

**Research on the Inhibition of Bacterial
Oxidation of Pyrite and the Concomitant
Acid Mine Drainage:**

**MA Loos. JM Conradie. PA Whillier
J Mare and Bosch**

WRC Report No.132/1/90

**M A LOOS
J M CONRADIE
P A WHILLIER
J MARÉ
C BOSCH**

**RESEARCH ON THE INHIBITION OF BACTERIAL OXIDATION
OF PYRITE AND THE CONCOMITANT ACID MINE DRAINAGE:
PART 1. INVESTIGATIONS ON GOLD MINE SAND DUMPS**

**Report to the
WATER RESEARCH COMMISSION
by the Department of Microbiology
UNIVERSITY OF STELLENBOSCH**

WRC Report No 132/1/90

ISBN 1 874858 01 2

ISBN SET 1 874858 04 7

ACKNOWLEDGEMENT

The Steering Committee and referees for this project consisted of the following persons:

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Mr PW Weideman	Water Research Commission	(Secretary)
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Prof FDI Hodgson	University of the Orange Free State	
Prof MA Loos	University of Stellenbosch	
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Mr TI Cannon	BTR Sarmcol
Dr L Bednarik	Cape Technikon

The financing of the project by the Water Research Commission, the contributions and collaboration of the Chamber of Mines and the contributions by the members of the Steering Committee are acknowledged gratefully.

**RESEARCH ON THE INHIBITION OF
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This project was funded by the Water Research Commission and carried out in terms of a tripartite agreement between the Water Research Commission, the Chamber of Mines and the University of Stellenbosch.

The report is presented in three parts:

- Part 1.** Investigations on gold mine sand dumps by MA Loos, JM Conradie, PA Whillier, J Maré and C Bosch (Department of Microbiology)
- Part 2.** Investigations on coal waste dumps by MA Loos, C Bosch and J Maré (Department of Microbiology)
- Part 3.** Development and testing of slow release systems by RD Sanderson and E Immelman (Institute of Polymer Science)

RESEARCH ON THE INHIBITION OF BACTERIAL OXIDATION OF PYRITE AND CONCOMITANT ACID MINE DRAINAGE

PART 1. INVESTIGATIONS ON GOLD MINE SAND DUMPS

EXECUTIVE SUMMARY

Concern about the production in mine waste dumps of acid drainage water containing high levels of sulphate and metal ions initiated the research of this project into the possibility of chemically inhibiting the bacteria responsible for the acid formation in the dumps. Research in the U.S.A. indicated that such treatment of dumps might be effective. Two types of dump have been investigated, namely, gold mine sand dumps and coal waste dumps. The project involved a contract of the Water Research Commission (responsible for finances) with the Department of Microbiology and the Institute for Polymer Science of the University of Stellenbosch (responsible for the research of the project) and the Chamber of Mines of South Africa (responsible for providing pilot scale experimental coal waste dumps and support services, including routine chemical analyses). The investigation was divided into two phases, namely, Phase I, comprising laboratory studies at the University of Stellenbosch, and Phase II, consisting of the pilot scale tests of chemical inhibitors on coal waste dumps, involving the Department of Microbiology at the University of Stellenbosch and the Chamber of Mines. The present final project report has been divided into three parts: Part 1 comprises all studies on the gold mine sand dumps (Phase I, Department of Microbiology), Part 2 all studies on coal waste dumps (Phases I and II, Department of Microbiology and Chamber of Mines) and Part 3 all studies on the development of slow-release systems for the inhibitor sodium lauryl sulphate (Phase I, Institute for Polymer Science). The remainder of this executive summary refers only to Part 1 of the report; Parts 2 and 3 have their own executive summaries.

The following are the main findings and conclusions from our evaluation of the possibility of inhibiting acid drainage-producing *Thiobacillus ferrooxidans* or other chemolithotrophic iron-oxidizing bacteria in gold mine sand dumps by treating the dumps with chemicals active against these bacteria.

1. The seepage of acid water from Witwatersrand gold mine sand dumps, which released an estimated 50 000 t of salts into the catchment of the Vaal Barrage during 1985 (Jones et al., 1988), showed the characteristics of acid drainage from mine waste deposits elsewhere in the world. The pH of the water was below pH 3, sulphate comprised a large proportion of the tonnage of salts in the acid drainage (Jones et al., 1988) and the brown colour of the seepage water and

precipitates in and around seepage pools indicated the presence of oxidized iron. That bacterially catalyzed iron oxidation was occurring in the seepage was indicated by the presence of populations of chemolithotrophic iron-oxidizing bacteria of ca. 10^6 to 10^7 /ml in drainage water and ca. 10^3 to 10^6 /g in the brown-coloured soil of a seepage area. It is presumed that the bacteria were *T. ferrooxidans* or contained a large proportion of *T. ferrooxidans* in a mixed population. These bacteria are the major iron-oxidizing bacteria of acid drainage elsewhere and the HJJ growth medium and conditions of incubation for the MPN estimates of the iron-oxidizing bacteria in sand dump seepage were selected for their suitability for *T. ferrooxidans*.

2. The gold mine sand dumps appeared to be a not very favourable growth environment for *T. ferrooxidans* and other possible iron-oxidizing chemolithotrophic bacteria of acid seepage. Increasing concentrations of an oxidized sand (i.e. in which the pyrite had been oxidized) increasingly retarded the growth of *T. ferrooxidans* in a favourable culture medium. The drying of mine dump sand resulted in the rapid destruction of large inoculum populations of *T. ferrooxidans* and iron-oxidizing bacteria from acid seepage, the destruction being more rapid in an oxidized sand than in an unoxidized sand containing pyrite substrate. However, high populations of *T. ferrooxidans* were maintained when the sands were kept moist.
3. Of the compounds tested in laboratory cultures as possible inhibitors of *T. ferrooxidans* and mixed populations of chemolithotrophic iron-oxidizing bacteria from sand dump acid drainage, the anionic detergent sodium lauryl sulphate (SLS) was the most effective. Inhibition of the most resistant cultures was achieved with 8 mg SLS/l in cultures containing no mine dump sand and with 20 mg SLS/l in cultures containing mine dump sand at 500 g/l. However, when the sand concentration was raised to 1000 g/l, 20 mg SLS/l only retarded ferrous iron oxidation by a less resistant *T. ferrooxidans* strain. Adsorption of the SLS by the sand can explain, or partly explain, this result. The sand-inhibitor interaction must receive further attention as a major factor determining the inhibitor dose and method of application for the control of acid drainage from sand dumps.
4. An anionic linear alkylbenzenesulphonate detergent (LAS) was almost as effective as SLS as an inhibitor of *T. ferrooxidans* and iron-oxidizing acid drainage bacteria in cultures without mine dump sand (LAS at 8 mg/l inhibited all cultures), but with 500 g/l sand in the cultures was less effective than SLS in that not all cultures were inhibited by 30 mg/l LAS. This result provides no grounds for considering LAS as an alternative inhibitor to SLS for the control of

acid drainage formation in sand dumps, but attention has not been given to economic considerations, which would also necessitate further comparison of the effectiveness of LAS and SLS.

5. Among the cationic surface-active compounds tested, the low pH antimicrobial quaternary pyridinium compound Ceepryn was more effective than SLS against *T. ferrooxidans* ATCC 19859 in cultures without sand (2 mg Ceepryn/l inhibited all cultures), but was considerably less effective in cultures containing 500 g/l sand, with 20 to 40 mg/l the minimum inhibitory concentration (in contrast to 8 mg SLS/l for the same *T. ferrooxidans* strain). The quaternary ammonium formulation Hyamine 3500 showed only partial or temporary inhibition of *T. ferrooxidans* and the acid drainage bacteria at the highest concentration tested (128 mg/l), even in cultures without mine dump sand. However, acidity is known to be highly detrimental to the activity of quaternary ammonium compounds. Adsorption to surfaces is a general characteristic of the quaternary pyridinium and ammonium compounds, reducing their antimicrobial activity, thus the reduction of the effectiveness of Ceepryn shown in the cultures with 500 g/l sand would likely be greatly enhanced in a sand dump. Evidence available to date provides no grounds for considering Ceepryn as a possible alternative to SLS for the combatting of acid drainage in sand dumps.
6. The food preservative organic acid inhibitors, benzoic and sorbic acid, are inherently less effective inhibitors of *T. ferrooxidans* in respect of minimum inhibitory concentrations than the surface-active chemicals, SLS, LAS and Ceepryn. The minimum inhibitory concentrations of sodium benzoate and sorbic acid in cultures without mine dump sand were 15 to 30 and 15 to 20 mg/l, respectively; these concentrations increased to 25 to 35 and 20 to 30 mg/l when 500 g/l mine dump sand was included in the cultures. The limited reduction of their effectiveness in the presence of the sand indicates that they have the advantage of little adsorption to the sand. It appears that doses of benzoic or sorbic acid to inhibit *T. ferrooxidans* in sand dumps would need to be about four times that of SLS, making even the cheaper benzoic acid probably not an economical alternative inhibitor to SLS.
7. Sodium lignosulphonate and polyacrylic acid had such limited activity against *T. ferrooxidans* that they cannot be regarded as possible inhibitors of acid drainage formation in sand dumps.
8. Investigations of the distribution of chemolithotrophic iron-oxidizing bacteria in sand dumps showed large populations only in wet regions where acid drainage

water was seeping through the sand at the base of the dump and emerging at the surface or in the interior grey-coloured sand exposed on excavated faces where the pyrite had not yet been oxidized to sulphate and yellow or orange oxidized forms of iron. The outer orange-coloured layer of sand dumps away from seepage zones contained almost no iron-oxidizing bacteria nor sulphur, both observations indicating that the pyrite substrate on which the bacteria grow had been lost from this part of the dumps during more than half a century of oxidation. As this process has proceeded to depths of ca. 10 m in sand dumps, the iron-oxidizing bacteria must be catalysing pyrite oxidation at or beyond this depth. Bacterial inhibitors to control acid production in the dumps must therefore be delivered in inhibitory concentrations to at least this depth. The absence of iron-oxidizing bacteria from most winter samples from the exposed face of the excavated dump, in contrast to their abundance in similar (but moister) grey-coloured samples in summer, suggests that pyrite oxidation is seasonal. It is possibly negligible in winter as drying of the dump sand results in rapid death of the iron-oxidizing bacteria.

9. The delivery of an inhibitor such as SLS to the sites of pyrite oxidation at least 10 m deep in sand dumps and the effectiveness of the inhibitor in the presence of the sand will be influenced by adsorption of the inhibitor to the sand. Although mine dump sand (50 or 100 g) adsorbed most of the SLS supplied in 100 ml water at concentrations of 1,0 to 6,0 mg/g sand, less than half the SLS supplied at below ca. 0,1 to 0,6 mg/g sand was adsorbed. Inhibitory SLS concentrations in laboratory cultures were below the latter concentrations, thus in the presence of sand at 500 g/l were only about double the inhibitory concentrations in the cultures without sand. Inhibitor delivery strategy will have to take into account adsorption and movement of the inhibitor in the sand dump. Continuous dosing of the dump from a slow-release formulation (Immeiman, 1987; this report, part 3) is one possible strategy and dosing in repeated pulses another. Continuous dosing with SLS would require an estimated total application of more than 115 kg/ha mine dump surface and possibly more than 460 kg/ha. The cost of the SLS as priced in 1990 (transport and application excluded) would be at least R1113/ha and possibly greater than R4453/ha. Whether such applications are justified in view of the probable complete reprocessing of the sand dumps during the next 20 years, is questionable. However, more research is needed on the adsorption and movement of potential inhibitors in sand dumps for a reliable comparative assessment of the two strategies for any specific inhibitor.

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1. GENERAL INTRODUCTION

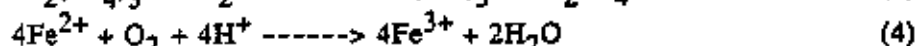
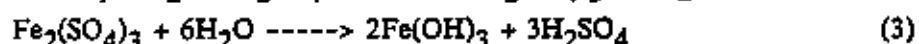
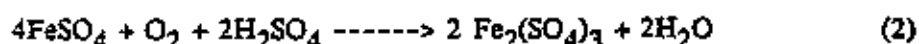
Disposal of the large volumes of ore waste generated by mining processes is achieved in most cases by forming dumps near the mine workings. These dumps are exposed to natural weathering agents and ores high in pyrite support microbial populations capable of producing acid water with high iron and sulphate contents, hence severe environmental pollution (Walsh, 1978). Acid water production is widespread in coal fields, for example, in the United States (Dugan, 1975; Good et al., 1970; Kleinmann et al., 1981; Kleinmann and Erickson, 1983; Walsh, 1978), but has also been shown and even utilized for bioleaching in dumps associated with the mining of gold (Clausen, 1973; James and Mroost, 1965; Jones et al., 1988; Marsden, 1986; Matic and Mroost, 1964; Mroost and Lloyd, 1970; Moshnyakova and Karavaiko, 1979; Thompson, 1980; Van Staden, 1970), copper (Brierley, 1982), uranium (Harries and Ritchie, 1981, 1983 a,b) and zinc (Goodman et al., 1983).

Microorganisms primarily responsible for acid water pollution from mines are chemolithotrophic bacteria of the genus *Thiobacillus* (Harrison, 1984; Lundgren and Silver, 1980; Lundgren et al., 1972; Walsh 1978). Of these, the acidophilic species *Thiobacillus ferrooxidans* is found in a variety of dumps throughout the world and appears to be the most common bacterium actively causing the pollution. *Thiobacillus ferrooxidans* converts the ferrous iron (Fe^{2+}) of pyrite (FeS_2) to its ferric form (Fe^{3+}) in an energy-yielding system, either during direct oxidation of the pyrite or following Fe^{2+} production in a more significant indirect oxidation cycle where ferric ions oxidize the pyrite (Kleinmann et al., 1981; Walsh, 1978).

The initial reaction of the direct oxidation of pyrite, either by bacterial action or abiotically, can be formulated (Lundgren and Silver, 1980; Walsh, 1978) as:



Subsequent biotic and abiotic reactions (depending on the conditions), which lead to the final abiotic oxidation of pyrite by ferric ions (indirect oxidation mechanism), can be represented (Lundgren et al., 1972; Kleinmann et al., 1981) as follows:



Reaction 1 shows the initiation of pyrite oxidation, either abiotically (autooxidation) or biotically by *T. ferrooxidans*, at high pH (pH >4.5) in the presence of moisture and oxygen. However, above ca. pH 5, biotic pyrite oxidation is slower than its abiotic oxidation (Kleinmann et al., 1981). Although *T. ferrooxidans* is acidophilic, Kleinmann and Crerar (1979) demonstrated that it was capable of colonizing crushed coal or overburden in a near neutral pH environment and acidifying it to pH 4 by initial direct oxidation of the pyrite. The formation of sulphuric acid in the initial oxidation reaction and concomitant decrease in the pH make conditions more favourable for the biotic oxidation of the pyrite by *T. ferrooxidans*. The biotic oxidation of pyrite is four times faster than the abiotic reaction at pH 3.0 (Pugh et al., 1984).

The next oxidation step (reaction 2), in which the ferrous sulphate is oxidized to ferric sulphate, proceeds abiotically at high pH (pH >4.5). However, as the pH decreases to pH <4.5, the abiotic reaction slows down (Lundgren et al., 1972; Kleinmann et al., 1981) and later the reaction becomes biotic as the pH decreases further towards pH 2.5, where it is completely biotic.

In reaction 3, the ferric sulphate is hydrolysed abiotically to ferric hydroxide and acid. This reaction occurs above pH 2.5, with the ferric hydroxide being visible as yellow boy in acid mine drainage. As the pH drops to pH <2.5, the ferric hydroxide dissolves releasing ferric iron into solution.

Reaction 4, which takes place at pH <2.5, is the biotic oxidation of ferrous iron, released mainly by the chemical oxidation of pyrite by ferric iron in reaction 5 (Kleinmann et al., 1981). Noike et al. (1983) noted that the maximum rate of production of ferric iron at 30°C occurred at pH <2.0. This oxidation of the ferrous iron provides electrons for the electron transport chain of *T. ferrooxidans*, and thereby energy and reducing power for the organism (Walsh, 1978). As a catalyst of reaction 4, *T. ferrooxidans* increases the reaction rate over that of the abiotic reaction 4 by a factor larger than 10^6 (Singer and Stumm, 1970). The ferric iron produced by reaction 4 then oxidizes the pyrite by reaction 5.

Reaction 5 is abiotic and proceeds at pH <2.5 with its optimum between pH 1 and 2 (Atkins, 1978). The ferric iron oxidizes the pyrite producing sulphate and ferrous iron, which is oxidized to ferric iron by *T. ferrooxidans* in reaction 4 (Lundgren et al., 1972; Kleinmann et al., 1981). A vicious cycle of abiotic pyrite oxidation and bacterial oxidation of ferrous iron thus arises (Singer and Stumm, 1970). The rate of the cyclic reactions is determined by the rate of reaction 4 (Nebgen et al., 1981). Oxygen was

shown to be rate-limiting for the oxidation of pyrite (Davidson et al., 1981; Pugh et al., 1984), although, surprisingly, Goodman et al. (1983) reported that *T. ferrooxidans* could oxidize pyrite under anaerobic conditions. Moshnyakova and Karavaiko (1979) reported that pyrite oxidation decreased as the temperature decreased and also that low pH promoted iron oxidation and the ferric iron attack on pyrite.

