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THE MUTUAL EFFECT OF MIXED THIOBACILLI AND LEPTOSPIRILLI POPULATIONS ON PYRITE BIOLEACHING

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ABSTRACT

Although current bio-oxidation processes with mesophilic bacteria result from the occurrence of mixed populations, the mutual effect of the various species has not been studied very extensively to date.

Mixed cultures made up of pure Thiobacillus ferrooxidans, Thiobacillus thiooxidans *and* Leptospirillum ferrooxidans *strains of the DSM collection were batch tested for their ability to oxidize a cobaltiferous pyrite ore. The most efficient population for pyrite oxidation was composed of the three bacterial species. The influence of the relative abundance of the different strains in the inoculum was studied. The cobalt solubilization rate obtained with* T. ferrooxidans *increased when* L. ferrooxidans *was present but was not affected by the initial concentration of* L. ferrooxidans. *The bioleaching with* T. ferrooxidans *was only improved by adding* T. thiooxidans *when the initial concentration of* T. thiooxidans *was higher than the initial concentration of* T. ferrooxidans.

During continuous bioleaching of the cobaltiferous pyrite at 20% solids with a natural mesophilic mixed population, rod-shaped and Leptospirillum-*like bacteria were enumerated in the liquid phase. The 100 l bioleaching unit is made up of 3 or 4 reactors arranged in cascade. The concentration of* Leptospirillum-*like organisms rose exponentially versus dissolved ferric iron, whereas the concentration of rod-shaped bacteria did not change from the value obtained in the first reactor, providing the solution contained less than 60 g.l^{-1} ferric iron. At higher Fe^{3+} concentrations, the rod-shaped population in the liquid phase decreased. These results suggest that the rod-shaped bacteria performed the earlier steps of pyrite oxidation, whereas* Leptospirillum-*like organisms participated in the later phase of bioleaching. The effluent from the last reactor was treated with $CaCO_3$ in order to precipitate iron. Recycling of partially neutralized bioleach solution to the feed seemed to increase the concentration of* Leptospirillum-*like bacteria in all reactors. Two hypotheses are proposed to explain this phenomenon: some bacteria may have been brought into the first tank with the recycled liquid, or the cobalt concentration may have affected the distribution of* Leptospirillum-*like bacteria between liquid and solid phases.* © *1998 Published by Elsevier Science Ltd. All rights reserved*

Keywords
Sulphide ores; Hydrometallurgy; Bacteria; Biooxidation; Mineral processing.

INTRODUCTION

To date, *Thiobacillus ferrooxidans* is the sulphur and iron-oxidizing bacterium that has been studied the most. This organism is usually associated, in natural environments, with several other autotrophic and heterotrophic species. Recent studies have demonstrated the diversity of microorganisms that are able to grow under the drastic conditions encountered during ore bioleaching. Sand *et al.* [1] found *Thiobacillus* and *Leptospirillum* species, chemoorganotrophic bacteria, and fungi in soil and acid mine drainage. Several experiments comparing the efficiency of pure and mixed cultures for bioleaching showed both the advantages of mixed cultures and the complexity of interactions between species. The association of *Leptospirillum* and a sulphur-oxidizing bacteria was shown to provide a more rapid and complete oxidation of sulphides than the individual pure cultures of the same strains [2–5].

Different results were obtained by mixing *T. ferrooxidans* and *Thiobacillus thiooxidans*. However, the contribution of *T. thiooxidans* to the biological degradation of pyrite is still controversial. Some authors report that *T. thiooxidans* competes with *T. ferrooxidans* for adsorption on solid surfaces and for consumption of the sulphidic substrate [6,7], but the presence of *T. thiooxidans* did not improve the bioleaching process. Other studies have shown that *T. thiooxidans* can cooperate with an iron-oxidizing bacterium through complementary metabolism [8,9].

The present work compares the ability of several mixed populations to degrade a cobaltiferous pyrite. Batch experiments were performed with culture collection pure strains of *T. ferrooxidans*, *T. thiooxidans,* and *Leptospirillum ferrooxidans*. Subsequently, a natural mixed population containing *Thiobacillus*- and *Leptospirillum*-like bacteria was used in two continuous experiments at 20% solids. The same inoculum, grown on arsenopyrite, had already been analysed by Collinet-Latil [8]. Some strains of *T. ferrooxidans* and *T. thiooxidans* were isolated, but no *Leptospirillum*-like bacteria were observed on the arsenopyrite substrate. Vibrio-shaped bacteria only appeared during continuous bioleaching of pyrite. Such a phenomenon has already been described in the literature [10]. Some strains of these *Leptospirillum*-like bacteria were isolated [11]; their characteristics were in agreement with the genus *Leptospirillum* [12–14].

However, the identification of some isolated strains in the bioleaching pulp does not provide much information about the activity of the different organisms during the reaction. It is not easy to enumerate bacteria in commercial leaching systems because the bacteria involved in bioleaching do not grow readily on solid media, and the organisms attached to the solid particles are not counted using direct microscopic methods. Hallmann *et al.* [5] estimated the number of ferrous ion or sulphur oxidizing bacteria with a three-tube most-probable-number technique, allowing a differentiation between *T. ferrooxidans* and *L. ferrooxidans* based on morphological criteria. Jerez and Arredondo [15] described a sensitive and specific immunobinding assay to enumerate *T. ferrooxidans* and *L. ferrooxidans*. However, a limitation of this method was detected by Hallberg and Lindström [16]: two strains of the same organism, namely *Thiobacillus caldus*, displayed distinct serotypes. This result was attributed to differences in LPS structures.

The present article describes the evolution, based on morphological criteria, of a mixed population during the continuous bioleaching of a cobaltiferous pyrite. The respective contribution of rod-shaped and vibrio-shaped organisms to the bioleaching, in relation to the different steps of the reaction, is discussed.

