	+ Glycerol	– Glycerol
Core 1	1.6×10^5	6.2×10^6
Core 2	1.1×10^5	1.0×10^3

tion of iron oxidation. However, the iron-oxidizing bacteria in both cases were unable to grow on the more acidic (pH 2.5–2.6) overlay media but did grow on the pH 3.5 iron/thiosulphate overlay medium. Similar bacteria (classed as unidentified 'moderately acidophilic' iron-oxidizing chemolithotropes) were also found in enrichment cultures of Bullhouse and Wheal Jane AMDs.

Numbers of sulphate-reducing bacteria in sediment cores from the main stream (the 'Afong Goch') draining the Parys mine site are shown in Table 2. There was some variation in the plating efficiencies of media with and without glycerol, though colonies tended to be larger on media with glycerol. Non-overlay plates were found to be ineffective at promoting the growth of sulphate-reducing bacteria colonies at low pH. There was considerable diversity in the morphologies of the sulphate-reducing bacteria colonies and also in the cell morphologies of isolates obtained, which included sporulating and non-sporulating rods. About 50% of isolates were able to grow on solid medium poised initially at pH 3.0 rather than 3.5.

Figure 2 compares the rate of ferrous iron oxidation in two metal mine AMDs (Parys and Cae Coch) in the presence and absence of indigenous microorganisms. In both cases, there was very little abiotic oxidation of iron over a three-day incubation period. In the presence of iron-oxidizing bacteria (predominantly *At. ferrooxidans* at both sites[19,20]) all of the ferrous iron present was oxidized within 2–3 days. The addition of inorganic nutrients to AMD samples (to ascertain whether the activities of iron-oxidizing bacteria were limited by N, P, etc.) produced ambiguous results, with iron oxidation being stimulated in Cae Coch AMD and slightly retarded in Parys AMD. Figure 2 also shows abiotic iron oxidation in AMD from one of the coal mines (Bullhouse). In this case, there was rapid and substantive oxidation of iron, so that after one day of incubation only 24% of the original ferrous iron remained. Very little of the remaining iron oxidized over the following two days, however, and even during extended incubation of up to one week (data not shown). This was seemingly due to the acidification of the AMD, which occurred during the first 24 hours of incubation (pH fell from 5.9 to 5.0), owing to the oxidation and hydrolysis of iron. Similar data (not shown) have been obtained with Ynysarwed AMD.

One way to accelerate iron removal from AMD is to promote oxidation and precipitation by microorganisms using immobilized iron-oxidizing bacteria. Figure 3 shows performance data of a column bioreactor containing biofilms of the uncharacterized 'moderately acidophilic' iron-oxidizing bacteria obtained by enrichment culture of Ynysarwed AMD. The reactor was assessed over a period of several months with influents from pH 2.5 to 4.0 and ferrous iron concentrations from 1 to 10 mM (56–560 mg l^{-1}). The efficiency of the bioreactor at removing iron from influent liquor without added nutrients, and in the absence of active aeration, was also monitored. Each data point shown in Fig. 3 represents the result of a single experiment carried out with this bioreactor. The data show that the system was both

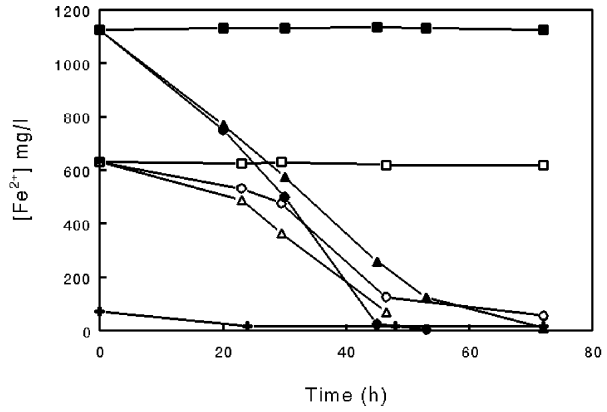

Fig. 2. Ferrous iron oxidation in native and filter-sterilized AMD. Symbols: ■ Cae Coch AMD (sterilized); ▲ Cae Coch AMD (native); ● Cae Coch AMD (+ nutrients); ❑ Parys AMD (sterilized); Δ Parys AMD (native); ❍ Parys AMD (+ nutrients); ✚ Bullhouse AMD (native).