There are different approaches to dealing with the problem of acid mine drainage. Attempts have been made to reduce the volume of acid drainage water by reducing the infiltration of water to mine workings (Kim et al., 1982; Walsh, 1978). Also, water is treated before discharge into rivers by liming (Bosman, 1983; Kim et al., 1982; Thompson, 1980; Van Staden, 1979) and can possibly be treated by passage through wetlands coupled with limestone treatment (Kleinmann, 1985), or by the addition of waste organic matter, such as sawdust, to the water in dams to promote the development of heterotrophic anaerobic sulphate-reducing bacteria (Atlas and Bartha, 1981). Tuttle et al. (1969) described such a system in which the biologically produced hydrogen sulphide reduced the ferric iron and precipitated it as FeS, at the same time causing the pH of the water to rise. Alternative approaches involve suppression of Fe^{2+} oxidation by *T. ferrooxidans* at the sites in mines or dumps where it is producing the acid water. Abandoned sub-surface mines can be sealed to restrict the availability of O_2 for pyrite oxidation (Kim et al., 1982); however, Walsh (1978) has discussed serious problems with this approach. In strip mines the acid mine drainage can be effectively controlled by spreading topsoil over the mine dumps and the establishment of a vegetation cover (Atlas and Bartha, 1981). The activity of *T. ferrooxidans* can also be suppressed by various organic compounds (Erickson et al., 1985; Kleinmann, 1980; Kleinmann et al., 1981; Kleinmann and Erickson, 1983; Onysko et al., 1984a,b; Torma and Itzkovitch, 1976; Torma et al., 1976; Tuovinen, 1978; Tuttle and Dugan, 1976) and Kleinmann and co-workers (Erickson et al., 1985; Kleinmann, 1980; Kleinmann et al., 1981; Kleinmann and Erickson, 1982, 1983) have had considerable success with the inhibition of *T. ferrooxidans* in coal discard dumps under field conditions by the application of solutions of sodium lauryl sulphate (SLS) or SLS incorporated in rubber.

In South Africa, gold mine slimes and sand dumps on the Witwatersrand have been estimated to cover 80 km^2 (Thompson, 1980). Other mining areas, including those of the Orange Free State, will greatly increase this figure for the country as a whole. Important questions that arise are:

- (i) What is the extent of acid drainage from gold mine sand dumps and slimes dams?
- (ii) What is the impact of acid drainage from these deposits on the environment?
- (iii) What can be done to control or minimise acid drainage from gold mine deposits?

Question (i) has been addressed for the Witwatersrand area in the survey of Jones et al. (1988) entitled "Research on the contribution of mine dumps to the mineral pollution load in the Vaal Barrage". From intensive studies on two slimes dams and a sand dump, they concluded that slimes dams with their low permeability contributed little to the salt load of water in the studied catchment area of the Vaal Barrage. The more porous, better aerated and drained sand dumps contributed most of the pollutant salts originating from mine deposits, mainly in seepage rather than runoff water. The intensively studied 3A17 dump contributed ca. 2300 t of salts (ca. 1600 t as sulphate) in 1985; by extrapolation, the sand dumps of the Vaal Barrage catchment area were estimated to have discharged about 50 000 t salts, mainly in seepage water, in that year. Estimates made for two other sand dumps in 1981 (T.D. Brown, Rand Mines Milling and Mining Company Limited, Johannesburg, personal communication) indicate leaching of sulphuric acid from the dumps at rates of 7065 and 1780 t/year for 25 and 40 years, respectively. Pyrite in Witwatersrand gold mine sand dumps has been oxidized, with much of the sulphur lost as sulphate, to depths of about 5 to 10 m (Marsden, 1986). The total annual discharge of acid drainage from dumps should be declining as a result of the slowing of pyrite oxidation as it proceeds deeper into the dumps where oxygen is increasingly limiting, as well as factors such as the construction of toe-dams, vegetation of dumps and slimes dams and the removal of sand dumps during reprocessing (Jones et al. 1988; Marsden, 1986).

Question (ii), concerned with the impact of acid drainage from gold mine deposits on the environment, is unresolved. The water in the Vaal Barrage has shown continually and rapidly increasing salts concentrations since about 1965 (Stewart, Sviridov and Oliver, 1981). Salt loads monitored at discharge points in the Vaal Barrage catchment (point source discharge and discharge to the Barrage) and estimates of salt loads discharged by diffuse or non-point sources from 1977 to 1987 (Department of Water Affairs; Directorate of Planning, 1986, 1990) are shown in Table I. It is clear that there is a huge problem with salts pollution of the water in the catchment, with gold mine deposits making a contribution to the salts from diffuse sources, but the proportion of the mine deposit salts (50 000 t in 1985) which reach the Vaal Barrage is unknown (Jones et al., 1988).

Question (iii) initiated the present investigation. Strategies so far to reduce the discharge of acid drainage from sand dumps and slimes dams have been vegetation of their surfaces and the construction of toe-dams where possible. These measures, particularly toe-dam construction, have by no means been universally applied, according to the inventory of the Chamber of Mines for 1987 in the Jones et al. (1988) report. Liming technology has been developed (Bosman, 1983; Thompson 1980) and widely applied (Van Staden 1979) for the treatment of acid water in the South African gold-mining

TABLE 1. Discharge of salts from point and diffuse (non-point) sources into surface water in the catchment of the Vaal Barrage and from the catchment into the Vaal Barrage^a

Hydrological year	Salts discharge from point sources	Estimated salts discharge from diffuse sources ^b	Total salts discharge from point and diffuse sources	Salts discharge from catchment into the Vaal Barrage
	t x 10 ³	t x 10 ³	t x 10 ³	t x 10 ³
1977/78	300	398	698	627
1978/79	262	124	386	250
1979/80	320	218	538	436
1980/81	322	191	513	473
1981/82	293	144	437	296
1982/83	272	94	366	198
1983/84	249	134	384	229
1984/85	202	157	359	217
1985/86	198	171	368	237
1986/87	232	258	489	363

^aData compiled by Stewart, Sviridov and Oliver, Consulting Engineers, for reports of the Department of Water Affairs: Directorate of Planning (1986, 1990). All values are rounded to the nearest thousand t.

^bCalculated from salts discharge into the Vaal Barrage plus estimated salts diversion elsewhere (e.g. removal in irrigation water, movement into aquifers, etc.) minus salts discharge from point sources.

industry, but not in the case of the seepage from sand dumps contributing to the salts from diffuse sources in the Vaal Barrage catchment. The research in Part 1 of the present report is an evaluation of the possibility of using antimicrobial chemicals (active against ferrous iron-oxidizing bacteria) to inhibit the production of acid drainage at source in gold mine sand dumps. It formed part of Phase I of the joint project "Inhibition of the bacterial oxidation of pyrite and concomitant acid mine drainage" involving the Department of Microbiology and Institute of Polymer Science of the University of Stellenbosch, the Chamber of Mines of South Africa and the Water Research Commission.

Initial studies, described in section 2 of this report, involved the establishment of suitable laboratory procedures for culturing the iron-oxidizing bacterial species *T. ferrooxidans* and monitoring its development in culture quantitatively. The iron-oxidizing bacteria in seepage from sand dumps were then investigated in respect of their numbers in soil and water samples and their growth characteristics in enrichment cultures (section 3). Growth of *T. ferrooxidans* in liquid cultures containing mine dump sand was then investigated (section 4), as were the inhibitory effects of various organic compounds on *T. ferrooxidans* and enrichment cultures of mine seepage iron-oxidizing bacteria in liquid medium, in the absence and presence of mine dump sand (sections 5 to 8). The compounds tested were antimicrobial anionic and cationic surface-active agents, antimicrobial organic acids used as food preservatives, sodium lignosulphonate and polyacrylic acid. Having established the inhibitory potential of several of the compounds, we turned our attention to the target bacteria in the sand dumps, with several studies to determine where they were located (section 9), where oxidation of the pyrite in the sand dumps occurred (section 10) and how moisture conditions and the availability of pyrite substrate in the dumps could be expected to affect them (section 11). Finally the adsorption of SLS, considered one of the most promising inhibitors, by mine dump sand was studied to assess the potential for its delivery to the sites of the target bacteria at a concentration able to inhibit them (section 12).

2. MEDIUM AND INCUBATION CONDITIONS FOR *T. FERROOXIDANS* CULTURES

2.1 Introduction

2.1.1. Medium composition

Liquid inorganic media containing ferrous iron as energy source have usually been used for the chemolithotrophic bacterium *T. ferrooxidans*. The 9K liquid medium of Silverman and Lundgren (1959a) has been used extensively. These workers reported doubling times in aerated cultures of 5,3 to 9,6 h. Ferrous iron at a concentration of 9 g/l appeared to be optimum for the most rapid production of high yields of cells. Above this level, progressive increases in the initial Fe^{2+} concentration in 9K medium failed to increase final cell yields and progressively decreased growth rates. However, even with Fe^{2+} at 9 g/l, the utilization of Fe^{2+} as a primary energy source was not very efficient, according to the calculations of Lundgren et al. (1972). Lundgren et al. (1972) and Margalith et al. (1966) reported that the iron-oxidizing species *T. ferrooxidans* was capable of growth in the basal salts of the 9K medium containing 5 g/l sulphur, substituted for iron as the energy source. Cell yields (dry weight) were about 150 to 180 mg/l for sulphur-grown cells, compared to 60 to 70 mg/l for iron-grown cells. McGoran et al. (1969) used the basal salts of the 9K medium and ferrous iron, a chalcopyrite concentrate or sulphur as an energy source. By using total bacterial nitrogen as the measure of growth, they showed that *T. ferrooxidans* had a minimum doubling time of 6,5 to 10 h on ferrous iron as substrate, 14 to 17 h on chalcopyrite and 7 to 8 days on sulphur.

An ammonium concentration of 3,6 to 18 mg/l was reported to be optimal for the oxidation of iron by *T. ferrooxidans* (Tuovinen et al., 1979). Amino acid nitrogen could replace the $(\text{NH}_4)_2\text{SO}_4$ in the inorganic medium normally required for growth (Lundgren et al., 1964); alanine, glutamic acid and lysine were comparable to $(\text{NH}_2)_2\text{SO}_4$, whereas arginine and histidine could not completely replace the ammonium. Neither proline, methionine, nor tryptophan was able to support growth.

The ability of *T. ferrooxidans* to grow in the absence of added nitrogen was reported by Eccleston and Kelly (1978). Such growth could have been due to either the ability of the organism to fix atmospheric nitrogen, as demonstrated by Mackintosh (1978), or by its ability to scavenge trace amounts of atmospheric ammonia that were absorbed into the acid medium (Hill and Postgate, 1969).

The ingredients in the 9K medium of Silverman and Lundgren (1959a) were (/l) 44,22 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3,00 g $(\text{NH}_4)_2\text{SO}_4$, 0,50 g K_2HPO_4 , 0,50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0,10 g KCl and 0,01 g $\text{Ca}(\text{NO}_3)_2$. The medium of Harrison et al. (1980) contained the same salts, but at different concentrations, namely (/l) 44 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2,00 g $(\text{NH}_4)_2\text{SO}_4$, 0,50 g K_2HPO_4 , 0,50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0,10 g KCl and 0,01 g $\text{Ca}(\text{NO}_3)_2$. The medium of Tuovinen and Kelly (1973) contained fewer salts (33,3 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0,40 g $(\text{NH}_4)_2\text{SO}_4$, 0,40 g K_2HPO_4 and 0,40 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /l). The salts in the standard medium 64 used by the American Type Culture Collection (1982) for *T. ferrooxidans*, were the same as those of the medium of Tuovinen and Kelly (1973), but the concentrations were (/l) 20 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0,80 g $(\text{NH}_4)_2\text{SO}_4$, 0,40 g K_2HPO_4 and 0,16 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. On the basis of preliminary trials not included in this report, we chose the medium of Harrison et al. (1980) for our studies. (The *T. ferrooxidans* ATCC 19859 and WLR test strains grew much better in this medium in the trials than in the medium of Tuovinen and Kelly).

2.1.2. Aeration

The iron-oxidizing thiobacilli have usually appeared strictly aerobic and limitations in oxygen concentration affect the rates of ferrous iron oxidation and metal extraction in leaching processes (Guay et al., 1975). Beck (1960) showed that during the oxidation of iron by resting-cell suspensions of *T. ferrooxidans*, the rate of oxygen consumption was high. Thus, during active metabolism, the O_2 in solution could be depleted faster than it could be replaced, resulting in a slowing of culture activity and growth. This danger is particularly great with stationary flask cultures where the only mechanism for oxygen transfer is diffusion. Silverman and Lundgren (1959a) found that sufficient O_2 and CO_2 were dissolved in the medium of stationary cultures to support growth of at least 3×10^7 cells/ml. When this population was attained, the growth rate fell rapidly, but it was stimulated again when proper aeration was supplied.

Shaking is widely used to aerate bacterial cultures (Freedman, 1970). Starkey et al. (1956) observed poorer oxidation of sulphur by *T. thiooxidans* on a reciprocal shaker than on a rotary shaker. Possibly the snaplike motion of the reciprocal shaker limited the contact time of the bacteria on the sulphur particles. A basic difference between reciprocal and rotary shaking is the consistent swirl pattern set up in rotary shaking (Freedman, 1970). Compared with the reciprocal shaker, the swirl pattern set up in the rotary shaker is not nearly as affected by media, viscosity, machine start-up or flask volume. In reciprocating shaking the geyser effect may wet the flask's stopper and contaminate the culture. These are the reasons why the rotary shaker is used more

frequently in culturing bacteria (Starkey et al., 1956). Starkey et al. (1956) used 100 ml of medium in a 250-ml Erlenmeyer flask shaken at 200 rpm on a rotary shaker for culturing *T. thiooxidans*. Schnaitman et al. (1969) emphasized the importance of CO₂ availability to *T. ferrooxidans*, as CO₂ is the source of all cellular carbon for this bacterium growing on mineral sulphides. Schnaitman and Lundgren (1965) found that CO₂ levels higher than ambient increased the growth of *T. ferrooxidans*.

Although most studies of growth and activity of *T. ferrooxidans* have been carried out in highly aerated systems, there have been several reports on the behaviour of *T. ferrooxidans* under conditions of low O₂ concentrations or even anaerobic conditions. Pugh and Umbreit (1966) showed that *T. ferrooxidans* could continue to fix CO₂, using ferrous iron as the energy source, in the absence of O₂. Brock and Gustafson (1976) found that under anaerobic conditions *T. ferrooxidans* was able to reduce ferric to ferrous iron in the presence of inorganic sulphur. Mackintosh (1978) showed that *T. ferrooxidans* formed colonies containing ferric iron and fixed N₂ efficiently in an atmosphere of 1:94:5 parts O₂:N₂:CO₂, and that the N₂-fixing system was sensitive to O₂. Goodman et al. (1983) showed the release of iron and zinc from iron-zinc sulphide ores under anaerobic or almost anaerobic conditions, particularly when CO₂ was passed into the cultures at suitable flow rates.

In our own studies there was no reason to consider anaerobic rather than aerobic conditions. We therefore compared three aerobic systems for the growth of two strains of *T. ferrooxidans*, namely, stationary cultures in Erlenmeyer flasks, shaken (rotated) cultures in Erlenmeyer flasks and cultures in test tubes aerated by a stream of pumped air. Our objective was the selection of a system for the aeration of large numbers of cultures under constant conditions.

2.1.3 Temperature

Lundgren and Silver (1980) have reviewed the effects of temperature on the metabolic activity of the iron-oxidizing bacteria. Active bacterial iron oxidation in soils and mines was noted at about 10°C and, although the lower temperature limit for this activity was not established, it was generally accepted to be the freezing point of water. Optimal leaching of metal sulphide ores and the oxidation of Fe²⁺ by iron-oxidizing bacteria was found to occur between 25°C and 45°C. The upper temperature limit for such biological oxidation was 55°C and only chemical oxidation occurred above this temperature. Increases in temperature resulted in the usual augmentation in reaction rate associated, within limits, with microbial as well as chemical reactions. Thus, between 23°C and

32°C, the temperature coefficient (Q_{10}) for the biological oxidation of ZnS , FeSO_4 , Cu_2S and CuS was approximately 2.

In their investigation of the growth of *T. ferrooxidans* and the oxidation of Fe^{2+} in relation to culture pH, Moshnyakova and Karavaiko (1979) studied these activities at 12°C and 28°C. At 28°C both the growth of *T. ferrooxidans* and the oxidation of Fe^{2+} occurred rather rapidly, and the growth curves showed an exponential form. The doubling times of the three test strains were 5, 7 and 9 h, respectively. The maximum rates of oxidation of Fe^{2+} by the three strains were 4,0 to 4,1, 4,3 to 6,1 and 3,1 to 3,7 g/l/day, respectively. At 12°C, the doubling times of the three strains in the second rapid phase of growth were 22, 32 and 36 h, respectively, while the maximum rates of oxidation of Fe^{2+} were correspondingly 1,1, 1,3 and 1,2 g/l/day. Thus, the rates of growth of *T. ferrooxidans* and the oxidation of Fe^{2+} decreased 4 to 5-fold and 3 to 5-fold, respectively, when the temperature was lowered from 28°C to 12°C. Temperature coefficients (Q_{10}) just larger than 2 are indicated.

Silverman and Lundgren (1959b) found that the optimal temperature for growth of *T. ferrooxidans* did not agree with that for iron oxidation. The bacteria grew well at 28°C but not at all at 37°C. The most rapid oxidation of Fe^{2+} took place at 37°C, with the rate over 80% of the maximum within the range 28°C to 40°C. Heat-labile constituents of the cell not involved in Fe^{2+} oxidation must have been responsible for the lack of growth at 37°C.

Our own tests of growth at different temperatures were conducted at 26°C and 31°C. The former was the temperature of our main incubator and the latter a higher temperature below 37°C, which seemed from the literature to be high for growth of *T. ferrooxidans*.