MATERIALS AND METHODS

Microorganisms

Pure strains were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM): *T. ferrooxidans* 583, *T. thiooxidans* 504, *L. ferrooxidans* 2705 and *L. ferrooxidans* 2391. The indigenous

inoculum was collected by BRGM from a mining site and initially cultivated on arsenopyrite [8], then on cobaltiferous pyrite in a continuous bioleaching unit.

Energy substrate

The cobaltiferous pyrite contains 40.3% iron and 1.4% cobalt, mostly disseminated in the pyrite matrix, resulting in the pyrite oxidation yield and cobalt solubilization yield having the same value. Iron and cobalt are dissolved simultaneously during the bioleaching process, the efficiency of which is evaluated with respect to cobalt dissolution kinetics.

Culture conditions

For the batch experiments, the pure strains from DSM were first sub-cultured in 250 ml erlenmeyer flasks containing 50 ml of medium. A 9K medium [17] was used for *T. ferrooxidans* and *L. ferrooxidans*, whereas *T. thiooxidans* was maintained on a Waksman medium [18]. The bioleaching experiments were carried out in air-lift tubes maintained at 35°C in a thermostated bath and with a culture volume of 200 ml. The basal medium contained $(NH_4)_2SO_4$ (3.7 $g.l^{-1}$), 85% H_3PO_4 (0.8 $g.l^{-1}$), KOH (0.48 $g.l^{-1}$) and $MgSO_4 7H_2O$ (0.52 $g.l^{-1}$) [8]. The quantities of cobaltiferous pyrite added to this nutrient solution were increased from 5.0% to 7.5% or 10% (w/w). The equipment, solutions and substrates were sterilized by autoclaving at 110°C for 30 minutes.

Two continuous bioleaching experiments (CBE) were performed. The first (CBE1) ran for 6 months, and the second (CBE2) for 3 months. The bioleaching system was made up of a series of 3 or 4 stainless steel reactors. The total volume of pulp in each reactor was 22 l. The cultures were mechanically agitated and aeration was performed by injecting 1% (v/v) CO_2-enriched air beneath the impellers. The first reactor was fed with fresh nutrient medium and concentrated pyrite slurry. The bioleaching slurry passed continuously from one reactor to the next by overflowing. Solids concentration in the feeding flow was 20% (w/w). In each reactor, $CaCO_3$ was continuously added in order to maintain the pH value close to 1.4. The main differences between CBE1 and CBE2 were the operating temperature and the cobalt concentration in the feed. During CBE1, temperature was progressively increased from 35°C to 41°C, and the feed contained recycled cobalt. The pH of the bioleached pulp from the last reactor was raised to 2.6 with $CaCO_3$ in order to precipitate iron without removing cobalt from the solution. The resulting liquor was then introduced into the nutrient solution so as to obtain a feed containing cobalt in the concentration range 1.2 to 1.5 $g.l^{-1}$. CBE2 was performed at 35°C, and a cobalt-free nutrient medium was used. The theoretical retention time in each tank was 54 hours during CBE1, and in the range 24 to 54 hours during CBE2. The detailed description of the operating conditions for CBE2 is given by d'Hugues *et al.* [19].

Microbiological analysis

For the enumeration of rod-shaped and *Leptospirillum*-shaped bacteria in the mixed population, the microorganisms were immobilized in gelatinous films according to the following method. Samples of bioleaching pulp (30 ml) were taken from the reactors, and left for 15 minutes to allow the largest ore particles to settle. Some supernatant (20 µl) was deposited on a glass slide. A solution of 1% agar was prepared in a 20 ml glass tube, heated up to boiling, cooled down to 60°C, and one drop was added to the supernatant spot. This preparation was immediately covered with a cover-glass and pushed firmly into place so as to obtain a thin gel bed. The slides were studied under an optical microscope at x1000 magnification. Rod-shaped and vibrio-shaped bacteria were counted in 4 optical fields for each preparation. The total number of bacteria enumerated on a slide was in the range 150 to 500. Two slides were studied for each sample.

The total concentration of microorganisms (free in solution) was determined using a Thoma counting-cell under the optical microscope at x400 magnification. The bioleaching supernatant was diluted in 2 $g.l^{-1}$ H_2SO_4.

Chemical analysis

Samples of pulp were centrifuged for 15 minutes at 1000 g. Ferrous and total iron in solution were determined volumetrically with $K_2Cr_2O_7$. Ferric iron reduction was performed with $SnCl_2$ [20]. Cobalt concentrations were determined by atomic absorption spectrophotometry (Varian SpectrAA-300).

RESULTS AND DISCUSSION

Oxidation of the cobaltiferous pyrite by DSM bacterial strains

The first experiment on pyrite was carried out at 5% solids (w/w) with strains that were not adapted to the sulphidic substrate. The kinetics of cobalt solubilization (Figure 1) showed that the bioleaching rate was greatly influenced by the bacterial composition of the inocula. All cultures contained at least *T. ferrooxidans* 583. The kinetics were clearly accelerated when *L. ferrooxidans* 2391 was also present. No improvement was observed when only *T. thiooxidans* was added to the inoculum, although *T. thiooxidans* was able to improve bioleaching efficiency when *Leptospirillum* species were also added.

