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THE FORMATION OF BIOFILMS OF IRON-OXIDISING BACTERIA ON PYRITE

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ABSTRACT

Iron-oxidising bacteria, notably Thiobacillus ferrooxidans and Leptospirillum ferrooxidans, are capable of oxidising sulphide minerals either directly at the point at which the bacteria are attached to the mineral, or indirectly by the production of ferric ions in the solution which then leach the mineral. In this study, the attachment of a mixed culture of these bacteria to a coupon of pyrite was studied. The bacteria form biofilms that are between 30 and 50 μm thick. In the earlier stages of biofilm growth, the coverage of the surface of the pyrite by the bacteria was not uniform; instead, clusters of microcolonies were separated by water channels. After ten to twelve days, a significant amount of a ferric oxide corrosion product formed on the surface of the pyrite, and the coverage of the surface by the biofilm was more uniform with a thickness of about 25 μm. During this stage of growth, the bacterial population was more concentrated at the solution interface. This work has shown that the attachment of these bacteria to the pyrite surface can occur as a biofilm, and the results suggest that the individual bacterium does not need to be attached to the pyrite for it to harness the mineral as an energy source. It is proposed that one of the functions of the biofilm may be to increase the concentrations of ferrous and ferric ions within the biofilm compared to the bulk solution. This hypothesis may be tested by using a microelectrode to determine the redox potential within the biofilm. Copyright © 1996 Elsevier Science Ltd

Keywords
Bioleaching; bacteria; redox reactions; biotechnology

INTRODUCTION

Bacterial oxidation of sulphide minerals has been exploited in the dump-leaching process for the recovery of copper and uranium, and in the tank-leaching process for the pre-treatment of refractory gold ores. The factors influencing the bacterial oxidation process have been studied extensively, and several reviews of the literature have been published [1–6].

Iron- and sulphur-oxidising bacteria, notably *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, are known to attach to the surface of the mineral particles. Etch patterns form where the bacteria are located [7], indicating that the oxidation of the mineral occurs at the point of attachment.

These bacteria are also capable of oxidising ferrous ions. The product of this reaction, ferric ions, is a strong oxidising agent, and is capable of oxidising sulphide minerals.

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Therefore, the bacteria are able to affect leaching both directly at the point of attachment to the sulphide mineral and chemically by the production of ferric ions. This dual role of these bacteria has resulted in the debate concerning the dominant mechanism of the bacterial leaching. As a result, the bacterial interaction with sulphide minerals has often been separated into two processes: the direct and the indirect mechanism. The direct mechanism is the mechanism in which the bacteria attach to the mineral surface and oxidise the sulphide surface, possibly by enzymatic activity. The indirect mechanism is that in which the bacteria oxidise ferrous ions that are present in solution, and the product of this reaction, ferric ions, oxidises the mineral. The conditions under which of these two mechanisms dominates the process has not been established, and much of the evidence is contradictory. For example, Espejo and Ruiz [8] determined that the total activity associated with the direct mechanism to be between 1 and 10%, while Boogerd *et al.* [9] determined that only 8 to 17% of the pyrite was oxidised chemically (i.e., by the indirect mechanism).

Populations of bacteria that are attached to a surface exist in a matrix of extracellular polymeric substances (EPS) that are produced by the bacteria. The cells and the EPS matrix is referred to as a 'biofilm'. Sessile, or attached, bacteria are different from planktonic, or unattached, bacteria [10]. These differences range from the activation of the algC gene to the loss of flagella [11]. In addition, the matrix of EPS protects the bacteria from toxins while still supplying the cells with sufficient nutrients [12].

Previous work has not examined the formation and structure of biofilms of iron-oxidising bacteria on the surface of pyrite. In this paper, we report the formation of biofilms of iron-oxidising bacteria on the surface of pyrite in a flow reactor.

EXPERIMENTAL

The experimental apparatus is schematically illustrated in Figure 1. Bacteria were grown in a batch culture vessel, and a pump circulated solution containing the bacteria from the culture vessel to the flow cell and back into the culture vessel.