2.1.4 pH

Thiobacillus ferrooxidans is active in the pH range 1,5 to 5,0 according to Silverman (1967) or pH 1,4 to 6,0, with an optimum between pH 2,5 and 5,8, according to Bergey's Manual (Buchanan and Gibbons, 1974). Silverman and Lundgren (1959b) studied the effect of the initial pH of the medium on Fe^{2+} oxidation and showed that optimal activity occurred over the range pH 3,0 to 3,6 with rates greater than 80% of the maximum oxidation rate within the range pH 2,5 to 4,2. However, according to Razzell and Trussell (1963), the optimum pH for the oxidation of ferrous iron, chalcopyrite and pyrite was between pH 1,0 and 2,5. At the lower pH values within the range for growth

of *T. ferrooxidans*, the formation of the basic ferric sulphate, jarosite, decreases (Tuovinen and Kelly, 1973).

Petrova et al. (1979), using computer modelling techniques, studied the relationship between culture pH and the rates of oxidation of Fe^{2+} by *T. ferrooxidans* at two temperatures. The range of pH values associated with optimum rates of Fe^{2+} oxidation by their two *T. ferrooxidans* strains was wider at 28°C (from pH 2,5 to 2,8) than at 12°C (from pH 2,3 to 2,4). Both growth of the bacteria and the enzymatic processes of Fe^{2+} oxidation appeared from the experiment at 12°C to be highly sensitive to changes in the medium pH. When the pH was maintained at pH 2,3 to 2,4 by the addition of sulphuric acid, the bacterial oxidation of Fe^{2+} in the exponential phase of growth at 12°C achieved the relatively high rate of 2,7 g/l/day, only 23% lower than at 28°C (Moshnyakova and Karavaiko, 1979).

The pH of the various inorganic media used for culturing Fe^{2+} -oxidizing *T. ferrooxidans* is: 9K medium of Silverman and Lundgren (1959a), pH 3,0 to 3,6; medium of Harrison et al. (1980), pH 3,4; medium of Tuovinen and Kelly (1973), pH 1,3; and medium 64 (American Type Culture Collection, 1982), pH 2,8. We tested growth of two *T. ferrooxidans* strains in the medium of Harrison et al. (1980) from pH 1,50 to 3,00, after culturing them initially in this medium at pH 1,50.

2.2. Materials and Methods

2.2.1. Bacterial strains

Two pure cultures of *T. ferrooxidans* were obtained from Prof. D.E. Rawlings of the Department of Microbiology, University of Cape Town. The strains were the American Type Culture Collection strain, ATCC 19859, and a South African isolate, WLR, from a water leach residue from the Fairview Mine, Paterson, eastern Transvaal. The WLR strain was a pure culture according to the DNA homology test of Harrison et al. (1980) for heterotrophic contaminants (D.E. Rawlings, personal communication).

2.2.2. Medium

The medium used in all *T. ferrooxidans* growth studies was that of Harrison et al. (1980), designated HJJ medium (see section 2.1.1. for the composition of the medium). The FeSO_4 in 500 ml water was filter-sterilized using a 0,20 μm Sartorius SM 11107

filter (Sartorius-Membranfilter GmbH, Göttingen, West-Germany), then added to the balance of the salts in 500 ml water after the latter had been autoclaved at 121°C for 15 min. Before sterilization the pH of each solution was adjusted to pH 1,50 with 15% (v/v) H_3PO_4 -15% (v/v) H_2SO_4 solution.

All growth studies were carried out using 100 ml volumes of medium in 250-ml Erlenmeyer flasks plugged with cotton wool.

2.2.3. Maintenance of cultures

All cultures were subcultured weekly in HJJ medium and were incubated on a rotary shaker at 120 rpm and 26°C.

2.2.4. Dichromate titration of ferrous iron

The growth of *T. ferrooxidans* populations in cultures was determined indirectly by monitoring the disappearance of ferrous iron compared with that in uninoculated control medium incubated under the same conditions. Dichromate titrations for ferrous iron were performed according to Vogel (1961), with modifications from Prof. D.E. Rawlings (Department of Microbiology, University of Cape Town). Ten ml 15% (v/v) H_3PO_4 -15% (v/v) H_2SO_4 solution and 6 drops of redox indicator were pipetted into a 100-ml Erlenmeyer flask. The indicator was the supernatant of 0,32 g diphenylamine sulphonate barium salt (Sigma Chemical Company, St. Louis, Mo., U.S.A.) in 100 ml water acidified with 1 ml concentrated H_2SO_4 . Five ml of sample from a culture were added and titrated with 0,01492 M $\text{K}_2\text{Cr}_2\text{O}_7$ (4,389 g $\text{K}_2\text{Cr}_2\text{O}_7$ /l water). The titration endpoint was indicated by a change in the colour from green to purple. Ferrous iron metabolized by a culture was calculated as a percentage of that remaining in the uninoculated control from the formula:

$$\text{Metabolized ferrous iron (\%)} = 100 - \frac{\text{ml dichromate titrated against culture sample} \times 100}{\text{ml dichromate titrated against control sample}}$$

Log percentage values were used for plotting growth curves.

2.2.5. Growth of *T. ferrooxidans* in HJJ medium

All cultures in the experiments were started from 10% (v/v) inocula. Initially the *T. ferrooxidans* ATCC 19859 inoculum was a culture that had been grown in 9K medium (Silverman and Lundgren, 1959a) for 9 days. The initial WLR inoculum had been cultured in the medium of Tuovinen and Kelly (1973), then stored for approximately 10 weeks at 4°C. The cultures in HJJ medium were incubated at 31°C with rotary shaking at 100 rpm. Growth of the cultures was monitored by daily titrations of ferrous iron. Flasks with sterile HJJ medium were incubated as controls. The experiments comprised studies of the growth of the thiobacilli in the initial cultures in HJJ medium and one or two successive subcultures with three or four replicate flasks per treatment in each experiment. The inocula were fully grown cultures from the preceding experiment.

2.2.6. Effect of aeration procedures and two temperatures on *T. ferrooxidans* growth

Cultures of *T. ferrooxidans* strains ATCC 19859 and WLR in 100 ml HJJ medium in 250-ml Erlenmeyer flasks were incubated as stationary cultures at 26°C and 31°C, or with rotary shaking at 120 rpm at 26°C or 150 rpm at 31°C, or air was bubbled from 10-ml pipettes through 100 ml cultures in 33 x 200 mm glass test tubes at 26°C using an aquarium airpump. Inocula (10%, v/v) were fully grown cultures in HJJ medium. The experiment was performed twice, with duplication of the different treatments on each occasion.

2.2.7. Effect of pH on *T. ferrooxidans* growth

The growth of *T. ferrooxidans* strains ATCC 19859 and WLR in HJJ medium brought to pH 1,50, 1,75, 2,00, 2,25, 2,50, 2,75, and 3,00 by the addition of 15% (v/v) H₃PO₄-15% (v/v) H₂SO₄ solution was studied. Inocula were 10% (v/v) of fully grown cultures in HJJ medium. The cultures were incubated at 31°C with rotary shaking at 150 rpm. The experiment was performed twice with duplication of the different treatments on each occasion.

2.3. Results

2.3.1. Growth of *T. ferrooxidans* in HJJ medium

Growth of *T. ferrooxidans* strains ATCC 19859 and WLR in HJJ medium was satisfactory, after an initial lag in the case of strain WLR (Fig. 1, 2). Heavy orange-brown sediment of crystals presumed to be jarosite ($\text{KFe}_3(\text{OH})_6(\text{SO}_4)_2$) formed on the bottoms and sides of the flasks containing active cultures. The times for complete Fe^{2+} oxidation by the initial cultures of ATCC 19859 and WLR were 142 and 355 h, respectively. Lag phases were almost eliminated in the first subcultures and completely eliminated in the second, reducing these times to 42 and 120 h, respectively, in the case of the second subcultures. The doubling times in the exponential phase of the initial cultures and two subcultures ranged from 8 to 11 h for strain ATCC 19859 and 12 to 30 h for strain WLR. On the basis of these results, HJJ medium was used for maintenance of the *T. ferrooxidans* cultures and as the growth medium in further experiments.

2.3.2. Effect of aeration procedures and two temperatures on *T. ferrooxidans* growth

Growth of *T. ferrooxidans* strains ATCC 19859 and WLR with different aeration methods, at 26°C and 31°C, is shown in Fig. 3 and 4. Overall best growth occurred in cultures grown at 26°C and shaken at 120 rpm and slightly slower growth (longer lag of WLR) in cultures grown at 31°C and shaken at 150 rpm. Cultures aerated with the aquarium airpump at 26°C also grew rapidly. Stationary cultures took approximately four times longer than shaken cultures to oxidize Fe^{2+} completely. With these cultures too, 26°C was a slightly more favourable temperature than 31°C for the growth of *T. ferrooxidans*.

2.3.3. Effect of pH on *T. ferrooxidans* growth

The effects of pH on the growth of *T. ferrooxidans* strains ATCC 19859 and WLR in HJJ medium are shown in Fig. 5 and 6. Both *T. ferrooxidans* strains grew over the pH range 1,50-3,00. The mean specific growth rates in the exponential phase of growth of the cultures and the times for 100% Fe^{2+} oxidation are plotted against pH in Fig. 7 and 8. The pH for optimal growth was in the range pH 1,75 to 2,50 for both ATCC 19859 and WLR, the former showing no growth rate differences over this range and the specific growth rate of the latter a sharp maximum at pH 2,50. On the basis of these results, the pH of HJJ medium in all subsequent experiments was adjusted to pH 2,00.

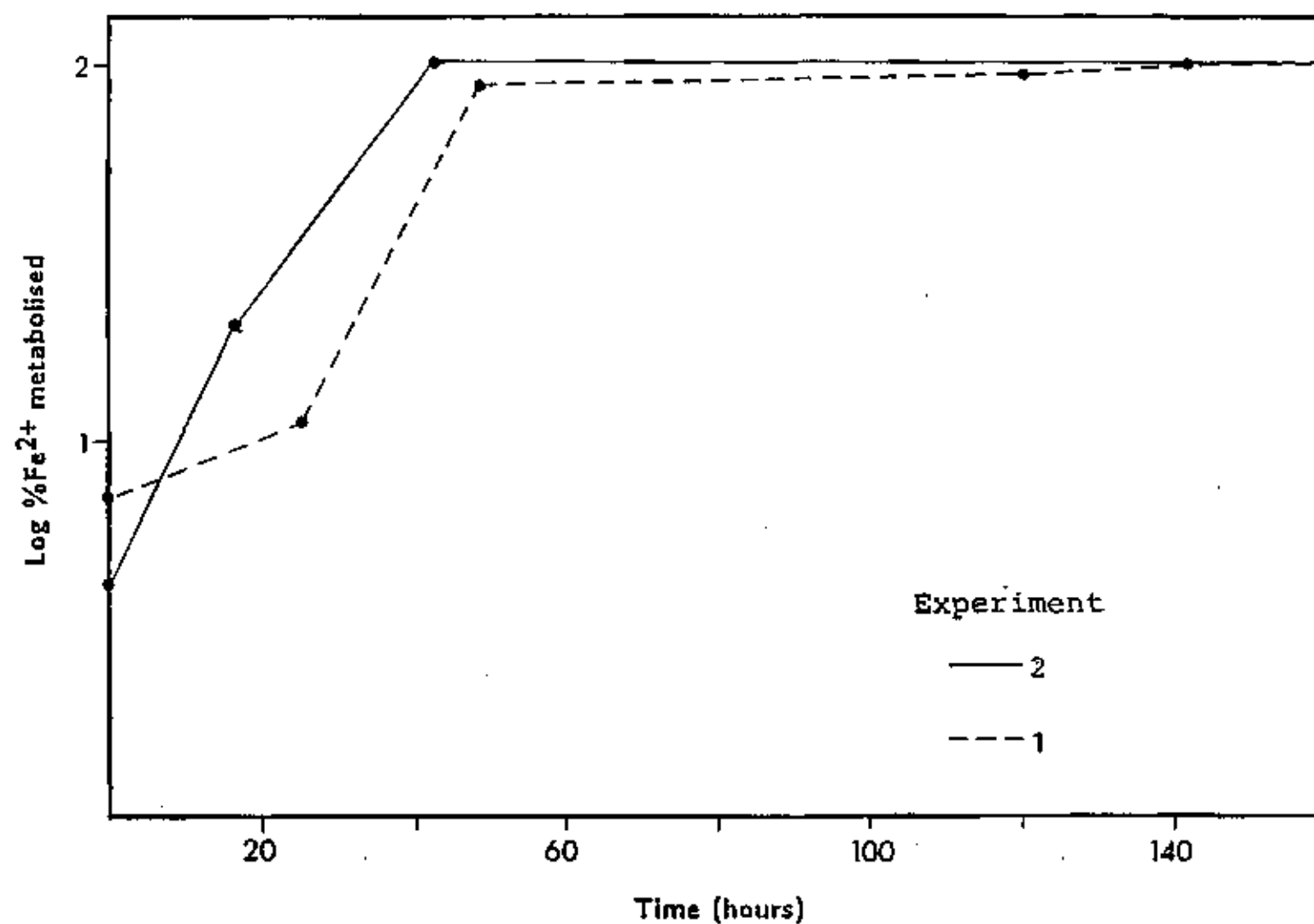


FIG. 1. Growth of *T. ferrooxidans* ATCC 19859 in HJJ medium at 31°C with rotary shaking at 100 rpm, as indicated by metabolism of ferrous iron.

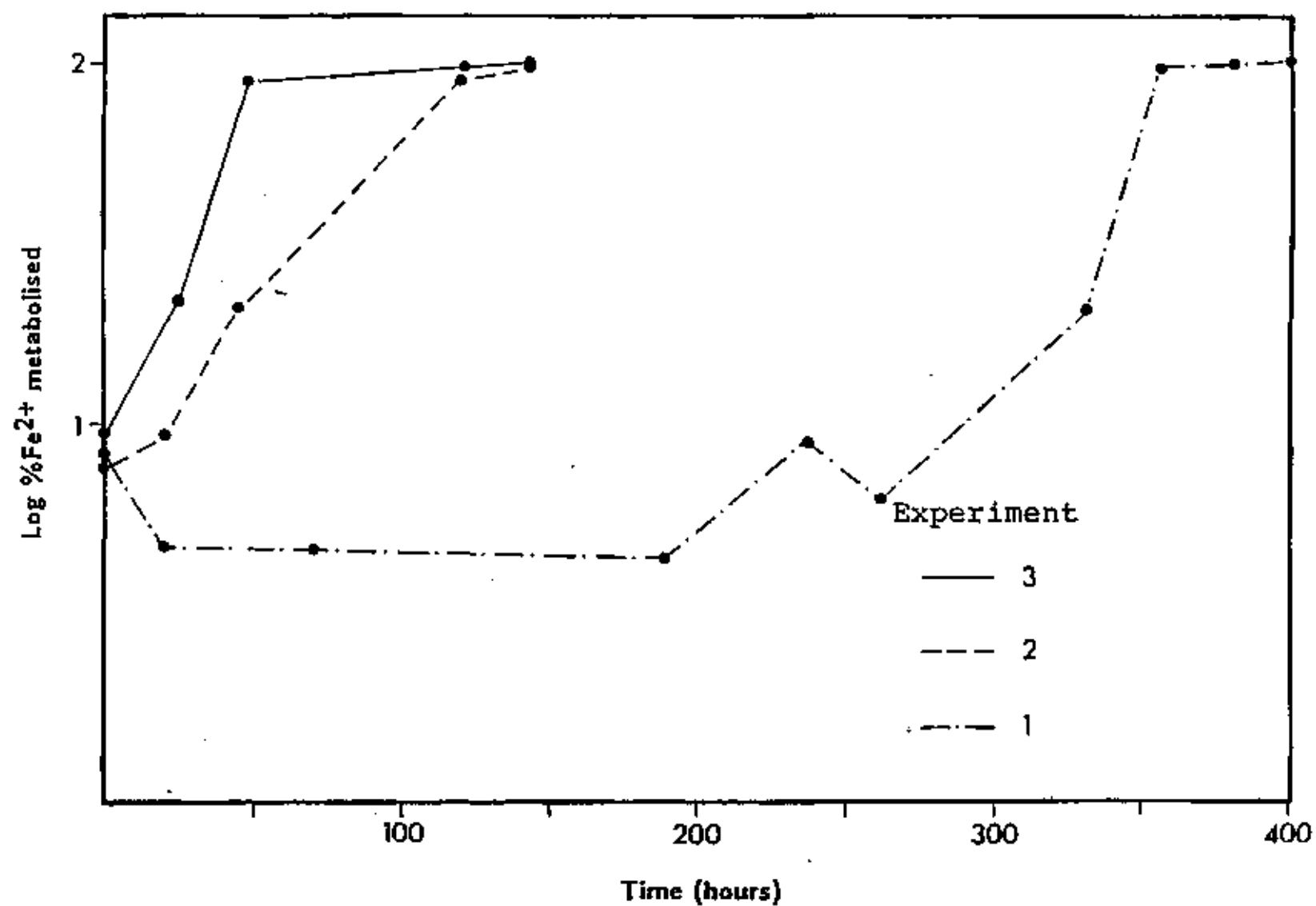


FIG. 2. Growth of *T. ferrooxidans* WLR in HJJ medium at 31°C with rotary shaking at 100 rpm.