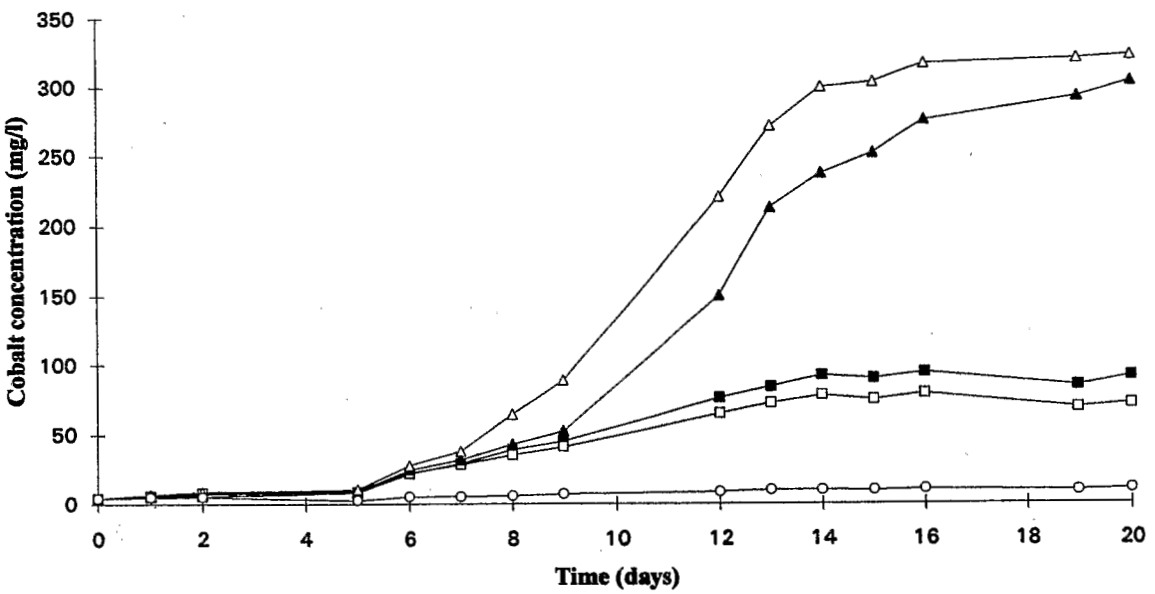

Fig.1 Kinetics of cobalt solubilization during the bioleaching of cobaltiferous pyrite with different bacterial populations. The cultures, performed in duplicate, were inoculated with strains that were not adapted to the pyrite substrate. Symbols: *Thiobacillus ferrooxidans* DSM 583 (■); *Thiobacillus ferrooxidans* DSM 583 + *Thiobacillus thiooxidans* DSM 504 (□); *Thiobacillus ferrooxidans* DSM 583 + *Leptospirillum ferrooxidans* DSM 2391 (s); *Thiobacillus ferrooxidans* DSM 583 + *Thiobacillus thiooxidans* DSM 504 + *Leptospirillum ferrooxidans* DSM 2391 (Δ); sterile control (O). Solids concentration was 5%.

The cultures obtained from the first experiment were used to inoculate another series of air-lift tubes containing fresh medium and 7.5% solids. The kinetics of cobalt solubilization (Figure 2) showed that cobalt release remained very slow when the inoculum contained only *T. ferrooxidans* 583. The lack of any increase in pyrite oxidation efficiency suggested that no adaptation of the strain had occurred after the first subculture on this sulphidic substrate. On the other hand, *T. thiooxidans* improved its positive contribution in comparison with the first experiment as higher values of cobalt recovery were obtained when this bacterium was present. Such bacterial adaptation to energetic substrates has often been described in the literature [21]. In the second experiment, the presence of *L. ferrooxidans* clearly shortened the "lag" phase.

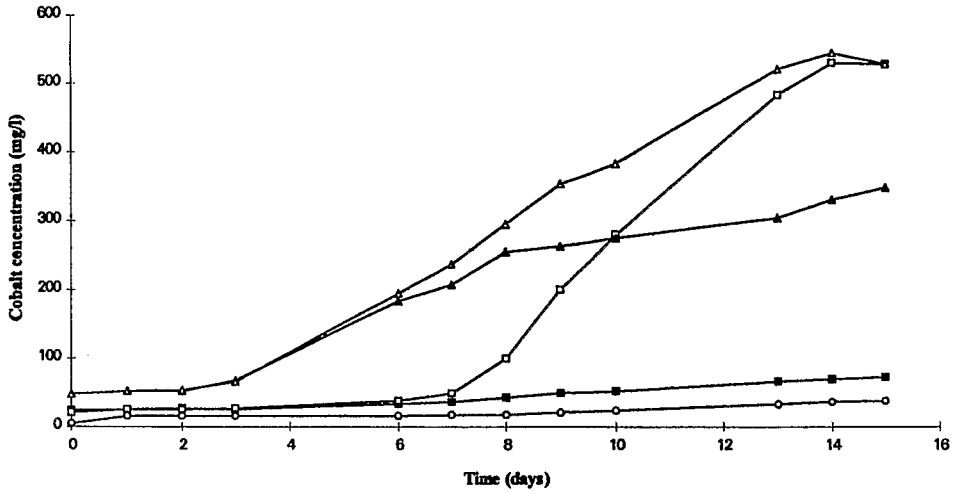

Fig.2 Kinetics of cobalt release with the second culture, performed in duplicate, of different bacterial populations on cobaltiferous pyrite. Symbols: *Thiobacillus ferrooxidans* DSM 583 (■); *Thiobacillus ferrooxidans* DSM 583 + *Thiobacillus thiooxidans* DSM 504 (□); *Thiobacillus ferrooxidans* DSM 583 + *Leptospirillum ferrooxidans* DSM 2391 (s); *Thiobacillus ferrooxidans* DSM 583 + *Thiobacillus thiooxidans* DSM 504 + *Leptospirillum ferrooxidans* DSM 2391 (Δ); sterile control (O). Solids concentration was 7.5%.