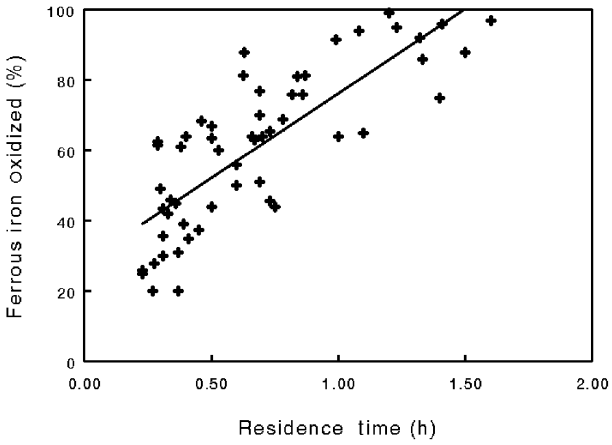

Fig. 3. Performance data of the packed-bed column bioreactor containing immobilized iron-oxidising acidophilic bacteria. Each symbol represents the result of a single experiment. A best linear fit for all data is also shown ($r = 0.80$).

robust and efficient at removing iron. About 90% of the influent iron was removed at a residence period of one hour. There were only relatively small fluctuations in bioreactor performance with changing influent chemistry, though 10 mM ferrous iron input and high flow rates did result in significant lowering of bioreactor efficiency. Most of the ferric iron precipitates

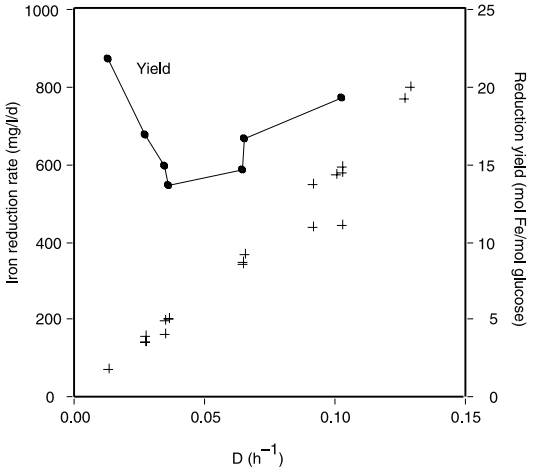

Fig. 4. Performance data of a packed-bed bioreactor containing immobilized iron-reducing acidophilic bacteria at different dilution rates. Symbols: ✚ iron reduction rate; ● iron reduction yield (mol glucose oxidized/mol ferric iron reduced).

remained in the reactor, and the design of the column (which had an inclined and integral drainage tap) meant that this material could be readily removed. Moreover, the ferric precipitates formed in the bioreactor appeared to be more dense than those formed abiotically, though no quantitative data were obtained.

Fixed-bed bioreactors used to reduce ferric iron in influent waters were also successful. Biofilms of *Acidiphilium* spp. established readily on Poraver beads, and the column reactors could be run for extended periods (>300 days) with minimal maintenance, as no inorganic precipitates accumulated in these systems. The iron-reduction bioreactors were not aerated, but there were no attempts to exclude oxygen. Figure 4 shows data from one of these bioreactors run at different dilution rates, using glucose as electron donor. The rate of ferric iron reduction increased with increasing dilution rate, reaching a maximum recorded value of 0.8 g Fe^{3+} reduced per litre per day, with a corresponding residence time was about 8 h (Fig. 4). The 'yield' values in Fig. 4 refer to the calculated stoichiometry between glucose oxidized (difference in influent and effluent concentrations) and ferric iron reduced. A net stoichiometry of 15–20 ferric iron ions reduced per molecule of glucose oxidized was obtained, which compares with a maximum figure (assuming complete oxidation of glucose to carbon dioxide coupled exclusively to ferric iron reduction) of 24.

Fixed-bed bioreactors containing acidophilic or acid-tolerant sulphate-reducing bacteria populations were shown to be capable of sulphate reduction and pH amelioration of acidic influent liquors. Figure 5 shows the performance of one of the sulphate-reducing bacteria bioreactors over a period of more than three months, during which the effects of different combinations of potential organic substrates were evaluated. While the initial change from a mixed to a single substrate (ethanol) produced a marked decline in the rate of sulphate reduction, the performance of the bioreactor subsequently recovered (Fig. 5). Average sulphate reduction rates of 0.3 g l^{-1} day^{-1} were achieved in the sulphate-reducing bacteria columns, which is considerably less than that reported (up to 5.3 g l^{-1} day^{-1}) for systems operating at circum-neutral pH. Analysis of column effluents showed that significant concentrations of acetic acid were present, indicating that the acidophilic/acid-tolerant sulphate-reducing bacteria populations were not capable of completely oxidizing the organic substrates in the influent liquors. When challenged with influent liquors of increasing acidity, rates of sulphate reduction were initially fairly constant (between pH 3 and 4) but showed a marked decline below pH 3.0 (Fig. 6)