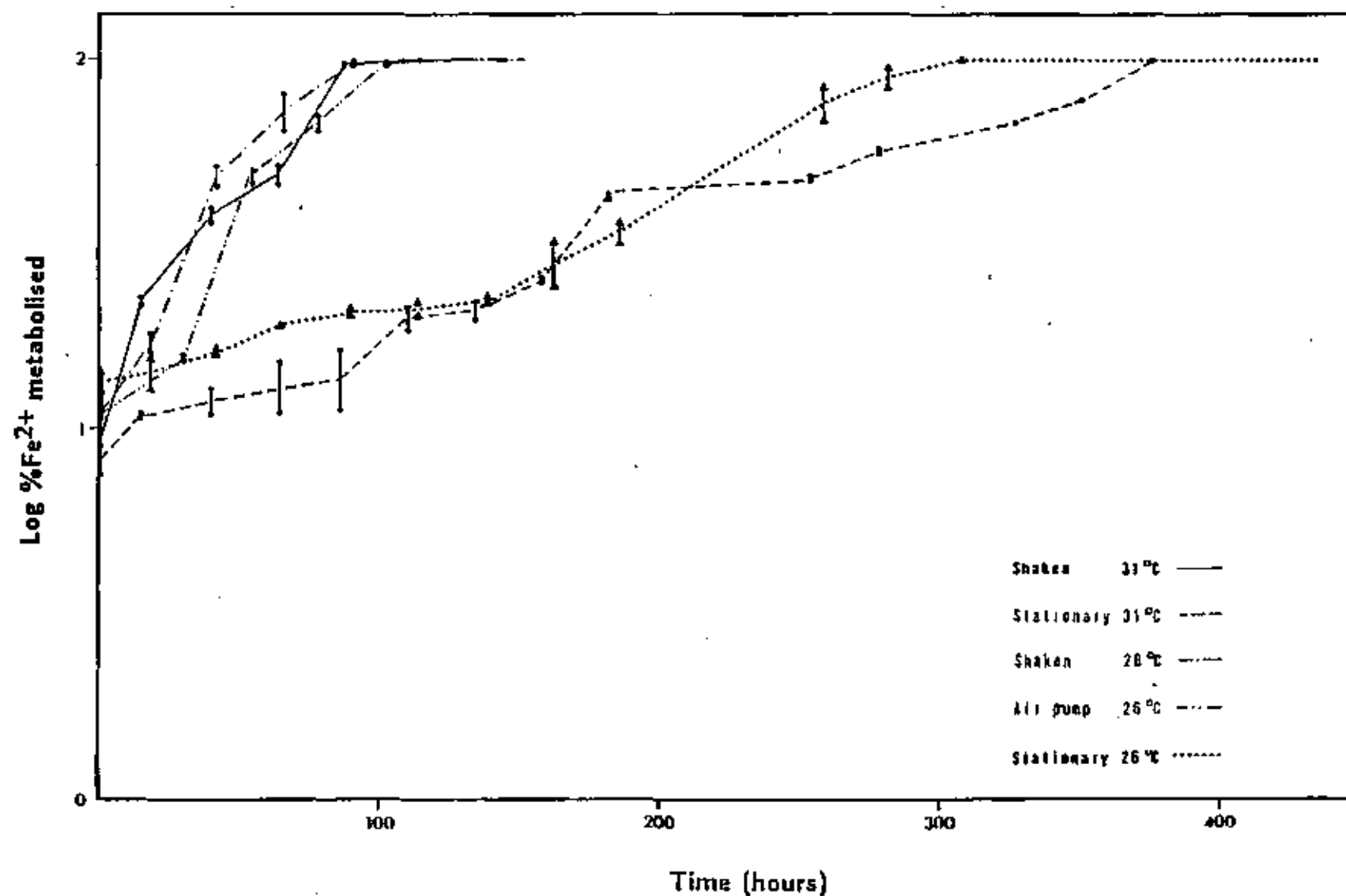


FIG. 3. Effect of aeration procedures and two temperatures on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. Vertical bars indicate Fe²⁺ metabolism in duplicate flasks; graph lines connect the means.

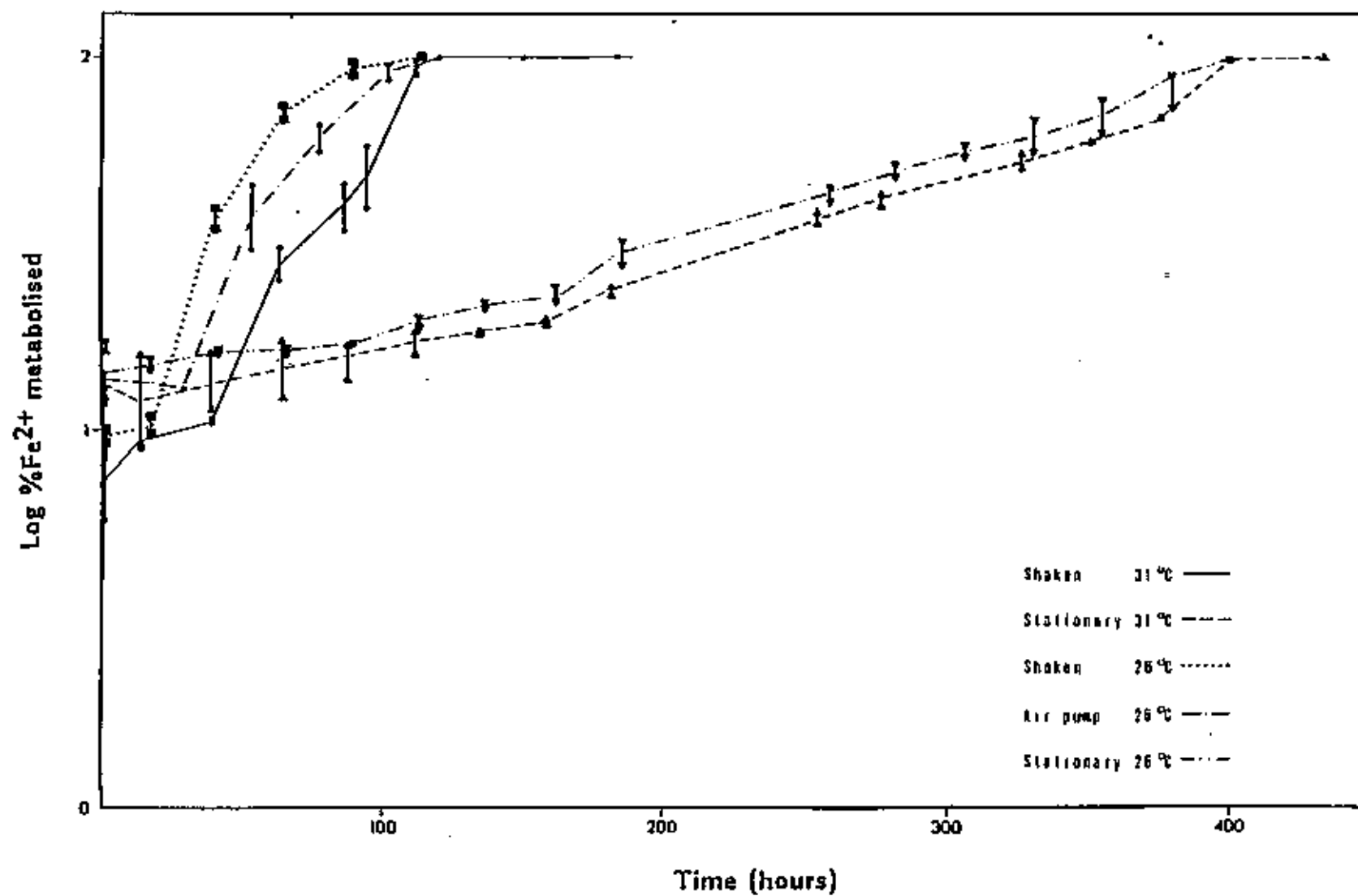


FIG. 4. Effect of aeration procedures and two temperatures on the growth of *T. ferrooxidans* WLR in HJJ medium.

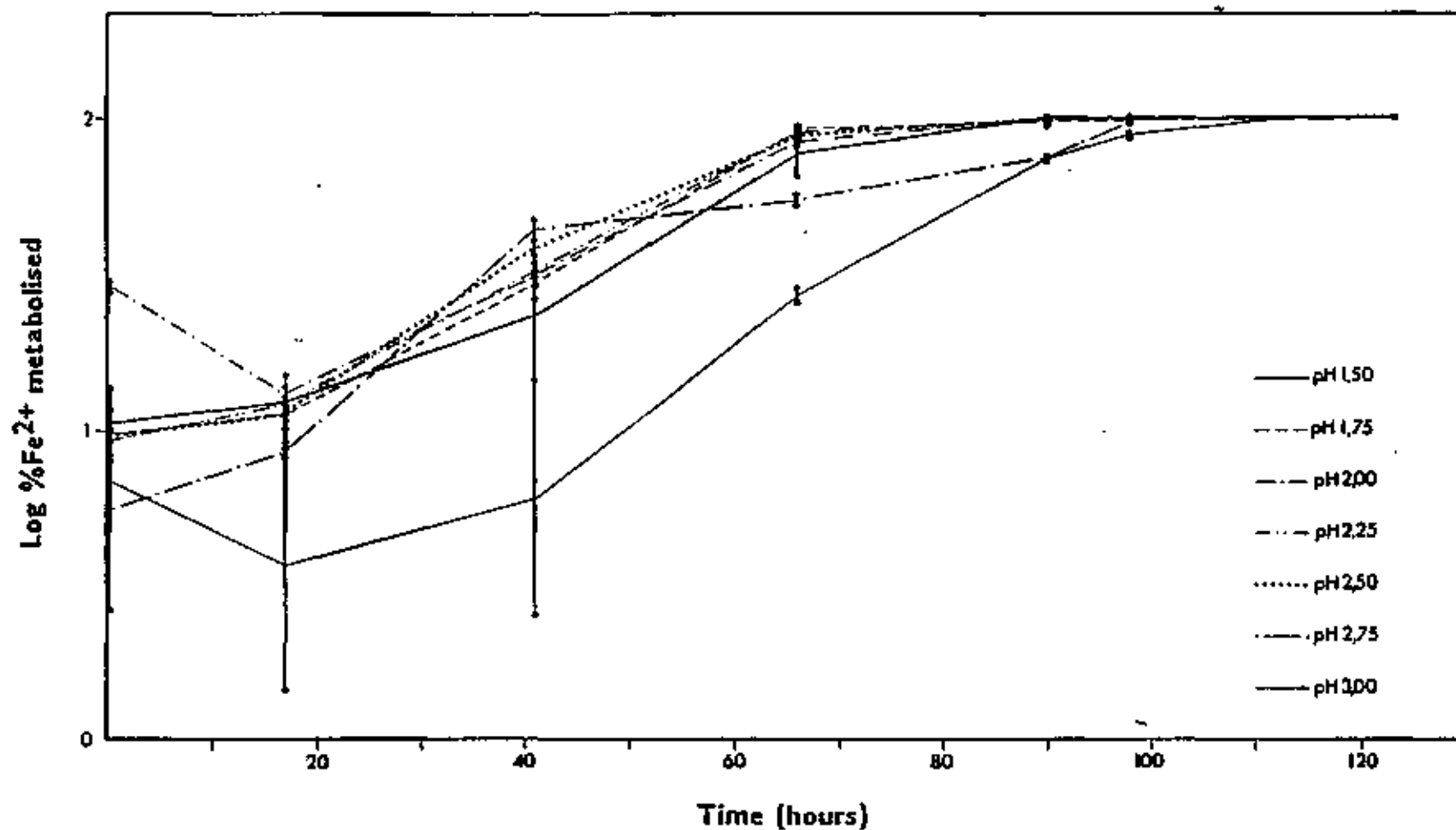


FIG. 5. Effect of pH on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium at 31°C with rotary shaking at 150 rpm.

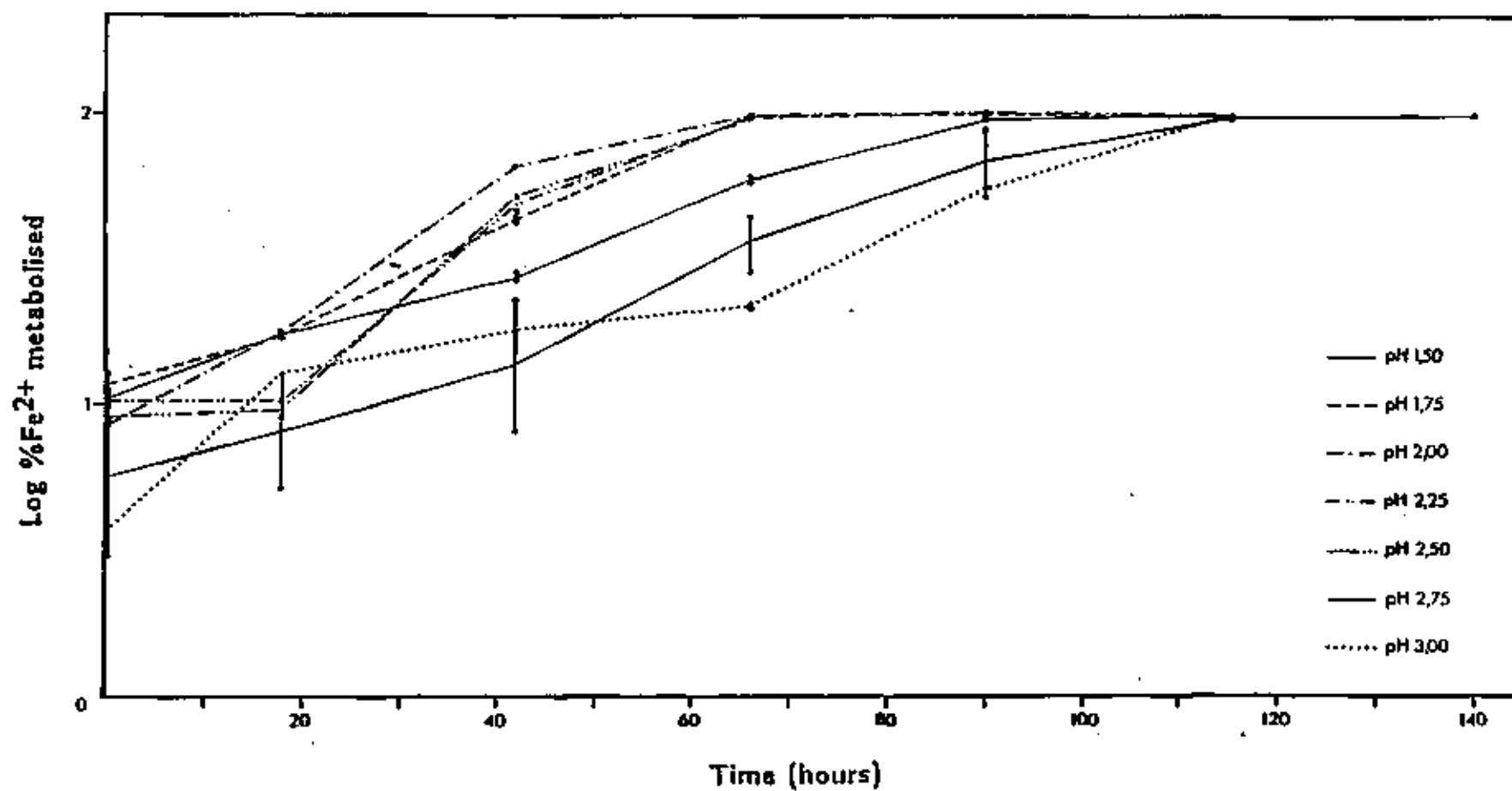


FIG. 6. Effect of pH on the growth of *T. ferrooxidans* WLR in HJJ medium at 31°C with rotary shaking at 150 rpm.

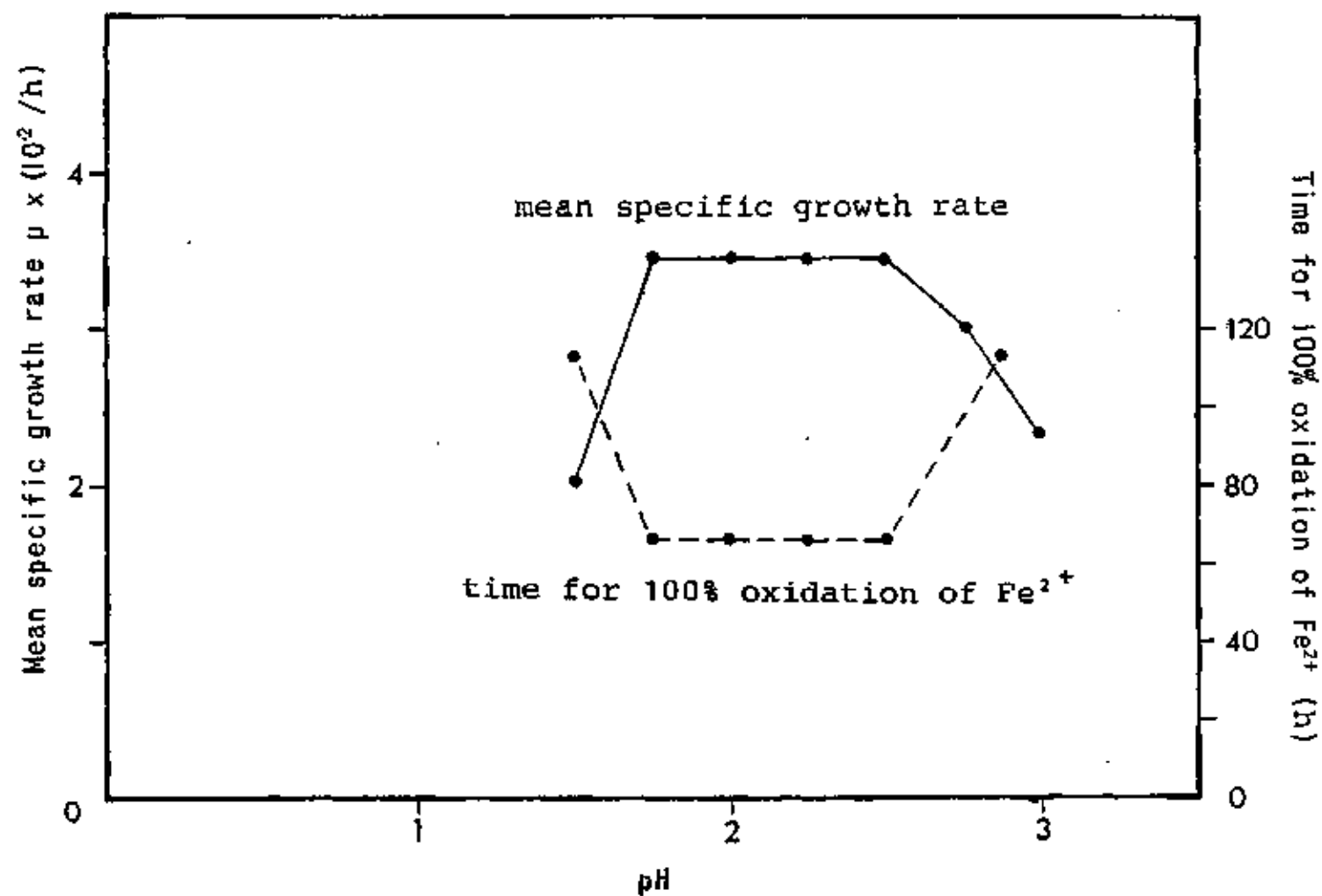


FIG. 7. Growth of *T. ferrooxidans* ATCC 19859 in HJJ medium at 31°C with rotary shaking at 150 rpm, as indicated by the mean specific growth rate in the exponential phase of growth and the time for complete oxidation of Fe^{2+} .