To explain the greater efficiency of mixed inocula, it could be considered that the different species have different affinities for the energetic substrates provided by pyrite (i.e. sulphide, sulphur and ferrous iron), and that pyrite bioleaching is the result of several steps:

$$FeS_2 + 3.5\ O_2 + H_2O \xrightarrow{\text{bacteria}} FeSO_4 + H_2SO_4 \quad (1)$$

$$2\ FeSO_4 + 0.5\ O_2 + H_2SO_4 \xrightarrow{\text{bacteria}} Fe_2(SO_4)_3 + H_2O \quad (2)$$

$$FeS_2 + Fe_2(SO_4)_3 \xrightarrow{\text{chemical step}} 3\ FeSO_4 + 2\ S^\circ \quad (3)$$

$$2\ S^\circ + 3\ O_2 + 2\ H_2O \xrightarrow{\text{bacteria}} 2\ H_2SO_4 \quad (4)$$

T. ferrooxidans is able to catalyse all the biological steps (Equations 1, 2 and 4). *T. thiooxidans* is involved in reactions 1 and 4. *L. ferrooxidans* does not oxidize inorganic sulphur compounds and its growth is supported only by Equation 2. Sulphur and sulphide provided much more energy than ferrous iron for *T. ferrooxidans* as shown by higher growth yields on sulphide than on ferrous iron [22]. Shrihari *et al.* [23] showed that iron-grown bacteria preferentially oxidized sulphur in chalcopyrite rather than ferrous sulphate in solution. Thus, a cooperative distribution of the different bioleaching steps between species could improve the global rate of the process.

The influence of the initial quantitative composition of the inoculum was studied at 10% solids. The kinetics of cobalt release obtained with mixed populations of *T. ferrooxidans* 583 and *L. ferrooxidans* 2705 (Figure 3a) showed that the bioleaching rate for these mixed cultures was independent of the initial concentration of *L. ferrooxidans*, in the range 0.0 to 1.5 x 10^8 cells.ml^{-1}. However, the bioleaching process started sooner when *L. ferrooxidans* was added in larger quantities (1.0 and 1.5 x 10^8 cells.ml^{-1}). *L. ferrooxidans* only uses ferrous iron, therefore, if *T. ferrooxidans* preferentially oxidizes the inorganic sulphur, no competition between *Thiobacillus* and *Leptospirillum* species would occur at the beginning of the experiment. Nevertheless, the concentration of *L. ferrooxidans* cells must be achieved to provide maximum oxidation of ferrous iron. Thus, the positive effect on the kinetics is delayed at the lowest initial concentration of *L. ferrooxidans*. The results of the experiment with *T. ferrooxidans* 583 and *T. thiooxidans* 504 (Figure 3b) showed that the effect of *T. thiooxidans* on the kinetics depends on its initial concentration

in the inoculum. When the concentration of *T. thiooxidans* in the inoculum was half of that of *T. ferrooxidans*, the bioleaching was not improved; cobalt solubilization was even slower than with *T. ferrooxidans* alone. When *T. thiooxidans* cells initially outnumbered *T. ferrooxidans* cells, the mixed culture was more efficient than *T. ferrooxidans* alone.

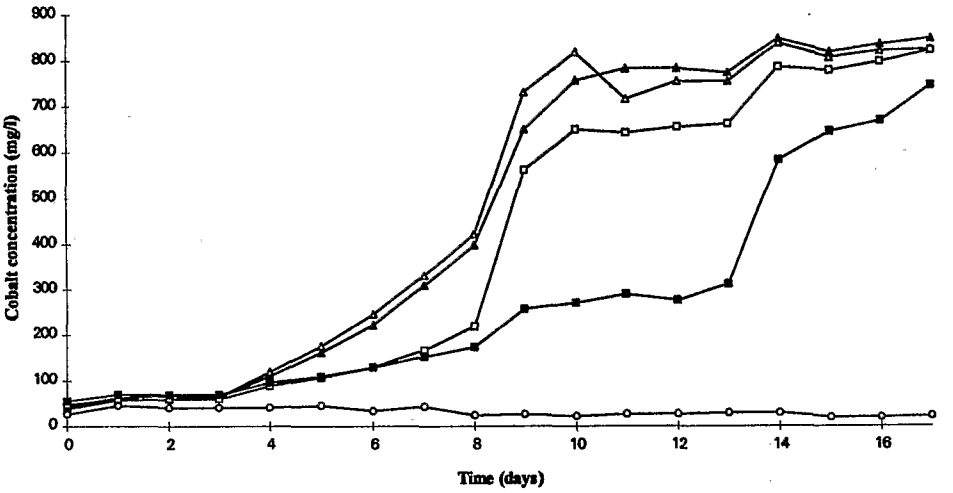

Fig.3a Influence of the ratio between different strains in the inoculum on the kinetics of cobalt release. Solids concentration was 10%.
Thiobacillus ferrooxidans DSM 583 / *Leptospirillum ferrooxidans* DSM 2705 with initial proportions between strains: 8 x 10^8 / 0 (■); 8 x 10^8 / 0.5 x 10^8 (□); 8 x 10^8 / 1 x 10^8 (s); 8 x 10^8 / 1.5 x 10^8 (Δ); sterile control (O).