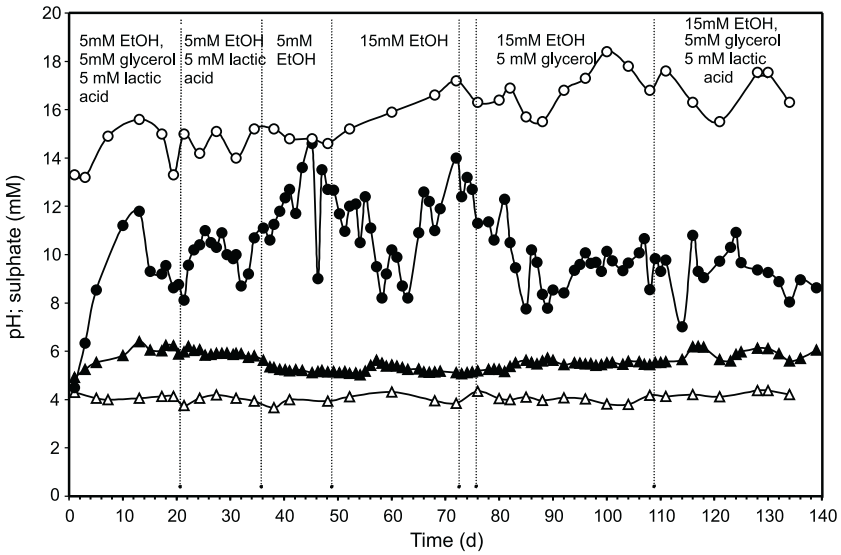

Fig. 5. Performance data of a packed-bed column bioreactor containing immobilized sulphate-reducing acid-tolerant bacteria percolated with influent AMD containing varying combinations of organic substrates. Symbols: △, pH (influent): ▲, pH (effluent); ○, sulphate (influent); ●, sulphate (effluent).

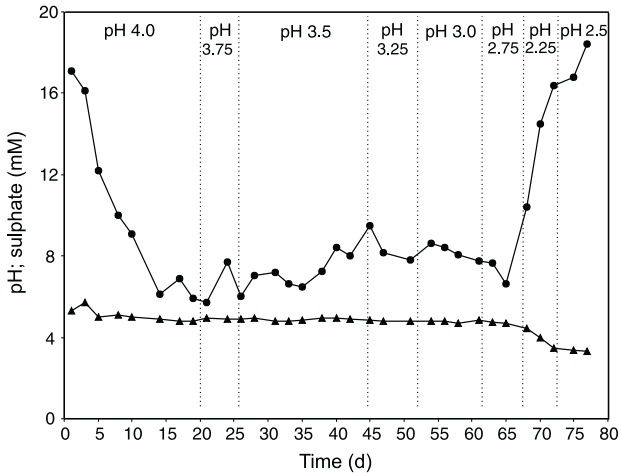

Fig. 6. Performance data of a packed-bed column bioreactor containing immobilized sulphate-reducing acid-tolerant bacteria percolated with influent AMD of varying pH and 15 mM sulphate. Symbols: ▲, pH (effluent); ●, sulphate (effluent).

Discussion

Waters draining mines and mine wastes can have variable chemical and biological profiles, as illustrated by the data we have presented here. The theory that AMD is formed by the actions of mineral-oxidizing bacteria that accelerate the dissolution of sulphidic minerals was supported by the widespread occurrence of these microorganisms in acidic effluents draining the five metal mines studied. The situation with the two coal mine effluents was less clear. Though iron-oxidizing bacteria were below detectable limits in AMD from Ynysarwed, and <100 ml^{-1} in samples from the Bullhouse site, they could be readily isolated from enrichment cultures of these waters. The iron-oxidizing bacteria isolated from the coal mine AMDs appeared to be less acidophilic than those from the metal mines (with the exception of Wheal Jane), where strains of *L. ferrooxidans* and *At. ferrooxidans* were dominant. These 'moderately acidophilic' iron oxidizers appear to be chemolithotrophic, though whether they are novel species or genera of bacteria is yet to be established. It is possible that iron-oxidizing bacteria of this type are widespread in mildly acidic AMD, particularly those draining coal mines, and that they play an important role in effecting redox transformations in these ferruginous waste waters.

The importance of microorganisms in iron transformations in AMDs waters was illustrated by comparing rates of ferrous iron

oxidation in native and filter-sterilized waters. At pH 2.5 (Parys and Cae Coch AMDs) there was minimal abiotic oxidation over a period of three days in the sterilized samples; the indigenous microbial community in the unsterilized samples oxidized virtually all of the ferrous iron originally present within this time. In the higher pH AMD waters, there was, initially, significant abiotic oxidation of ferrous iron; thereafter the acidification of AMDs following ferric iron hydrolysis caused residual fractions of ferrous iron to be stable over protracted incubation periods, despite active agitation and aeration of samples. This suggests that iron-oxidizing bacteria have a critical role in removing iron from ferruginous mine waters that are moderately acidic to neutral at source. Kirby *et al.* examined the relative contributions of microorganisms and abiotic factors in promoting ferrous iron oxidation in AMD, and concluded that, at pH 5 and above, iron oxidation is primarily abiotic, but below pH 5 microbial oxidation is of greater importance.[22] A similar scenario was seen with AMD from the Bullhouse coal mine site in the present study.