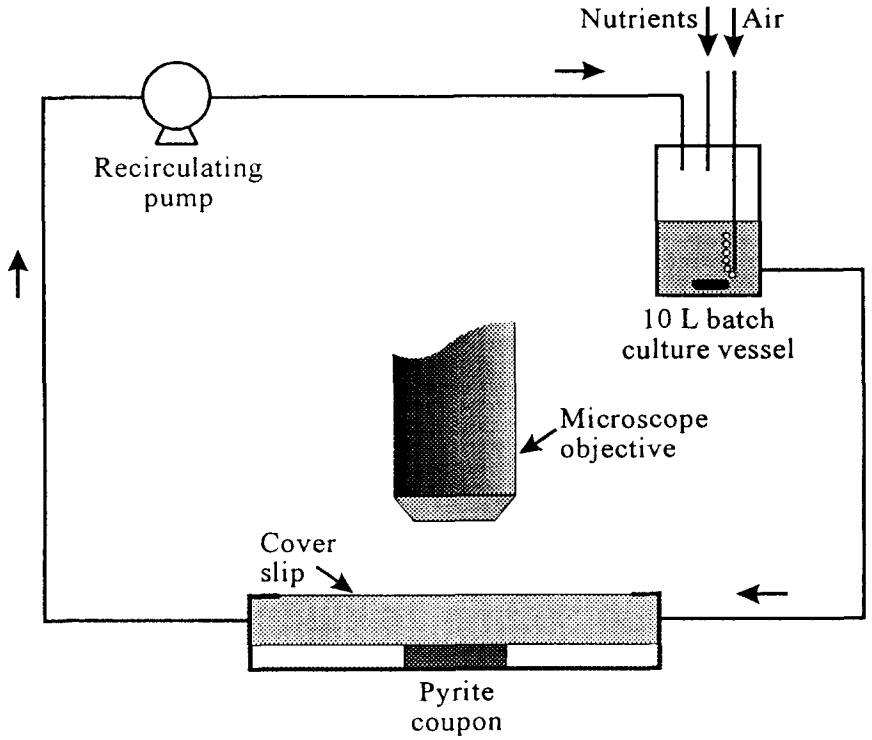

Fig.1 Schematic diagram of the experimental apparatus

The response of bacteria that occur in natural ecosystems differs from those of laboratory cultures [13]. The bacterial sample used in this study was a combination of three samples that originated from natural sources in Southern Africa.

The bacteria were grown in a 10L batch culture vessel, which was maintained at 32°C. The bacterial population was maintained in a suspension of pyrite that was milled to 75% passing 75μm. The culture was harvested once a week, and the volume made up with 0K solution, which supplies essential nutrients, but has no ferrous sulphate [14]. This ensured that the bacteria utilised pyrite as their main source of energy.

The flow cell consisted of a rectangular chamber. A glass cover slip on one side formed an observation window, and the opposite side contained the pyrite sample as a coupon in a recessed well. The dimensions of the chamber were 40 mm long by 12 mm wide by 0.5 mm deep. The coupon of pyrite was 20 mm by 5 mm wide. The flow rate through the chamber was 30 ml/min. The flow cell is illustrated in Figure 2.