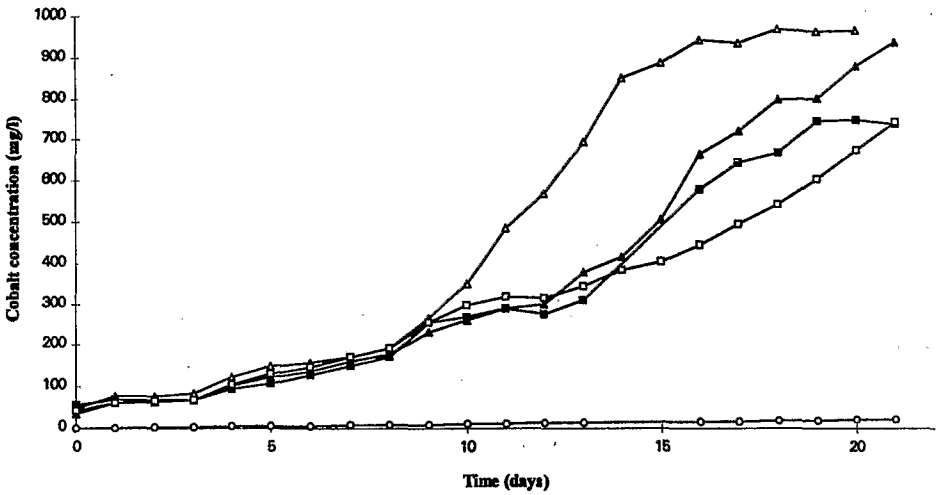

Fig.3b Influence of the ratio between different strains in the inoculum on the kinetics of cobalt release. Solids concentration was 10%.
Thiobacillus ferrooxidans DSM 583 + *Thiobacillus thiooxidans* DSM 504 with initial proportions between strains: 8 x 10^8 / 0 (■); 8 x 10^8 / 4 x 10^8 (□); 8 x 10^8 / 8 x 10^8 (s); 8 x 10^8 / 12 x 10^8 (Δ); sterile control (O).

The interactions between the two *Thiobacillus* species were different from those between *L. ferrooxidans* and *T. ferrooxidans*. When *T. ferrooxidans* and *T. thiooxidans* were associated (Figure 3b), the two species were likely to compete for the pyrite substrate. In this case, if there were sufficient *T. thiooxidans* cells to withstand the competition of *T. ferrooxidans*, the bioleaching rate could be improved because the two

Thiobacillus species could grow in cooperation. This hypothesis could explain why cobalt solubilization was accelerated when the inoculum contained more *T. thiooxidans* cells than *T. ferrooxidans* cells.

Numeration of rod-shaped and *Leptospirillum*-like bacteria during the continuous bioleaching of cobaltiferous pyrite by a natural mixed population

The evolution of two morphological groups, rod-shaped and *Leptospirillum*-like bacteria, was studied during continuous bioleaching. The analyses were only performed on the liquid phase, and the bacteria attached to the ore particles were not considered. No attempt was made to isolate and classify the microorganisms. The *Leptospirillum*-like group contained both vibrio and helicoidal organisms. Some small coccoid cells, which were occasionally observed, were not enumerated.

The maximum bacterial concentration was equivalent during the two continuous experiments. CBE1 and CBE2 contained approximately 10^{11} cell.ml^{-1}. In the case of CBE1 (Figure 4a), this maximum concentration was obtained in the second reactor, when iron reached 50 g.l^{-1}. In contrast, the highest bacterial concentration was often measured in the last reactor during CBE2, with iron between 60 and 70 g.l^{-1} (Figure 4b). However, it must be stressed that very high Fe^{3+} concentrations are not lethal for the entire bacterial population in solution, which holds at a very stable level.

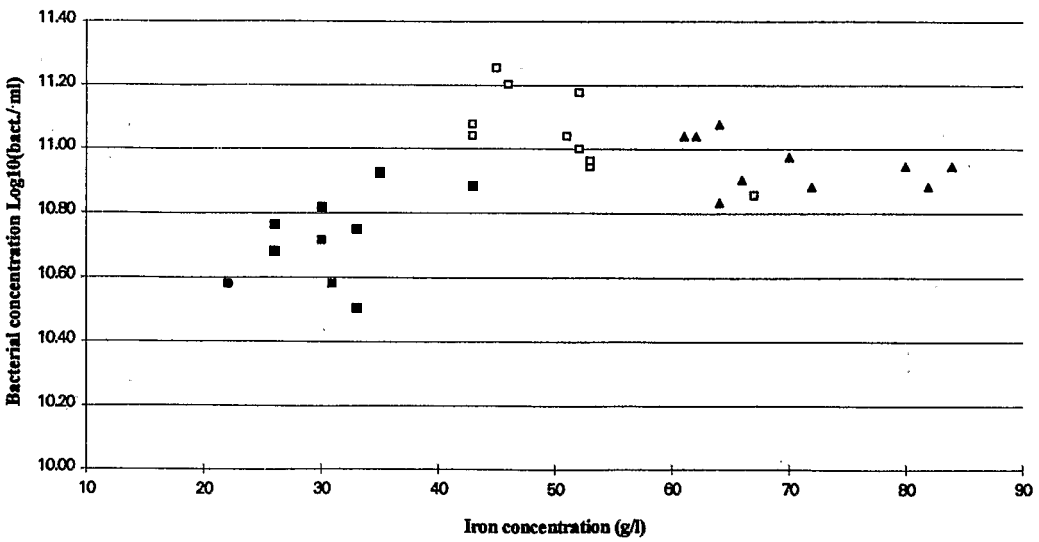

Fig.4a Evolution of bacterial concentration versus iron concentration during the continuous leaching experiments. Ferric iron systematically represented more than 95% of total iron.
CBE1 (with cobalt recycling): reactor 1 (■); reactor 2 (□); reactor 3 (s).

The concentration of *Leptospirillum*-like organisms increases exponentially up to 10^{11} cell.ml^{-1} versus iron concentration for both CBE1 and CBE2 (Figure 5a). Nevertheless, for iron concentrations in the range 10 - 50 g.l^{-1}, these bacteria were more numerous during CBE1 than during CBE2, and the maximum concentrations of *Leptospirillum*-like organisms were observed in the liquid phase at lower iron concentrations in the case of CBE1. On the other hand, the distribution of rod-shaped bacteria versus iron concentration had a very high reproducibility from one continuous experiment to the next (Figure 5b). The concentration of rod-shaped bacteria remained between 10^{10} and 10^{11} cell.ml^{-1} when the medium contained less than 60 g.l^{-1} iron. Above this concentration, the rod-shaped organisms were less numerous in the bioleaching liquor. The drop in rod-shaped bacteria at iron concentrations higher than 60 g.l^{-1} may be related to a greater toxicity of Fe^{3+} on these organisms. Several authors have reported that *Thiobacillus* species are more sensitive to ferric iron than are *Leptospirillum* strains [10, 24]. In contrast to *Leptospirillum*-like organisms, whose concentration clearly increased with the oxidation level, the maximum numbers of rod-shaped organisms were reached in the first reactor.