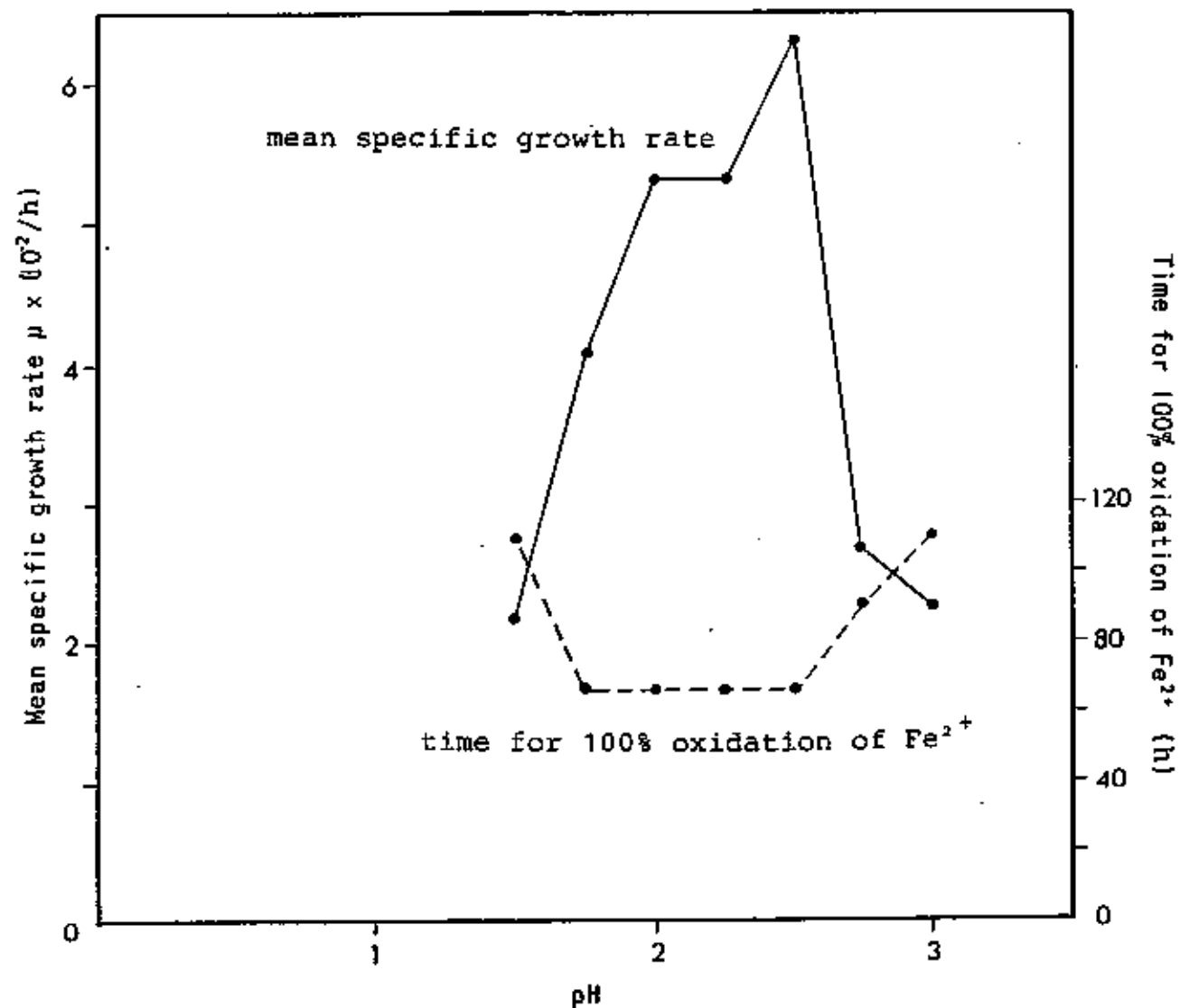


FIG. 8. Growth of *T. ferrooxidans* WLR in HJJ medium at 31°C with rotary shaking at 150 rpm, as indicated by the mean specific growth rate in the exponential phase of growth and the time for complete oxidation of Fe^{2+} .

2.4. Discussion

Figures 1 and 2 show good growth of *T. ferrooxidans* strains ATCC 19859 and WLR in HJJ medium. The composition of HJJ medium is close to that of the well-known 9K medium of Silverman and Lundgren (1959a) for *T. ferrooxidans*.

The bacteria grew much better in cultures aerated by shaking or by means of an aquarium pump than in stationary cultures (Fig. 3, 4). These observations correspond with the findings of Beck (1960), who showed that during metabolism of *T. ferrooxidans* in stationary cultures, the O_2 in solution was depleted faster than it was replaced. He therefore suggested that cultures should be aerated by shaking at a suitable speed (130 rpm). Although the cultures aerated with the aquarium pump showed a high rate of Fe^{2+} -oxidizing activity, this was not a practical method for aerating a large number of cultures under constant conditions and it was decided to shake all cultures on a rotary shaker at 120 rpm.

Growth of *T. ferrooxidans* ATCC 19859 and WLR seemed slightly better at 26°C than at 31°C under otherwise similar culture conditions, although it was good at both temperatures. Also for the practical reason of more available shaker space for cultures at 26°C, they were grown subsequently at this temperature. Likewise, Tuttle and Dugan (1976) incubated their cultures at $25 \pm 2^\circ C$ on a rotary shaker at 180 rpm.

According to Fig. 7 and 8, Fe^{2+} was oxidized to Fe^{3+} at the highest rate between pH 1,75 and 2,50. Only the WLR strain had a sharp optimum (at pH 2,50). It was decided to use media of pH 2,0 for all subsequent experimental work.

3. POPULATION SIZES AND GROWTH CHARACTERISTICS OF IRON-OXIDIZING BACTERIA IN ACID SEEPAGE FROM A GOLD MINE SAND DUMP

3.1 Introduction

The acid mine drainage seeping from sand dumps should be a good growth medium for acidophilic iron-oxidizing microorganisms, such as *T. ferrooxidans*. Acidity and high concentrations of sulphate have been demonstrated by Jones et al. (1988) and high concentrations of dissolved ferrous iron, resulting from reaction (5) in section 1, by Thompson (1980). Visual evidence for iron undergoing oxidation in the seepage water is the brown colour of the water and soil in the seepage areas.

Iron-oxidizing bacteria have been isolated from acid drainage water or high populations demonstrated by most probable number (MPN) count in the water after it has left the dumps where it was formed (Beck, 1967; Colmer and Hinkle, 1947; Dugan et al., 1970; Manning, 1975; Millar, 1973; Tuovinen and Kelly, 1973; Tuttle et al., 1968, 1969). These studies have been conducted mainly on acid drainage from coal waste dumps. The iron-oxidizing bacteria are presumed to represent bacteria involved in production of the acid drainage in the dumps. No such studies have been reported for South African gold mine dumps, although iron-oxidizing microorganisms have been counted in the upper layers of a slimes dam (Matic and Mroost, 1964) and isolated from gold dump material (for example, the *T. ferrooxidans* WLR strain supplied to us by Prof. D.E. Rawlings).

The main iron-oxidizing microorganism isolated from acid drainage water or acidifying dumps has been the acidophilic chemolithotrophic bacterium, *T. ferrooxidans* (Beck, 1969; Dugan, 1975; Dugan and Lundgren 1964; Lundgren and Silver, 1980; Lundgren et al., 1972; Walsh, 1978). However, other iron-oxidizing bacteria have been found in ores or acid drainage waters, for example, *Metalligenium* sp. (Walsh, 1978; Walsh and Mitchell, 1972) and *Leptospirillum ferrooxidans* (Balashova et al., 1974). To what extent iron-oxidizing bacteria other than *T. ferrooxidans* have a role in the formation of acid mine drainage is uncertain (Kleinmann and Crerar, 1979).

As a first step in our field investigations, we counted populations of iron-oxidizing bacteria, capable of growth in HJJ medium, in acid soil and water samples from a seepage area at the base of a sand dump. These determinations also served as a test of MPN procedures for environmental populations of these organisms. We were also

interested in the growth rates of the sampled populations of iron-oxidizing organisms in HJJ medium, and studied this aspect using enrichment cultures developed from iron-oxidizing MPN cultures. These cultures were also used later in tests of the effect of inhibitors on iron-oxidizing bacteria (sections 5 and 6). In addition, we investigated the development of iron-oxidizing cultures in HJJ medium containing, as inoculum, coarse and fine fractions of acid seepage soils or the fine fraction only.

3.2. Materials and Methods

3.2.1. Soil and water samples

Four soil (G1 to 4) and three acid drainage water (H1 to 3) samples were obtained on 22 June 1983 from a seepage area at the base of sand dump no. 3A19 on the Nourse Mine, on the eastern side of Johannesburg. The samples were taken at the southwestern corner of the mine dump, inside the toe-dam. The soil samples appeared to be the soil on which the dump had been deposited, although sand and other material washed from the dump could have been present. These samples were saturated with or moistened by the acid drainage water. All samples were collected in sterile, dark-brown, screw-cap bottles and stored at room temperature until used for MPN counts of iron-oxidizing bacteria (after 40 and 75 days) and growth studies (after 132 days).

The pH of each soil and water sample was determined using a Beckman pHTM43 pH meter with combination electrode 39820. Soil (10 g) was suspended in duplicate in 25 ml distilled water for the pH determinations.

Moisture content of the soil samples was determined by drying 50 g soil in duplicate for 5 days at 100°C and cooling it in a desiccator before weighing.

3.2.2. Most probable number estimates of iron-oxidizing bacteria

For the 10^{-1} dilutions for MPN counts, 10 g soil or 10 ml water (40 days after sampling) were suspended in 90 ml HJJ medium, lacking FeSO_4 . Tenfold dilutions to 10^{-9} were prepared in the same medium. From each dilution, 1.0-ml aliquots were inoculated into three 250-ml flasks, each containing 99 ml complete HJJ medium. The flasks were incubated as stationary cultures for 44 days at 26°C. Ferrous oxidation was indicated by development of a deep orange-brown colour. The positive iron-oxidizing and negative flasks were recorded, and iron-oxidizing bacterial populations calculated from the relevant MPN table of De Man (1983).

According to Cochran (1950), five tubes or flasks per dilution give greater accuracy than three. Consequently, 75 days after sampling, additional dilution series of the same soil and water samples were prepared as described above. One-ml aliquots of each dilution were mixed with 9 ml HJJ medium in five 15 x 200-mm test tubes. The test tubes were incubated at an angle of 20° from the horizontal at 26°C. After 57 days the positive iron-oxidizing and negative tubes were recorded, and iron-oxidizing bacterial populations calculated from the MPN table of De Man (1983).

3.2.3. Growth of iron-oxidizing bacteria in enrichment subcultures from MPN tubes

Enrichment subcultures were prepared from Fe^{2+} -oxidizing tubes at certain dilutions showing positive reactions in the MPN studies. Contents of tubes at the same dilution were pooled to produce inoculum. These tubes were from the samples and dilutions H1, 10^{-1} and 10^{-7} ; H2, 10^{-1} and 10^{-6} ; H3, 10^{-1} and 10^{-6} ; G1, 10^{-1} and 10^{-5} ; G2, 10^{-1} and 10^{-6} ; G3, 10^{-1} and 10^{-6} ; G4, 10^{-1} and 10^{-6} . Duplicate flasks containing 100-ml volumes of HJJ medium at pH 2.00 were each inoculated with 10 ml of pooled inoculum from MPN tubes and incubated with shaking at 120 rpm at 26°C. Growth of the iron-oxidizing bacteria was followed by titrations of Fe^{2+} with $\text{K}_2\text{Cr}_2\text{O}_7$ as described in section 2.2.4. After complete oxidation of the Fe^{2+} , further successive subcultures (second and third subcultures) were made in fresh HJJ medium.

3.2.4. Growth of iron-oxidizing bacteria in HJJ medium containing coarse plus fine or fine fractions of seepage soil as inocula

Soils G1 to G4 (20 g) were suspended separately in duplicate in 200 ml of HJJ medium in 250-ml Erlenmeyer flasks. The flasks were left at room temperature for approximately 1 h to permit settling of the larger soil particles, when 100 ml of the supernatant, containing the finer soil particles, were decanted into a sterile 250-ml Erlenmeyer flask. Both sets of flasks were incubated at 26°C as stationary cultures. The growth of iron-oxidizing bacteria in the cultures was followed by daily titration of Fe^{2+} with $\text{K}_2\text{Cr}_2\text{O}_7$.

3.3. Results

3.3.1. pH and moisture content of soil and water samples

The pH values of the soil and water samples from the seepage at the base of the Nourse Mine sand dump 3A19 are shown in Table 2. The pH of soil samples G1 to G4 differed very little from their mean of pH 2.60. The pH of the water samples (mean pH 2.26) was somewhat lower than that of the soil samples.

3.3.2. Most probable number estimates of iron-oxidizing bacteria

The MPN estimates of iron-oxidizing bacteria in the four soil and three water samples are presented in Table 3. The categorization of the MPN counts indicates more reliable counts from three flasks than from five tubes per dilution although the 95% confidence limits of the latter were narrower. Counts for all samples were high (1.3×10^4 to 1.7×10^7 /g or ml), with most water counts/ml higher than most soil counts/g.

3.3.3. Growth of iron-oxidizing bacteria in enrichment subcultures from MPN tubes

The growth of iron-oxidizing bacteria in enrichment subcultures from MPN tubes inoculated with dilutions of mine dump soil and water samples is shown in Fig. 9 to 15. Times for total Fe^{2+} oxidation in the subcultures and doubling times of the iron-oxidizing bacteria in the third subcultures are given in Table 4. The times for complete Fe^{2+} oxidation by the first G1, G2 and G4 subcultures were between 64 and 191 h, while for G3 it was from 472 to 500 h. These times decreased to between 64 and 93 h for the third subcultures, except G3 10^{-6} for which it decreased to 225 h. The doubling times for the iron-oxidizing bacteria in the exponential phase of the third soil subcultures were between 13 and 22 h, except in the case of G3 10^{-6} for which it was 50 h.

The times for complete Fe^{2+} oxidation in the first subcultures from the water MPN tubes, with the exception of the H2 10^{-6} subculture, were between 184 and 328 h, and the times for the third subcultures were between 69 and 188 h. The doubling times for the iron-oxidizing bacteria in the third subcultures were between 11 and 26 h. The time for complete Fe^{2+} oxidation in the first subculture of H2 10^{-6} was 472 h and in the second subculture 309 h; the doubling time for the iron-oxidizing bacteria in this subculture was 45 h. A third subculture of H2 10^{-6} was not grown.

TABLE 2. pH values and moisture contents of soil and water samples from seepage at the base of the Nourse Mine sand dump 3A19

Sample		pH	Moisture content ^a
Soil	G1	2,61 ^b	15,17
	G2	2,56 ^b	14,05
	G3	2,56 ^b	10,00
	G4	2,65 ^b	29,10
Water	H1	2,20	
	H2	2,21	
	H3	2,37	

^a g/100 g wet soil at time of first MPN determination of iron-oxidizing bacteria.

^b pH of 1:2,5 soil:water suspension.