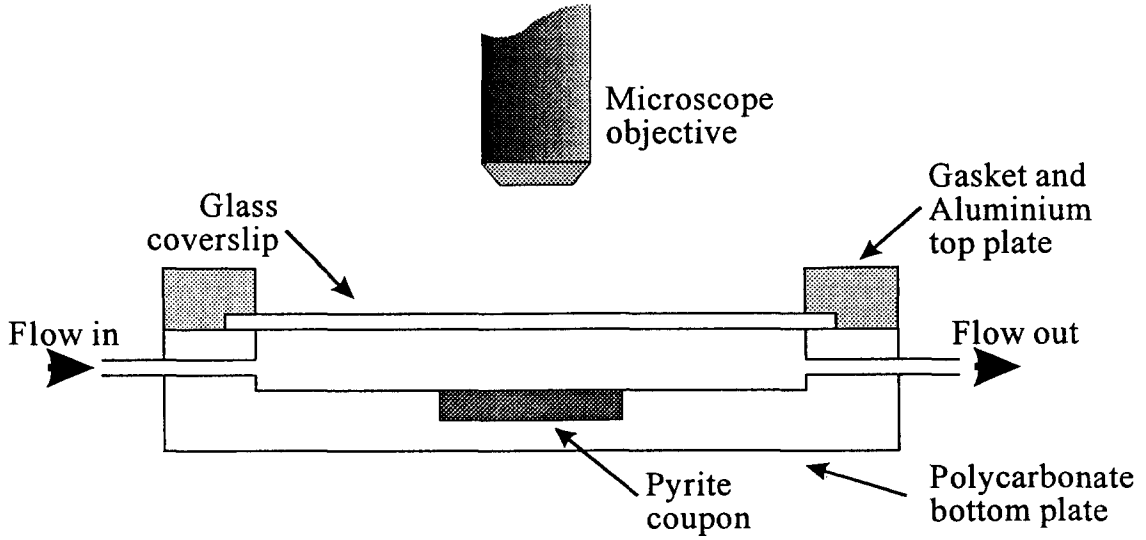

Fig.2 Diagram of the flow cell

The coupon was cut from an ore sample that was obtained from a Witwatersrand gold mine in South Africa. This ore sample consisted of large pyrite grains in a silica matrix. The coupon was polished using 600 grit silicon carbide powder.

The formation of the biofilms of iron-oxidising bacteria on the sample coupon was observed by reflected-light and fluorescent imaging using a BioRad MRC 600 confocal-scanning-laser microscope attached to an Olympus BH-2 microscope. The confocal microscope allows only light that is in the narrow focal plane through to the photo-multiplier tubes, so that the biofilm can be optically sectioned in a non-destructive manner [15]. The images were collected in digital form. The image analysis software allowed the construction of vertical images of the biofilm which allowed the determination of any vertical segregation within the biofilm. The images of the biofilm were collected by a Sony video display unit, and printed on a Sony colour printer.

The surface of the pyrite was observed in reflected red light at a wavelength of 647 nm. Fluorescien, which fluoresces at 518 nm, was used to stain the biofilm. The fluorescent image arising from the fluorescein was observed at 488 nm. Previous work indicated that fluorescein did not affect the functioning of bacteria [16]. The fluorescein was added to the feed to the flow cell at a concentration of 0.1 mmol/L. In this work, in which the pH of the solution was less than 2, it was found that the fluorescien stained the biofilm clusters, rather than staining the background solution which was found in previous work [15]. Ethidium bromide was used to stain individual cells.

RESULTS

After inoculation, a clearly observable biofilm formed on the surface of the pyrite coupon after two to four days. Cell clusters varied in size, mainly between 50 and 300 μm in size and between 30 and 50 μm in height. The clusters were separated by open spaces between them that were of similar size to the clusters. In the earlier stages of the biofilm growth (between 2 and 5 days), the biofilm seemed to be more concentrated on the pyrite grains than on the inert silica matrix. Towards the later stages (about 10 to 13 days), a significant amount of corrosion product formed on the surface of the whole coupon, and the biofilm covered this corrosion product.

Figure 3 shows the fluorescent and the reflected images of the sample coupon. The reflected image indicates the pyrite grains as a lighter image while the silica matrix, in which the pyrite grains are imbedded, is darker. Thick coverage of the surface of coupon by the biofilm produces a dark shadow on the reflected image, and a corresponding bright patch in the fluorescent image. The fluorescent image indicates that the coverage of the surface of the coupon is not uniform, but 'patchy'. This non-uniform coverage of the surface has been observed for other biofilms [17].