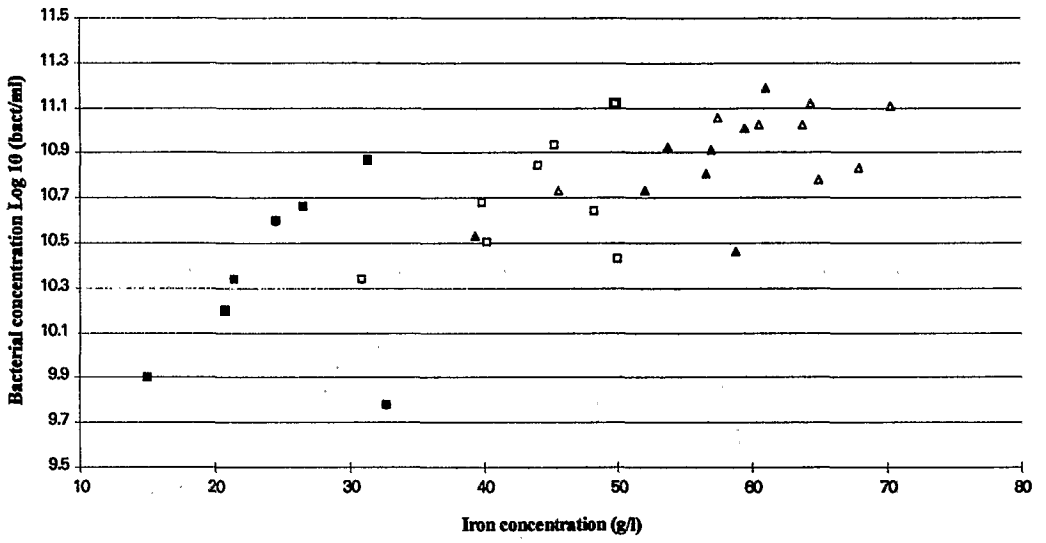

Fig.4b Evolution of bacterial concentration versus iron concentration during the continuous leaching experiments. Ferric iron systematically represented more than 95% of total iron.
CBE2 (without cobalt recycling): reactor 1 (■); reactor 2 (□); reactor 3 (s); reactor 4 (Δ).

Since the concentration of rod-shaped bacteria reached its maximum value in the first reactor, the rise in total bacterial concentration, as iron dissolution progressed, was closely related to the increase in *Leptospirillum*-like organisms. In the liquid phase, the growth of *Leptospirillum*-like bacteria was observed earlier during CBE1 than during CBE2. Consequently, the maximum bacterial concentration was obtained earlier during CBE1 than during CBE2 (Figure 4).

Concerning the morphological composition of bacterial populations, the main difference between CBE1 and CBE2 was the earlier appearance of *Leptospirillum*-like organisms during CBE1. The maximum growth rate of the *Leptospirillum*-like strains isolated from CBE1 was observed at 37.5°C [11], which is slightly higher than the optimum growth temperature of thiobacilli and *L. ferrooxidans*. Consequently, the increase in temperature during CBE1 may have promoted the growth of *Leptospirillum*-like organisms. However, the phenomenon of early appearance of these bacteria was observed before the increase in temperature. During the first phase of CBE1, the most important change in the operating conditions between CBE1 and CBE2 concerned the recycling of cobalt solution. Although the precipitation of iron with $CaCO_3$ removed most of the bacterial population, which was carried away with the solid phase, some organisms remained in the recycled solution. Furthermore, iron precipitated at pH 2.6, which is not an inhibiting value for acidophilic bacteria. Consequently, the first reactor was continuously inoculated with bacteria from the last reactor, mainly with *Leptospirillum*-like bacteria, which represented the largest part of the population at the end of the bioleaching reaction. Alternatively, a second hypothesis may be proposed to explain the early increase in the concentration of *Leptospirillum*-like bacteria in the liquid phase during CBE1. Several authors have reported the tendency of *L. ferrooxidans* for attachment to ore particles [4, 24, 25]. During CBE1, the *Leptospirillum*-like bacteria may have been affected by the high cobalt concentration, which increased from 1–2 to 5–6 $g.l^{-1}$ during the progress of bioleaching. Hallmann *et al.* [5] observed that *T. ferrooxidans* tolerated 10 $g.l^{-1}$ Co, whereas *L. ferrooxidans* was inhibited by 1 $g.l^{-1}$. Two strains of *Leptospirillum*-like bacteria, isolated from the CBE1 culture, were affected by 0.5 $g.l^{-1}$ Co when they grew on ferrous iron [11]. Thus, the attachment of *Leptospirillum*-like organisms to ore particles may have been partially inhibited by the recycled cobalt during CBE1. Consequently, the concentration of these bacteria in the liquid phase may have been greater than in the case of CBE2.