TABLE 3. MPN estimates of iron-oxidizing bacteria population in four soil and three water samples from seepage at the base of the Nourse Mine sand dump 3A19

MPN method (flasks or tubes per dilution) and sample		Iron-oxidizing bacteria per g (dry mass) soil or per ml water from tables of de Man (1983)			
		MPN	Category	Confidence limits ($\geq 95\%$)	
<u>3 flasks</u>					
Soil	G1	$2,4 \times 10^4$	1	$0,4 \times 10^4$	$9,9 \times 10^4$
	G2	$4,3 \times 10^5$	1	$0,9 \times 10^5$	$18,1 \times 10^5$
	G3	$9,3 \times 10^5$	1	$1,8 \times 10^5$	$36,0 \times 10^5$
	G4	$2,4 \times 10^5$	1	$0,4 \times 10^5$	$9,9 \times 10^5$
<u>5 tubes</u>					
Soil	G1	$5,8 \times 10^5$	0	$2,1 \times 10^5$	$14,9 \times 10^5$
	G2	$1,3 \times 10^4$	3	$0,6 \times 10^4$	$3,4 \times 10^4$
	G3	$2,2 \times 10^3$	1	$0,7 \times 10^3$	$4,4 \times 10^3$
	G4	$1,7 \times 10^6$	3	$0,6 \times 10^6$	$3,4 \times 10^6$
<u>3 flasks</u>					
Water	H1	$2,4 \times 10^6$	1	$0,4 \times 10^6$	$9,9 \times 10^6$
	H2	$9,3 \times 10^5$	1	$1,8 \times 10^5$	$36,0 \times 10^5$
	H3	$2,4 \times 10^6$	1	$0,4 \times 10^6$	$9,9 \times 10^6$
<u>5 tubes</u>					
Water	H1	$1,7 \times 10^7$	3	$0,6 \times 10^7$	$3,4 \times 10^7$
	H2	$3,4 \times 10^6$	2	$1,3 \times 10^6$	$10,0 \times 10^6$
	H3	$1,3 \times 10^6$	3	$0,6 \times 10^6$	$3,4 \times 10^6$

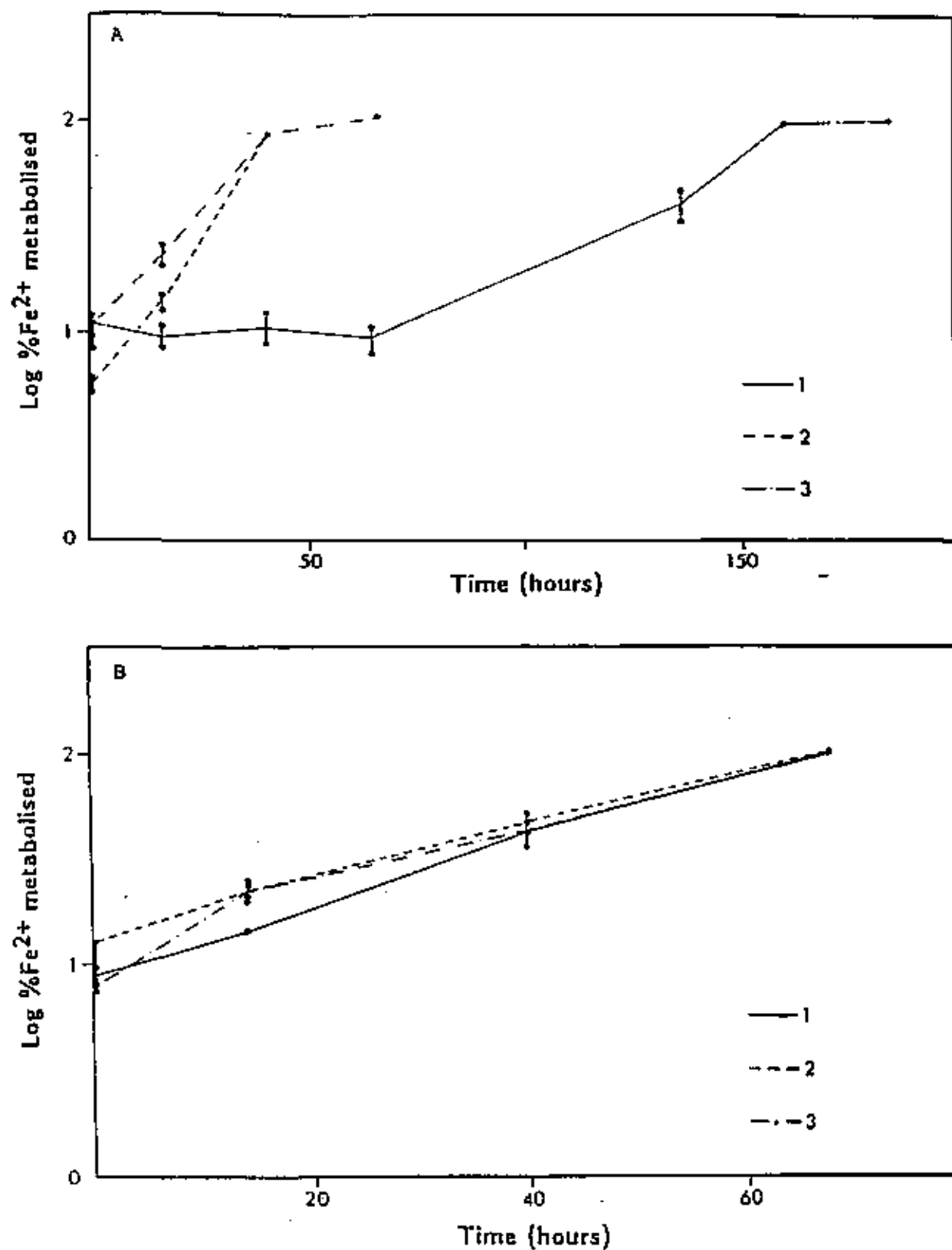


FIG. 9. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of mining dump soil G1. A, subcultures from 10^{-1} dilution; B, subcultures from 10^{-5} dilution.

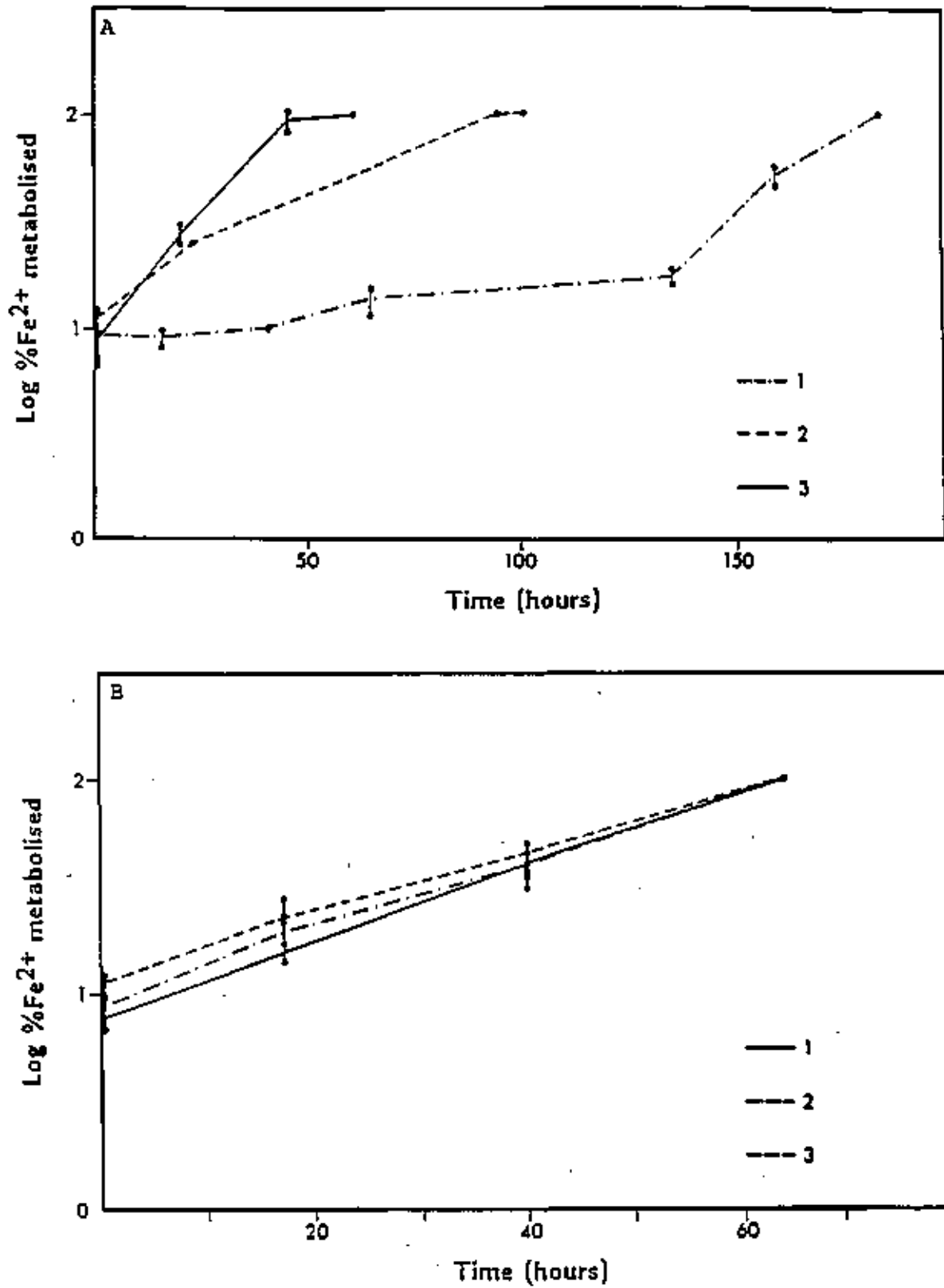


FIG. 10. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of mining dump soil G2. A, subcultures from 10^{-1} dilution; B, subcultures from 10^{-6} dilution.

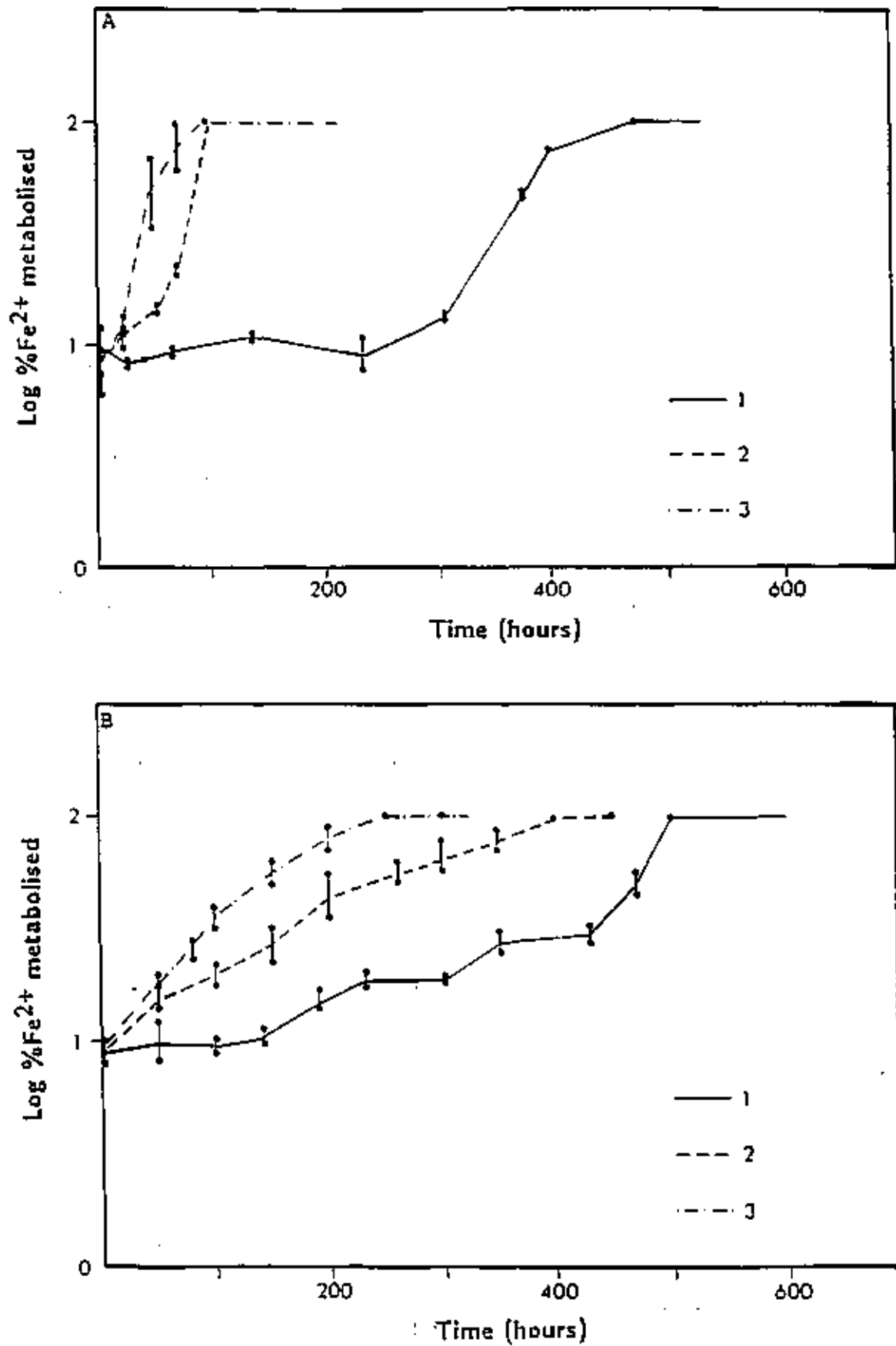


FIG. 11. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of mine dump soil G3. A, subcultures from 10^{-1} dilution; B, subcultures from 10^{-6} dilution.

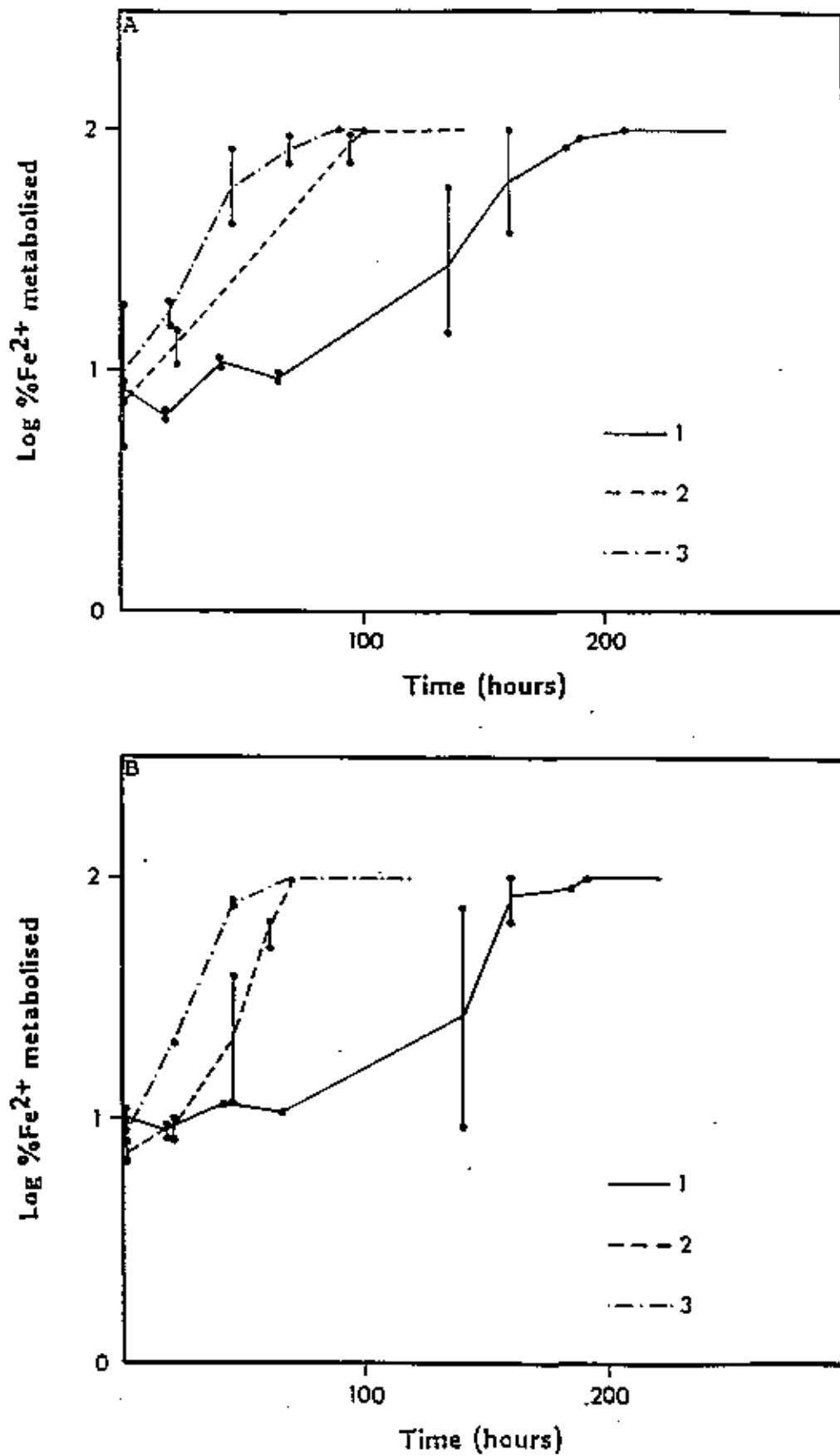


FIG. 12. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of mine dump soil G4. A, subcultures from 10⁻¹ dilution; B, subcultures from 10⁻⁶ dilution.