Fig.3 The fluorescent and reflected light images of the coupon of pyrite after 4 days. The fluorescent image, shown on the left-hand side shows the clusters of biofilm as lighter regions. The right-hand side shows the reflected light image of the same region of the coupon. The lighter regions represent the pyrite surface, while the dark regions are the silica matrix. Shadows on the reflected image are formed by thicker clusters of biofilm. One shadow is indicated by the arrow, and the corresponding position on the fluorescent image is also indicated. The length of the scale bar is 250 μm

The reflected image of a grain of pyrite in the silica matrix is shown in Figure 4, and the corresponding fluorescent image is shown in Figure 5. Both these Figures indicate that the bacterial biofilm does not form a uniform layer or a monolayer on the surface, but rather forms 'patchy' coverage. These microcolonies or patches produce dark shadows on the reflected image, indicating that they are thicker than a single monolayer. Figures 4 and 5 indicate that there is more biofilm formation on the surface of the pyrite grain than there is on the surface of the silica matrix.

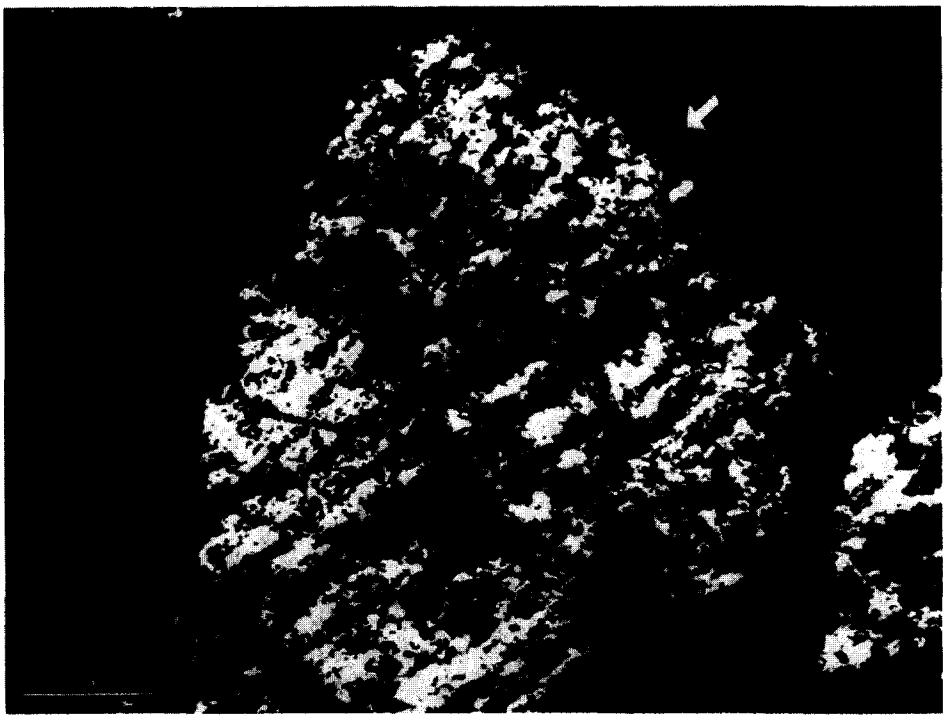

Fig.4 The reflected image of a pyrite grain. Dark regions on the grain are shadows formed by biofilm clusters. The length of the scale bar is 100 μm