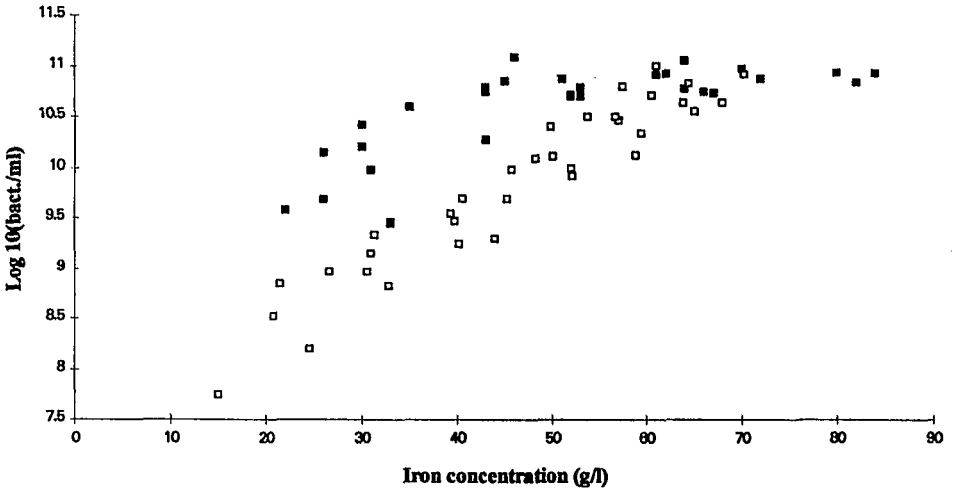

Fig.5a Evolution of bacterial concentrations (*Leptospirillum*-like bacteria and rod-shaped organisms) during the continuous leaching experiments.
Leptospirillum-like organisms: CBE1 (■); CBE2 (□).

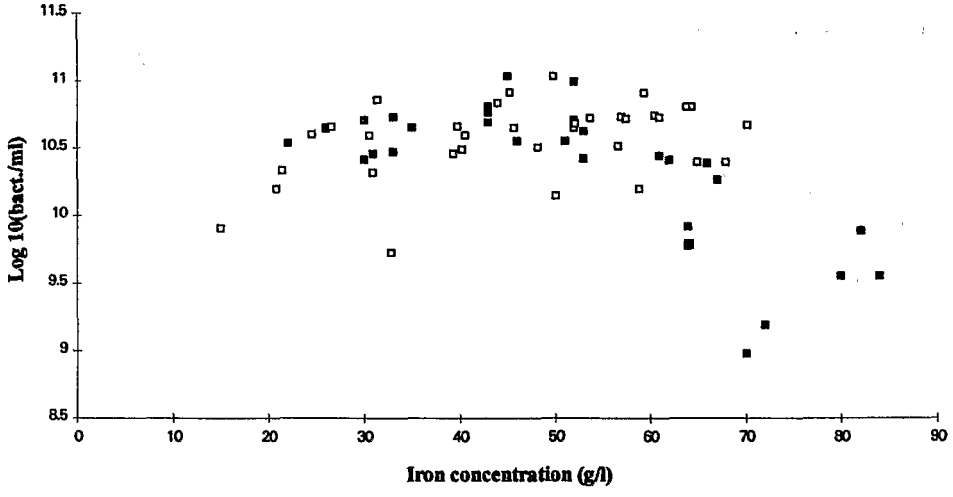

Fig.5b Evolution of bacterial concentrations (*Leptospirillum*-like bacteria and rod-shaped organisms) during the continuous leaching experiments.
Rod-shaped organisms: CBE1 (■); CBE2 (□).

CONCLUSION

The mixed population of the continuous bioleaching unit contained both *Thiobacillus* and *Leptospirillum*-like strains. Using pure strains from DSM, the association of *T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans* was demonstrated to be the most efficient population for the bioleaching of cobaltiferous pyrite. Similar results have already been reported [1]. Moreover, the relative abundance of the different organisms in the mixed population influenced the kinetics of bioleaching. Nevertheless, the best leaching rates obtained with the mixture of the strains from DSM were no higher than those obtained with the indigenous mixed population adapted to the substrate for one year in the continuous unit (data not shown). This suggests that the indigenous bacteria may have improved their ability to oxidize the cobaltiferous pyrite and increased

their resistance to acidity and metals in solution during the continuous experiments. Goebel and Stackebrandt [26] used enrichment cultures from a mine site to show that the operating conditions select a well defined mixed culture. These authors found that the continuous reactors at 40°C contained *L. ferrooxidans* and moderately thermophilic sulphur-oxidizers, but neither heterotrophic bacteria nor *T. ferrooxidans,* even though these can be isolated from the mine site and the batch reactors at 35°C.

The present study suggests that the composition of the mixed culture depends not only on the culture mode, batch or continuous, but also on the progress of the reaction when the continuous culture is performed using a series of several reactors. The rod-shaped bacteria represent the dominant morphological population in the first reactor. This population did not increase during the later steps of the process, whereas *Leptospirillum*-like bacteria seemed to increase in number as the bioleaching solution passed through the series of reactors. Although only free cell populations were studied, it can be assumed that the importance of indirect leaching, catalysed by the ferrous iron-oxidizing *Leptospirillum*-like bacteria, may increase with the progress of the treatment.