One drawback of using indigenous populations of iron-oxidizing bacteria to oxidize and precipitate iron is that their numbers in AMD might be very low (as in the coal mine samples in the present study) and that, even when present in reasonable numbers (as in Parys and Cae Coch AMD), rates of iron oxidation may be far slower than desirable. By contrast, packed-bed bioreactors containing immobilized iron-oxidizing bacteria have been used successfully to accelerate the process of iron removal from ferruginous waters.[9–11] The bioreactor described in the present study also demonstrated how the efficiency of iron removal could be improved greatly by such a system, with an average of figure of 90% of influent iron oxidized and a mean residence period within the bioreactor of one hour. This bioreactor differed from those described in other studies both in the support matrix and bacterial populations used. The porous synthetic glass beads were successful in promoting the development of bacterial biofilms. There were also no problems with flow of synthetic AMD through the bioreactor over a six-month testing period, owing to the high porosity of the system. The bacterial population used (a mixed culture obtained from Ynysarwed AMD) contained the 'moderately acidophilic' iron-oxidizers, and also *At. ferrooxidans*-like bacteria which became progressively dominant with time. Use of bioreactors containing mixed cultures of iron-oxidizing bacteria with different pH optima and (in the case of *L. ferrooxidans* substrate affinities could prove more advantageous for treating AMD of different and fluctuating compositions than previous systems which have tended to use *At. ferrooxidans* alone.

The reduction of soluble ferric iron present in mine waters, on the other hand, is desirable in certain situations. One way in which reduction is currently achieved is to percolate ferric iron-rich waters through columns containing ground pyrite, causing ferric iron to be reduced as it reacts with the sulphide mineral (as in Fig. 1). A disadvantage of this approach is that it results in increased concentrations of soluble ferrous iron. Bacterial ferric iron reduction is a novel and attractive alternative that avoids this problem. Iron-reducing acidophiles (principally *Acidiphilium* spp.) are widespread in acidic mineral-oxidizing environments.[16,17,23] These are facultative anaerobes which, in contrast to neutrophilic iron-reducers, do not require strictly anoxic conditions to reduce ferric iron. We have measured rates of up to 130 mg ferric iron reduced per litre per day in a fermenter maintained at 60% dissolved oxygen (M-A. Dziurla and D.B. Johnson, unpubl. data). This effect was also illustrated by the iron-reduction bioreactor described above, in which between 63 and 83% of the reducing equivalents (using glucose as electron donor) were transferred to ferric iron rather than molecular oxygen, even though no attempts were made to exclude oxygen from the system and dissolved oxygen was present in all effluent liquors sampled. The high redox potential of the ferrous/ferric couple (+770 mV, at pH 2) is not that much lower than that of the oxygen/water couple (+820 mV), which suggests that switching from oxygen to ferric iron as terminal electron acceptor is only moderately disadvantageous from an energetic standpoint.

In contrast to iron reducers, acidophilic bacteria that obtain their energy from the dissimilatory reduction of sulphate to sulphide have been notoriously difficult to isolate, even though there is ample evidence of sulphate reduction occurring in metal-rich acidic environments.[24–26] By using a different approach from those used earlier, Sen and Johnson[18] were able to enrich for acidophilic sulphate-reducing bacteria and to grow isolates under controlled acidic conditions in the laboratory. Packed-bed bioreactors containing mixed cultures of acidophilic sulphate-reducing bacteria were shown to reduce sulphate and generate alkalinity when challenged for over four months with synthetic AMD of pH 3 and above. The system could be adapted to use a single and cost-effective carbon and energy source (ethanol). Shortfalls in the system include the relatively low rates of sulphate reduction (compared with neutrophilic systems), incomplete oxidation of substrate and the inhibition of bacterial sulphate reduction by very low pH AMD. However, this bioreactor system represents a significant advance in the treatment of AMD using sulphate-reducing bacteria technology, without the requirement of pre-treating with alkali to create conditions favourable to neutrophilic sulphate-reducing bacteria. It is likely that, with the isolation of more acidophilic and efficient strains, bioreactor systems incorporating acidophilic sulphate-reducing bacteria will become increasingly important for bioremediating AMD.

Indeed, greater awareness of the biodiversity of acidic, metal-rich environments, and of the metabolic capabilities of acidophilic microorganisms, will continue to have a major impact on our understanding of the nature and genesis of AMD, and will increase the options that exist for using controlled and directed bioremediation for cleaning up these polluting waste waters.

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