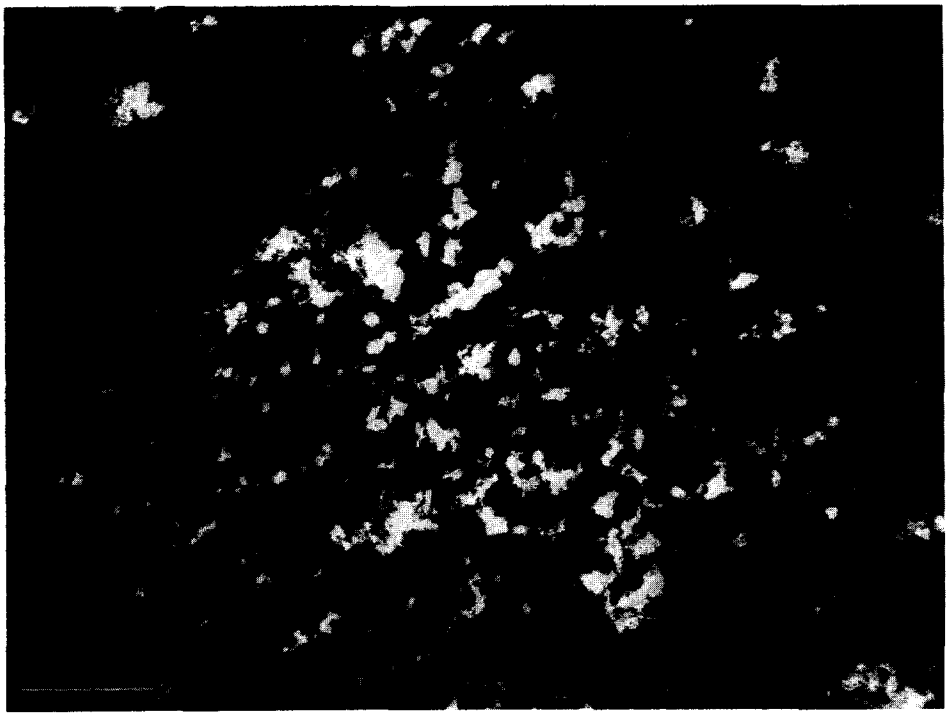

Fig.5 The fluorescent image of the biofilm, indicating the non-uniform coverage of the surface after two days for the same area shown in Figure 4. The length of the scale bar is 100 μm

Figure 6 shows the optical sectioning of the biofilm at various levels, indicating that the biofilm formation consists of microcolonies that are more cylindrical in shape. Previous work has found various shapes, including more mushroom-like formations [16].

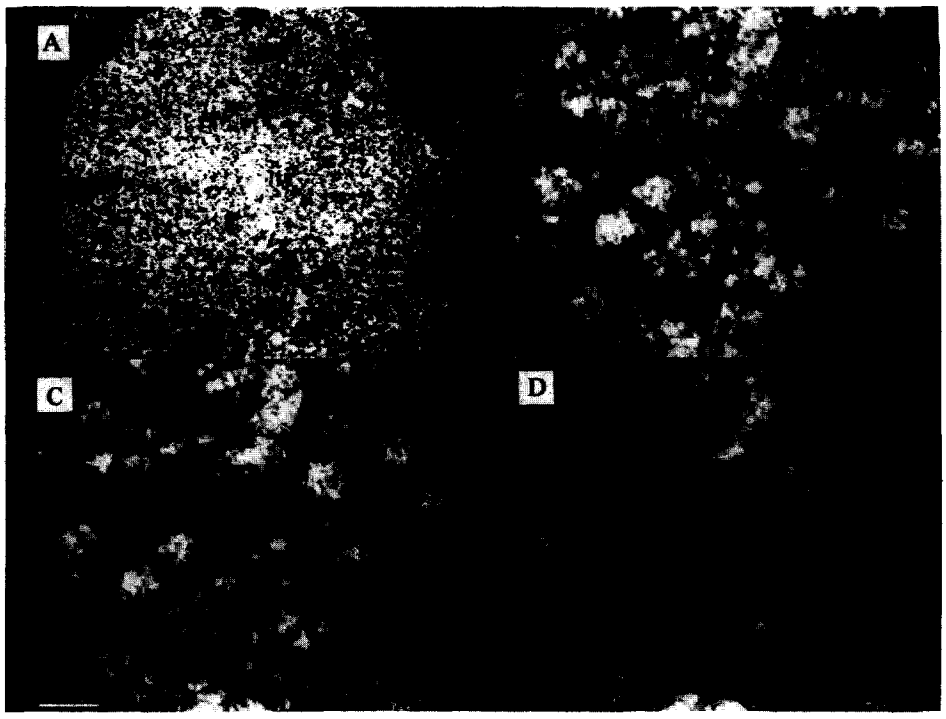

Fig.6 Optical sectioning of the biofilm. The reflected image of the pyrite grain is shown in A while the fluorescent images at 10 μm, 30 μm and 50 μm are shown as B, C, and D, respectively. The length of the scale bar is 100 μm