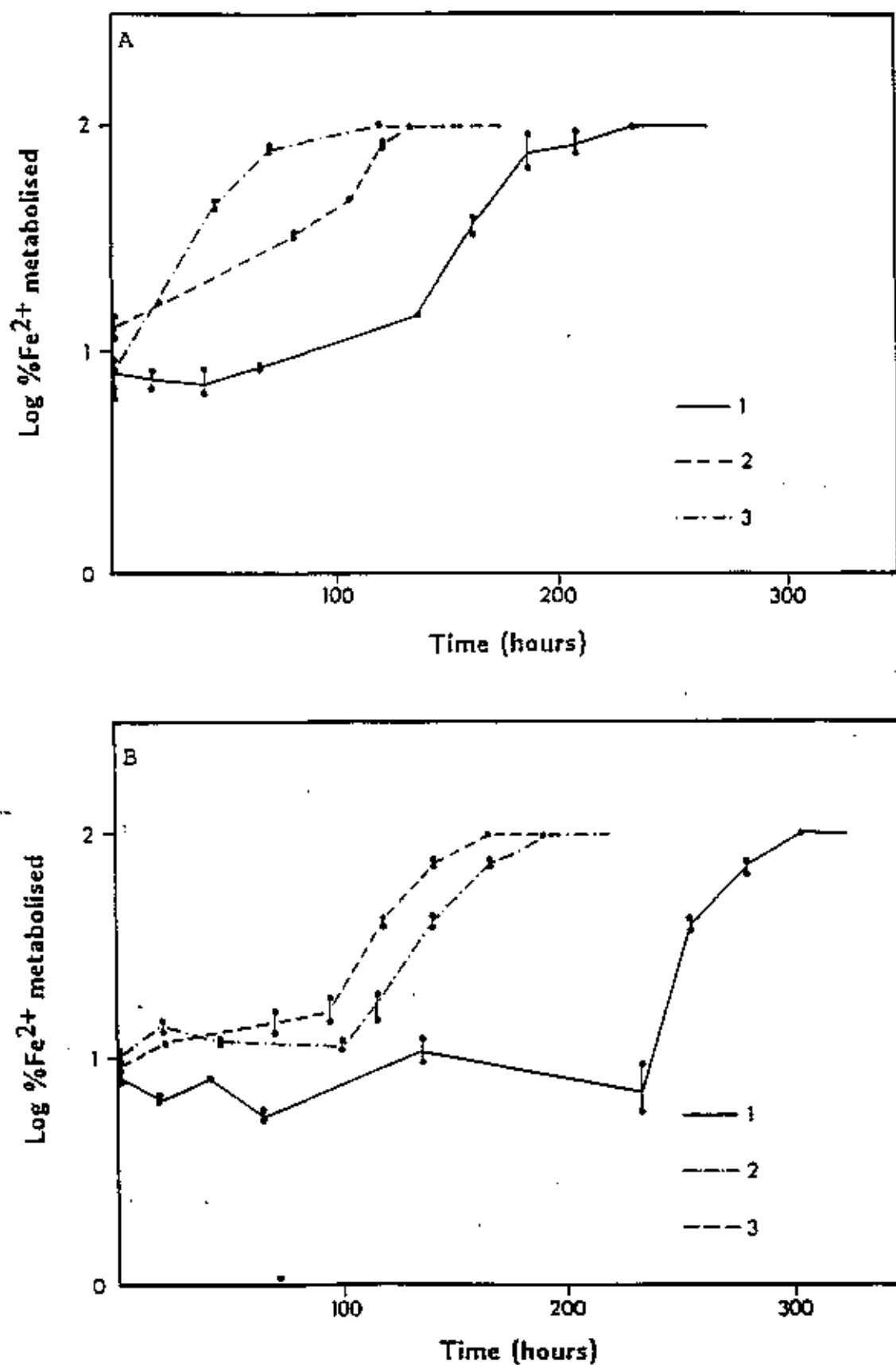


FIG. 13. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of acid mine drainage water H1. A, subcultures from 10^{-1} dilution; B, subcultures from 10^{-7} dilution.

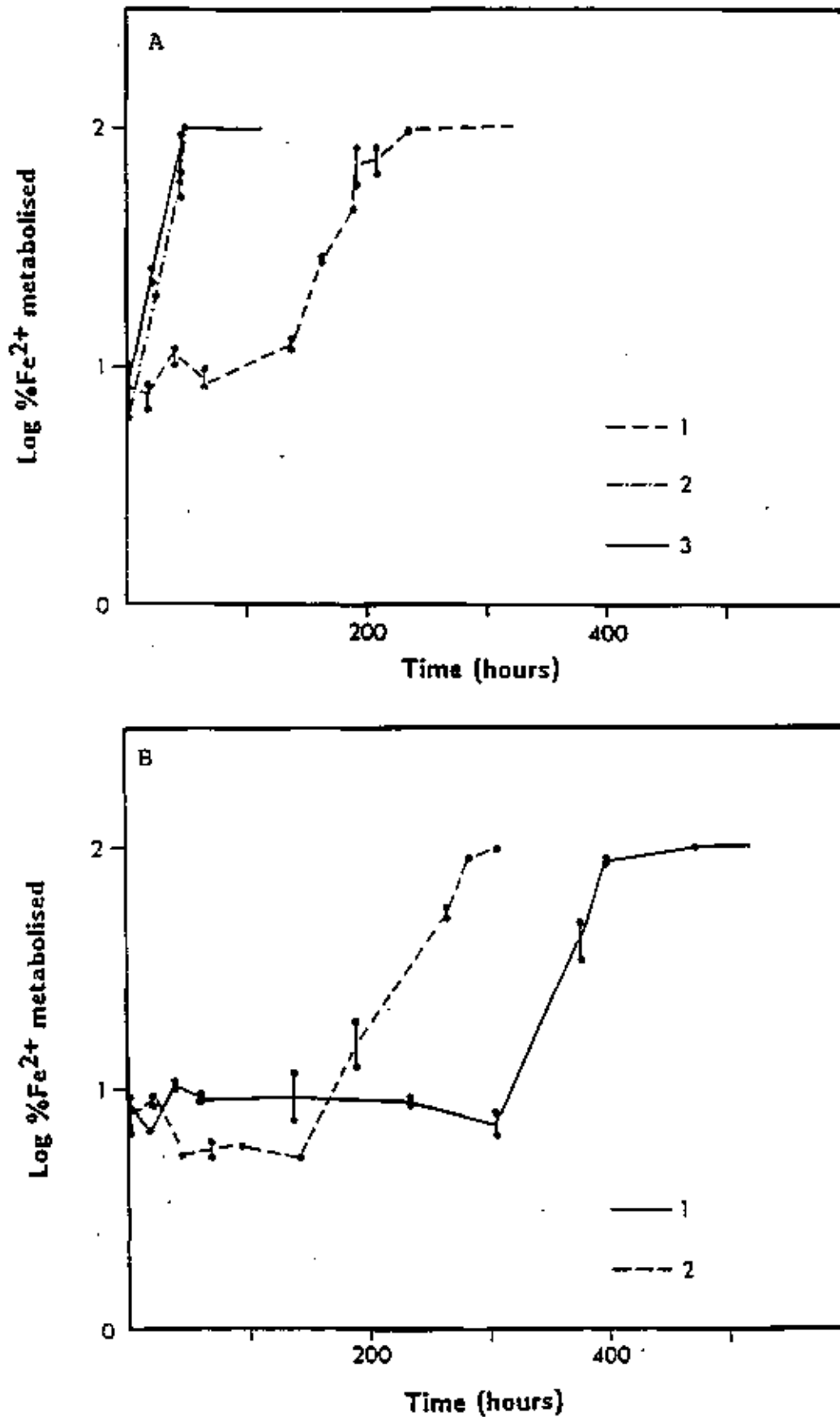


FIG. 14. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of acid mine drainage water H2. A, subcultures from 10^{-1} dilution; B, subcultures from 10^{-6} dilution.

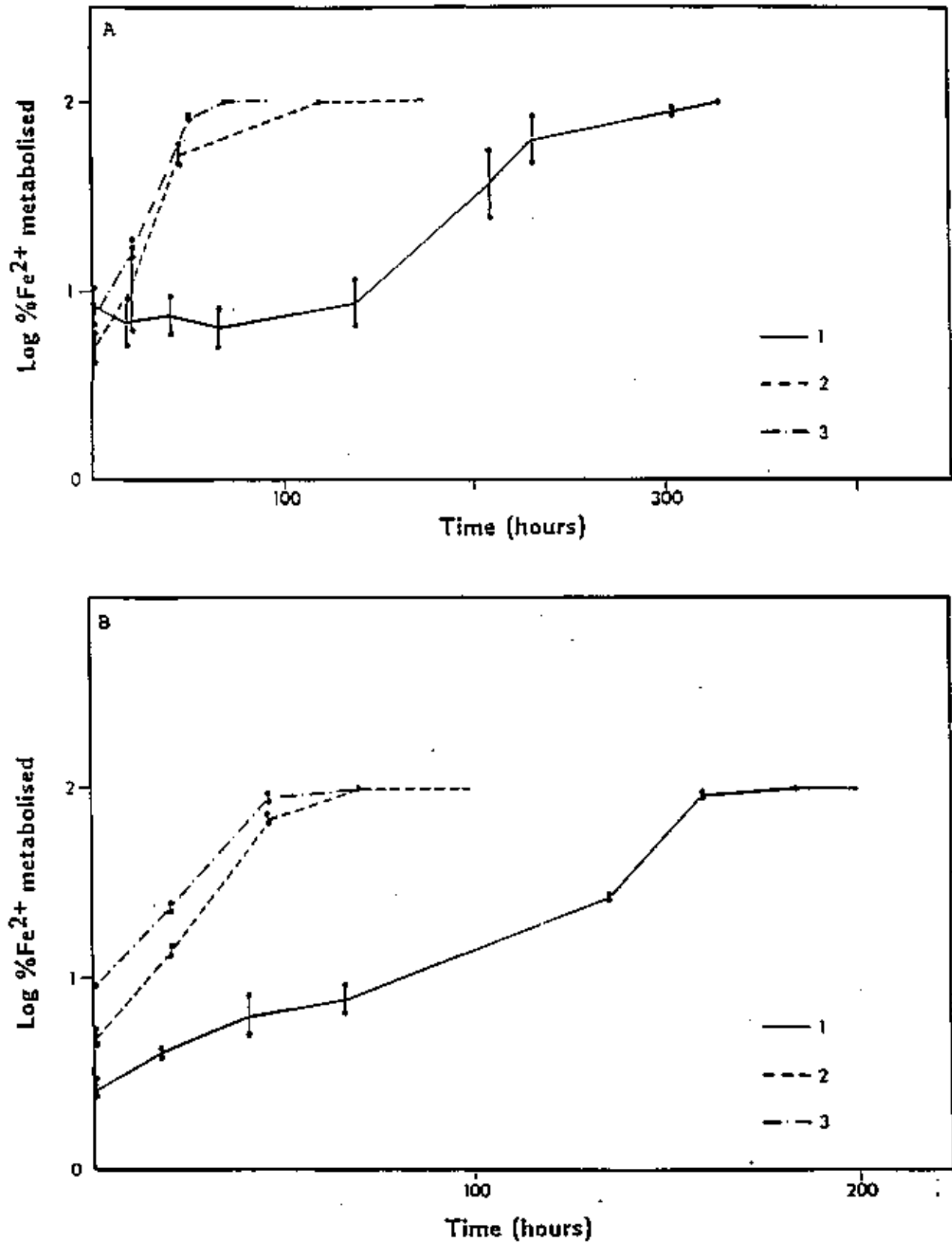


FIG. 15. Growth of iron-oxidizing bacteria in successive enrichment subcultures (1, 2 and 3) from MPN tubes inoculated with dilutions of acid mine drainage water H3. A, subcultures from 10⁻¹ dilution; B, subcultures from 10⁻⁶ dilution.

TABLE 4. Times for total Fe^{2+} oxidation by the first, second and third subcultures and doubling times during the exponential phase of the third subcultures of the iron-oxidizing bacteria from MPN tubes

Origin of cultures (sample and MPN dilution)		Time (h) for complete Fe^{2+} oxidation by subculture			Doubling time of Fe^{2+} -oxidizing bacteria in exponential phase of subculture 3 (h)
		1	2	3	
Soil	G1 10^{-1}	184	69	69	13
	G1 10^{-5}	64	64	64	22
	G2 10^{-1}	184	100	69	13
	G2 10^{-6}	64	64	64	21
	G3 10^{-1}	472	93	93	11
	G3 10^{-6}	500	400	225	50
	G4 10^{-1}	160-208	100	90	15
	G4 10^{-6}	160-191	69	69	13
Water	H1 10^{-1}	232	132	117	18
	H1 10^{-7}	304	165	188	10
	H2 10^{-1}	232	48	48	13
	H2 10^{-6}	472	309	-	45 ^a
	H3 10^{-1}	328	117	69	11
	H3 10^{-6}	184	69	69	26

^aDoubling time of subculture 2.

3.3.4. Growth of iron-oxidizing bacteria in HJJ medium containing coarse plus fine or fine fractions of mine dump soil as inocula

The coarse plus fine fractions of the soil inocula had a negligible or small stimulatory effect on the growth of iron-oxidizing bacteria in HJJ medium compared with their growth in this medium containing the fine soil fraction only (Fig. 16). The flask with only the fine fraction of soil G1 took 48 h longer to show complete Fe^{2+} oxidation than the flask containing coarse and fine fractions of this soil as inoculum. The difference in the case of the two fractions of soil G2 was 58 h. In the case of the G3 and G4 soils, there was no difference in the time for complete Fe^{2+} oxidation between the two treatments. The times for complete Fe^{2+} oxidation by the coarse plus fine fractions of soils G1, G2, G3 and G4 were 90, 90, 114 and 66 h, respectively.

3.4. Discussion

Large populations of iron-oxidizing bacteria were detected in the soil and acid drainage water samples from the seep at the southwestern corner of Nourse Mine dump 3A19; the soil of these samples (G1 to G4) represented the soil on which the mine dump had been deposited. This abundance of iron-oxidizing bacteria in all the highly acid soil and water samples (pH 2.20 to 2.65) was expected from the studies of Beck (1967), Colmer and Hinkle (1947), Dugan et al. (1970), Manning (1975), Millar (1973), Tuovinen and Kelly (1973) and Tuttle et al. (1968, 1969), showing iron-oxidizing bacteria in the highly acid drainage water from coal and copper mines. The bacteria were probably growing on Fe^{2+} released in high concentration in the seepage water during the oxidation of pyrite in the dump by bacterially produced Fe^{3+} (Kleinmann et al., 1981). The iron-oxidizing bacterial populations in 1 ml soil solution in soil samples G1 to 4 based on the MPN determinations with three flasks per dilution and the moisture associated with 1 g (dry weight) of soil, were 1.34×10^5 , 2.40×10^6 , 5.20×10^6 and 1.34×10^6 , respectively. They therefore did not differ much from the MPN values for the three acid drainage water samples (H1 to 3). Although the MPN results from the determinations with three flasks per dilution were in general in more reliable categories (de Man, 1983) than those determined with five tubes per dilution, the 95% confidence limits of the latter were narrower. Nevertheless, we decided to use three flasks per dilution in future MPN determinations (sections 9 and 11). The two sets of MPN counts for individual samples usually showed differences, but these could possibly have arisen from the longer storage of the samples used for the MPN counts with the test-tubes.

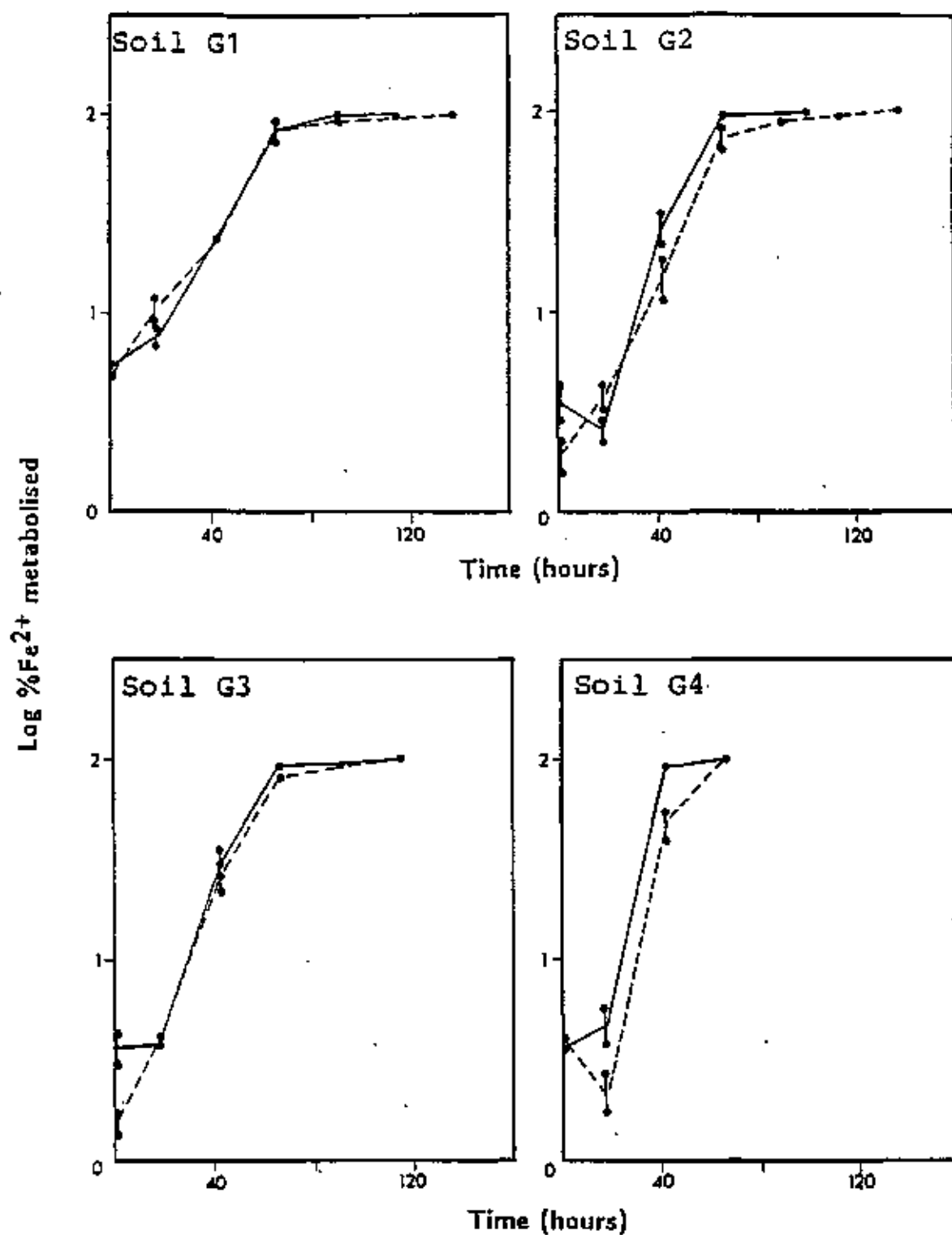


FIG. 16. Growth of iron-oxidizing bacteria in HJJ medium inoculated with coarse plus fine (—) or fine (-----) fractions of mine dump soils G1 to 4.