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REFERENCES

1. Sand, W., Rohde, K., Sobotke, B. & Zenneck, C., Evaluation of *Leptospirillum ferrooxidans* for leaching. *Appl. Environ. Microbiol.,* 1992, **58** (1) 85–92.
2. Norris, P.R. & Kelly, D.P., Dissolution of pyrite (FeS_2) by pure and mixed cultures of some acidophilic bacteria. *FEMS Microbiol. Lett.,* 1978, **4**, 143–146.
3. Norris, P.R., Iron and mineral oxidation with *Leptospirillum*-like bacteria, in *Recent Progress in Biohydrometallurgy,* eds. G. Rossi and A.E. Torma, Cagliari, Italy, 1983, 83–96.
4. Merretig, U., Wlotzka, P. & Onken, U., Removal of pyritic sulfur from coal by *Leptospirillum*-like bacteria, in *Biohydrometallurgy,* Proceedings of the International Symposium, eds. J. Salley, R.G.L. McCready and P.L. Wichlacz, Jackson Hole, Wyoming, 1989, 761–771.
5. Hallmann R., Friedrich A., Koops H.P., Pommerening-Röser A., Rohde K., Zenneck C., & Sand W., Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and Physicochemical actors influence microbial metal leaching. *Geomicrobiology J.*, 1993, **10**, 193–206.
6. Wakao, N., Mishina, M., Sakurai, Y. & Shiota, H., Bacterial pyrite oxidation. III. Adsorption of *Thiobacillus ferrooxidans* cells on solid surfaces and its effect on iron release from pyrite. *J. Gen. Appl. Microbiol.,* 1984, **30**, 63–77.
7. Rouas, C., Production de solutions de fer ferrique par *T. ferrooxidans* à partir de pyrite. Application à la lixiviation d'un minerai d'uranium. *Ph. D. Thesis*, University of Provence, Marseille, 1988.
8. Collinet-Latil, M.N., Lixiviation bactérienne par *Thiobacillus ferrooxidans* et *Thiobacillus thiooxidans* d'un concentré de flottation arsénopyriteux aurifère (réfractaire à la cyanuration directe). *Ph. D. Thesis*, University of Provence, Marseille, 1989.
9. Helle, U. & Onken, U., Continuous bacterial leaching of a pyritic flotation concentrate by mixed cultures, in *Biohydrometallurgy*, Proceeding of the International Symposium, eds. P.R. Norris and D.P. Kelly, Warwick, 1987, 61–75.
10. Helle, U. & Onken, U., Continuous bacterial leaching of a pyritic concentrate by *Leptospirillum*-like bacteria. *Appl. Microbiol. Biotechnol.,* 1988, **28** (6) 553–558.
11. Battaglia, F., Morin, D., Garcia, J.L. & Ollivier, P., Isolation and study of two strains of *Leptospirillum*-like bacteria from a natural mixed population cultured on a cobaltiferous pyrite substrate. *Antonie van Leeuwenhoek,* 1994, **66**, 295–302.
12. Balashova, V.V., Vedenina, I.Y., Markosyan, G.E. & Zavarzin, G.A., The auxotrophic growth of *Leptospirillum ferrooxidans. Mikrobiologiya, Engl. Tr.,* 1974, **43** (4) 491–494.
13. Pivovarova, T.A., Markosyan, G.E. & Karavaiko, G.I., Morphogenesis and fine structure of

Leptospirillum ferrooxidans. Mikrobiologiya, **50** (3), 1981, 482–486.

14. Harrison Jr., A.P. & Norris, P.R., *Leptospirillum ferrooxidans* and similar bacteria: some characteristic and genomic diversity. *FEMS Microbiol. Lett.*, 1985, **30**, 99–102.
15. Jerez, C.A. & Arredondo, R., A sensitive immunological method to enumerate *Leptospirillum ferrooxidans* in presence of *Thiobacillus ferrooxidans. FEMS Microbiol. Lett.*, 1991, **78**, 99–102.
16. Hallberg, K.B. & Lindström, E.B., Multiple serotypes of the moderate thermophile *Thiobacillus caldus*, a limitation of immunological assays for biomining microorganisms. *Appl. Environ. Microbiol.*, 1996, **62** (11), 4243–4246.
17. Silverman, M.P. & Lundgren, D.C., Studies on the chemoautotrophic iron-bacteria *Ferrobacillus ferrooxidans*: I. An improved medium and a harvesting procedure for securing high cell yields. *J. Bacteriol.*, 1959, **77**, 642–647.
18. Waksman, S.A. & Joffe, I.S., Microorganisms concerned with the oxidation of sulfur in soil. II. *Thiobacillus thiooxidans*, a new sulfur oxidizing organism isolated from the soil. *J. Bacteriol.*, 1922, **7**, 239.
19. D'Hugues, P., Cezac, P., Cabral, T., Battaglia, F., Truong-Meyer, X.M. & Morin, D., Bioleaching of a cobaltiferous pyrite: a continuous laboratory-scale study at high solids concentration. *Minerals Engineering*, 1997, **10** (5), 507–527.
20. Charlot, *Les méthodes de la chimie analytique, analyse quantitative minérale*, 5th ed. eds. Masson et Cie, Paris, France, 1966.
21. Suzuki, I., Takeuchi, L.T., Yuthasastrakosol, T.D. & Oh, J.K., Ferrous iron and sulfur oxidation and ferric iron reduction activities of *Thiobacillus ferrooxidans* are affected by growth on ferrous iron, sulfur, or a sulfide ore. *Appl. Environ. Microbiol.*, 1990, **56** (6), 1620–1626.
22. Attia, Y.A. & Elzeky, M., Bioleaching of non-ferrous sulfides with adapted thiophilic bacteria. *Chem. Eng. J.*, 1990, **44** (2), B31–B40.
23. Shrihari, Kumar, R., Gandhi, K.S. and Natarajan, K.A., Role of cell attachment in leaching of chalcopyrite mineral by *Thiobacillus ferrooxidans. Appl. Microbiol. Biotechnol.*, **36**, 1991, 278–282.
24. Johnson, D.B., Biological desulfurization of coal using mixed populations of mesophilic and moderately acidophilic bacteria, in *Processing and Utilization of High-Sulphur Coals, IV,* eds. P.R. Dugan, D.R. Quigley and Y.A. Attia, Elsevier Science Publishers BV, Amsterdam, 1991, 567–577.
25. Norris, P.R., Barr, D.W. & Hinson, U.K., Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite, in *Biohydrometallurgy,* Proceeding of the International Symposium, eds. P.R. Norris. and D.P. Kelly, Warwick, 1987, 43–59.
26. Goebel, B. & Stackebrandt, E., Cultural and phylogenetical analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl. Environ. Microbiol.*, 1994, **60**, 1614–1621.