The cross-section of the biofilm in the vertical direction was observed by staining the cells with ethidium bromide. The vertical section of a three-day-old biofilm is shown in Figure 7. This image was obtained after fluorescent microspheres had been added to the system which also fluoresce at the same wavelength as ethidium bromide. These microspheres have become lodged in the biofilm clusters. This Figure illustrates the sack-like growth of the individual clusters and the voids between the clusters. The top of the clusters are larger, forming structures that are not too dissimilar from the mushroom-like clusters that have been observed previously [16].

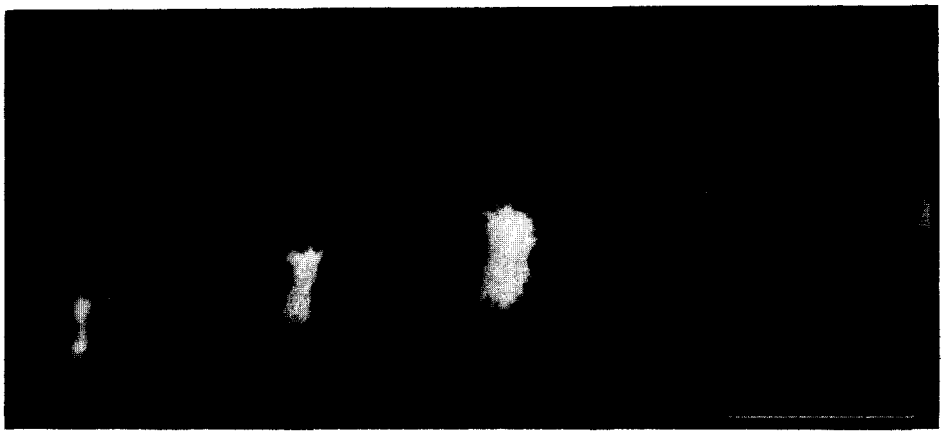

Fig.7 The vertical section of the biofilm clusters after three days. The cells were stained with ethidium bromide, and a large amount of fluorescent beads are lodged in the bacterial clusters. The length of the scale bar is 5 μm

Figure 8 shows a vertical section of the biofilm that had grown for thirteen days. At this point the biofilm had become more uniform, covering larger sections of the coupon. The cells were individually stained with ethidium bromide (no fluorescent beads were present in this experiment). This Figure indicates that the bacteria cells are more clustered towards the solution side of the biofilm, rather than at the solid surface.

During this experiment, a large amount of corrosion products formed on the surface of the coupon. Analysis by x-ray photoelectron spectroscopy (XPS) indicated that this product consisted mainly of ferric oxide, Fe_2O_3, or ferric hydroxide.

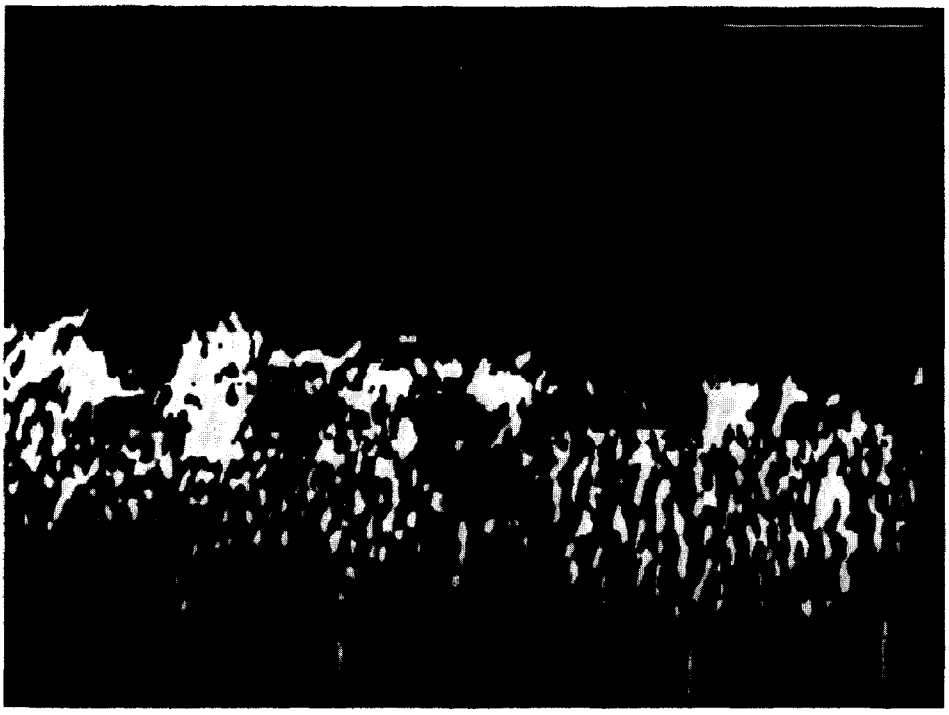

Fig.8 The vertical section of the biofilm after 10 days. The cells were stained with ethidium bromide. The bottom of the image corresponds with the surface of the coupon, while the top of the image corresponds with the bulk solution. The length of the scale bar is 10 μm

DISCUSSION

This work indicates that iron-oxidising bacteria readily form biofilms that are similar in structure and form to those formed from other bacteria [10,11]. The biofilm consists of clusters of bacteria and EPS separated by water channels or voids. The bacteria attach more readily to the pyrite grains in the earlier stages of the growth of the biofilm. In later stages of biofilm formation (at about 12 days) a large amount of corrosion product had formed and the bacterial clusters were much larger with less voids between them.

The structure of the biofilm indicates that direct attachment of the bacteria to the pyrite surface is not necessary for the bacteria to utilise the pyrite as an energy source. This is most clearly shown in the horizontal and vertical optical sections, in which it is shown that the bacteria form clusters that are between 30 and 50 μm thick, and that the bacteria in these experiments were concentrated at the interface with the solution, rather than at the pyrite surface.

De Beer *et al.* [16] measured the concentration of oxygen at various points in a biofilm grown from an undefined consortia of cells. The profiles of the concentration of oxygen within the biofilm indicated that there was a large depletion of oxygen within the bacterial clusters, while the voids between the clusters acted as conduits for the supply of oxygen to the lower regions of the clusters. The iron-oxidising bacteria

used in this study are aerobic and it is most likely that similar concentration profiles exist within the biofilm grown from them. In addition, it is also possible that the concentration of ferrous and ferric ions within the clusters is different to that in the bulk solution. If this is the case, the one of the functions of the biofilm may be to produce a microenvironment in which the growth of the bacteria is enhanced; consequently, the rate of dissolution of the pyrite substrate is enhanced above that achieved by chemical oxidation at the levels of ferrous and ferric ions found in the bulk solution.

Thus, the results of this work suggest a model in which the unreacted pyrite is covered by a corrosion product of ferric oxide or hydroxide which is in turn covered by a bacterial biofilm. This model of the growth and function of the bacteria could be examined by determining the profiles of the redox potential and concentration of oxygen throughout the biofilm.

CONCLUSIONS

This work has shown that iron-oxidising bacteria form biofilms on the surface of a coupon of pyrite. The biofilm formation is more developed on the grains of pyrite than on the silica matrix, suggesting that the wild strain used in this study may have a greater affinity for the pyrite than for an inert surface. The structure of the biofilm is similar to the biofilms grown from other bacteria on an inert substrate, indicating that, while there may be an affinity for the pyrite surface, direct attachment of all the individual bacteria to the pyrite surface is not necessary for them to be able to utilise the pyrite as an energy source. It is proposed that the bacteria may create their own microenvironment within the biofilm in which there are enhanced concentrations of both ferrous and ferric ions, such that the rate of growth of the bacteria and the rate of dissolution of the pyrite is enhanced. This could be verified by probing the biofilm with redox, pH and oxygen microelectrodes.

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