The growth of iron-oxidizing bacteria in enrichment subcultures from MPN tubes inoculated with dilutions of mine dump soil and water samples indicated the presence of different strains (Table 4). In the third subcultures of samples G1, 2 and 4 the times for total Fe^{2+} oxidation were more or less the same (64 to 93 h), while for the G3 10^{-6} third subculture the time was 225 h. The doubling time for this subculture was 50 h, whereas the times for the strains in samples G1, 2 and 4 were between 11 and 22 h. The doubling times for the iron-oxidizing bacteria in the water samples (H1 to 3) did not differ as much as those for the strains in the soil samples and were between 10 and 26 h. The strains in the water samples grew almost at the same rate. Silverman and Lundgren (1959a) reported doubling times of *T. ferrooxidans* strain TM in aerated cultures, using the 9K medium, of 5.3 to 9.6 h. McGoran et al. (1969) showed that *T. ferrooxidans* NCIB 9490 had a minimum doubling time of 6.5 to 10 h on 9K medium, 7 to 9 days on a sulphur substrate and 14 to 17 h on a chalcopyrite substrate.

The G1 to G4 soil inocula containing coarse plus fine soil had a negligible or small stimulatory effect on the growth of their iron-oxidizing bacteria in HJJ medium compared with the corresponding growth in the HJJ medium inoculated with the fine soil fraction only (Fig. 16). Duncan et al. (1964), Razzell and Trussell (1963a), Silverman et al. (1961), Ehrlich and Fox (1967), and Lundgren and Silver (1980) all studied the influence of particle size on the oxidation of pyrite and leaching of metals and found greater oxidation and extraction rates when the specific surface area of the ore was increased by reducing the particle size. Such oxidation of particulate ore material was not involved in the present study with soils G1 to 4, which were base soil below the mine dump material, but particulate soil materials such as montmorillonite clays may have various profound effects on microbial activities (Stotsky, 1974). In contrast to soils G1 to 4, the presence of mine dump sand G5 in *T. ferrooxidans* cultures in HJJ medium had a marked retarding effect on the bacterial oxidation of the ferrous iron (see section 4).

4. EFFECT OF MINE DUMP SAND ON GROWTH OF *T. FERROOXIDANS* IN HJJ MEDIUM

4.1 Introduction

When the first studies of the effects of potential chemical inhibitors on *T. ferrooxidans* in HJJ medium containing 500 g/l of mine dump sand (G5) were conducted (sections 5 to 8), it became evident that growth of the *T. ferrooxidans* test strains, as indicated by Fe^{2+} oxidation, in medium without inhibitor was slow by comparison with growth in similar cultures without sand. A minor study of the effect of increasing concentrations of the sand on the growth of the ATCC 19859 test strain in HJJ medium was therefore undertaken.

4.2. Materials and Methods

Growth of *T. ferrooxidans* ATCC 19859 was studied in a duplicate series of 100-ml cultures in HJJ medium supplemented with 0, 1, 5, 10, 20, 40, 60, 80 and 100 g air-dried mine dump sand. The sand, designated G5, was a yellow wind-blown sand from a mine dump vegetation research plot on the eastern side of the Nourse Mine 3A19 sand dump. The pH of a 1:2.5 suspension of the sand in water was pH 4.05. The G5 sand contributed negligible ferrous iron-oxidising microorganisms as shown by the failure of two flasks of uninoculated HJJ medium incubated with 100 g sand to oxidise ferrous iron faster than uninoculated medium without sand. Inoculum, added at 10% (v/v), was a 3- to 4-day ATCC 19859 culture grown in HJJ medium at 26°C with shaking at 120 rpm. The experimental cultures with the sand were also incubated under these conditions, sampled periodically and residual Fe^{2+} monitored by the $\text{K}_2\text{Cr}_2\text{O}_7$ titration (section 2.2.4).

4.3. Results

Figure 17 shows the retarding effect of the G5 mine dump sand on the rate of growth of *T. ferrooxidans* ATCC 19859 as indicated by Fe^{2+} oxidation. The 50 g/l sand concentration caused only a slight decrease in the growth rate. At 600 g/l, the sand resulted in an initial lag in growth as well as a retarded growth rate. Concentrations of 800 and 1000 g/l sand caused similar retardation to that caused by 600 g/l sand.

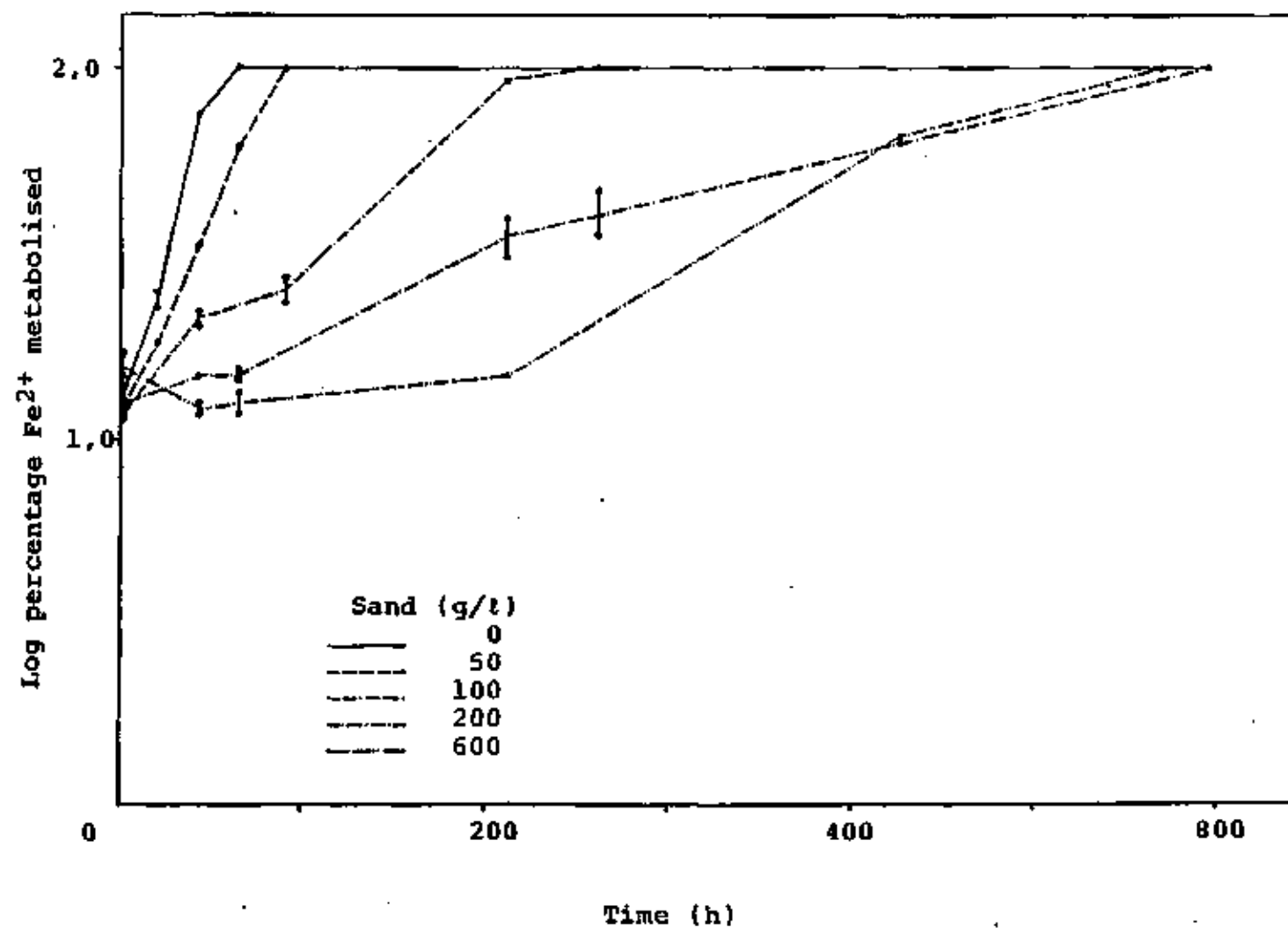


FIG. 17. Effect of mine dump sand G5 on growth of *T. ferrooxidans* ATCC 19859 in HJJ medium at 26°C with shaking at 120 rpm.

4.4 Discussion

The addition of mine dump sand to *T. ferrooxidans* ATCC 19859 cultures in HJJ medium markedly impeded oxidation of the FeSO_4 substrate. Although this implies a retarding effect on bacterial growth, oxidation of Fe^{2+} in FeSO_4 medium is not necessarily indicative of cell growth (Mahapatra and Mishra, 1984) and it may be more accurate to substitute the word 'activity' for 'growth'. It is possible that the sand slowed bacterial activity as a result of adsorption phenomena involving nutrients, the bacterial cells and the sand particles. Ivarson et al. (1980) suggested that cation adsorption to clay decreased Fe^{2+} oxidation by reducing the availability of the cations. On the other hand, chemicals that could affect the cultures adversely may have been introduced into the culture solution from the sand (Livesey-Goldblatt et al., 1983). Whatever the mechanism, these laboratory results suggest that both iron oxidation and growth of *T. ferrooxidans* in gold mine sand dumps may be slow, even slower than in the culture flasks with 100 g sand/100 ml medium. It should be noted that the G5 sand was in a highly oxidized state with little residual pyrite sulphur (section 10).

5. INHIBITION OF *T. FERROOXIDANS* AND IRON-OXIDIZING BACTERIA FROM MINE DUMP SEEPAGE BY SYNTHETIC CHEMICALS.

A. ANIONIC SURFACE-ACTIVE AGENTS

5.1 Introduction

Low concentrations of the following two anionic detergents were observed by Dugan and Lundgren (1964) and Dugan (1975) to be highly inhibitory to *T. ferrooxidans*:

Sodium lauryl sulphate $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3^-\text{Na}^+$
(SLS, sodium dodecyl sulphate)

Alkylbenzenesulphonate $\left[\begin{array}{c} \text{R} \\ | \\ \text{C}_6\text{H}_4 \\ | \\ \text{SO}_3^- \end{array} \right] \text{X}^+$
(ABS)

where R is a branched hydrocarbon (polypropylene) chain and X^+ a cation.

In addition, Dugan and Apel (1983) mentioned that linear alkylbenzenesulphonate had considerable potential as an inhibitor of *T. ferrooxidans*:

Linear alkylbenzenesulphonate $\left[\begin{array}{c} \text{R}' \\ | \\ \text{C}_6\text{H}_4 \\ | \\ \text{SO}_3^- \end{array} \right] \text{X}^+$
(LAS)

Where R' is a linear hydrocarbon chain and X^+ a cation.

Dugan (1975) found that shaken cultures of *T. ferrooxidans* in 9K medium were inhibited by 2 mg/l SLS. Dugan (1975) and Dugan and Lundgren (1964) reported that ABS was also a very effective inhibitor, although less inhibitory than SLS in that 5 mg/l or greater, depending on the cell population, was the inhibitory concentration. According to Dugan (1975), 5 mg/l ABS effectively inhibited iron oxidation by a *T. ferrooxidans* cell suspension containing 4×10^7 cells/ml. However, if the cell concentration was doubled or quadrupled, 5 mg/l ABS caused only partial inhibition.

Kleinmann (1980) and Kleinmann and Erickson (1983) showed inhibition of soluble iron production from and acidification of high sulphur coal by *T. ferrooxidans* by treatment of the coal with 25 to 50 mg/l SLS, ABS or alpha olefin sulphonate.

The molecules of these anionic surface-active agents have a polar sulphate or sulphonate and a non-polar (alkyl) end. Normally considered as cleaners rather than bactericides, anionic detergents do have antibacterial properties at low pH. Above all, anionic detergents are economical (Kleinmann et al., 1981). As summarized by Kleinmann and Erickson (1983), the mechanism of inhibition of *T. ferrooxidans* by such low concentrations of SLS and alkylbenzenesulphonates seems to be related to alteration of the semipermeable properties of the cytoplasmic membrane and disaggregation of the cell envelope by the interaction of SLS with the lipid, lipoprotein, and lipopolysaccharide components of the cell wall. *Thiobacillus ferrooxidans* possesses a multilayered cell envelope which allows it to maintain a neutral internal pH, despite the acid environment in which it lives. The anionic surfactants induce seepage into the cell of H^+ , which slows Fe^{2+} oxidation by decreasing the activity of acid-sensitive enzymes. Higher concentrations of surfactants kill the bacteria.

The water-soluble detergent, SLS, has been used successfully by the mining industry for acid drainage prevention in coal refuse (Dugan and Apel, 1983). Concentrations of 5-20 mg/l SLS were inhibitory and 25 mg/l bactericidal in laboratory experiments (Kleinmann and Erickson, 1983). Generally about 10 times more detergent was required to kill *T. ferrooxidans* in coal refuse than in 9K medium in the absence of coal. According to Onysko et al. (1984a), it may be uneconomical at sites where extensive rainfall occurs, resulting in rapid SLS washout. An important factor reducing SLS effectiveness in coal refuse is the adsorptive capacity of the refuse, which was determined by Kleinmann and Erickson (1983) to be about 45 mg SLS/kg waste coal. However, both laboratory and pilot-scale tests indicated that SLS had the potential to slow down pyrite oxidation in coal waste by inhibiting *T. ferrooxidans* (Kleinmann, 1979; Kleinmann et al., 1981; Onysko et al., 1984a).

To delay the leaching of SLS from dumps, Kleinmann (1979) incorporated SLS in elastomere formulations to obtain a slow release of the inhibitor for extended periods of time. Vulcanized rubber proved to be the best matrix for the slow release of SLS. The best release rates were obtained with an elastomere formulation containing 34% (m/m) SLS. The effectiveness of the SLS was not affected by release from the rubber. The SLS-elastomere formulations were tested on pilot scale coal refuse dumps of ca 25 t, containing 27 kg of SLS-rubber slabs (27.2% m/m SLS) placed below the surface to minimize interference with natural infiltration (Kleinmann, 1979, 1980; Kleinmann et al., 1981). Detergent concentrations of 2.0 to 12.0 mg/l were observed in the effluent. After 10 days, acidity and iron concentrations in the effluent from the treated piles were 80-fold lower than those in effluent from the control piles. However, acidification was only delayed, as after 5 months only a 3-fold difference in the acidity and iron values between the effluents from treated and control piles was seen. The treatment was thus

not totally effective, but showed that higher SLS concentrations were necessary for complete inhibition. This might be achieved by the use of SLS-rubber pellets for more rapid release of the SLS (Kleinmann et al., 1981).

In further field trials, Kleinmann et al. (1981) again achieved marked reductions in acidity of mine dump effluent from dumps treated with SLS-rubber slabs. For example, a 20 ha upper valley between two ridges of acid-producing overburden was treated with 90 kg of SLS-elastomere slabs of 0.3 m². The slabs were distributed in ponds along the valley, while a further 50 l of 280 g/l SLS solution was also applied there to provide an initial slug of detergent. After 7 months the acidity of the effluent drainage into the lower valley was reduced by half. The pH increased from pH 2.85 to pH 3.30. Only a low detergent concentration (0.1 mg/l SLS) was detected in the effluent. This trial proved that SLS effectively reduced acid formation in a mine dump and was released in the effluent water at sufficiently low concentrations not to pollute the area.

Full-scale field tests involving the application of SLS only in solution were conducted by Kleinmann and Erickson (1983) on an 11-acre inactive coal refuse pile in Raleigh County, West Virginia. The dump was treated during September 1981 with 10 drums (10 x 250 l) of 30% SLS, diluted 175:1. After an initial lag period, the effluent water quality improved, with a 60% decrease in acidity, sulphate and manganese, as well as a 90% decrease in iron. Acid production from a similarly treated 8-acre active coal refuse area in northern West Virginia fell approximately 95%, with an associated 95% decrease in iron concentration. Kleinmann and Erickson (1983) concluded that where the cost was justified, application of an anionic surfactant in solution, with applications at least three times a year, could be considered.

These promising results on the control of acid formation in coal dumps by the application of SLS, prompted our research into the possibility of using anionic surface-active agents (SLS or LAS) for the control of acid drainage formation in gold mine sand dumps. We gave attention to determining the concentrations needed to inhibit pure cultures of *T. ferrooxidans* or enrichment cultures of iron-oxidizing bacteria from acid sand dump seepage, before and while they were multiplying actively. The studies were conducted with cultures in HJJ medium, without and containing mine dump sand, which could be expected to influence the effectiveness of inhibition, particularly through adsorption of the inhibitor.

5.2. Materials and Methods

5.2.1. Inhibitor: Sodium lauryl sulphate (SLS)

Thiobacillus ferrooxidans ATCC 19859 and WLR (10 ml of 3-day-old shaken cultures in HJJ medium) were inoculated separately in duplicate into 90 ml HJJ medium, containing 0, 0,5, 1,0, 2,0, 4,0, 8,0, 16,0, 32,0, 64,0 and 128,0 mg/l SLS (supplied by Dr. Theodor Schuchardt, Munchen, West Germany). These media were prepared by mixing appropriate volumes of HJJ medium and a 256,0 mg/l stock solution of SLS in HJJ medium. The cultures were incubated at 26°C on a rotary shaker at 120 rpm, and Fe^{2+} metabolism monitored using the $\text{K}_2\text{Cr}_2\text{O}_7$ titration.

To investigate the effect of adding the inhibitor during culture growth, a stock solution of 1280 mg/l SLS was added to *T. ferrooxidans* ATCC 19859 and WLR cultures in HJJ medium during the exponential phase of growth (24 to 25 h) to yield, in duplicate, the same series of concentrations from 0 to 128,0 mg/l as in the previous paragraph. This was achieved by removing 20 ml of culture and adding appropriate volumes of the SLS stock solution and fresh HJJ medium. Culture conditions were otherwise unchanged.

The effect of SLS on a mixed *T. ferrooxidans* population from the Nourse Mine sand dump acid seepage was also investigated. Enrichment cultures of iron-oxidizing bacteria were prepared in 1983 from MPN tubes of the soil and water samples collected at the base of dump 3A19 (see section 3.2.1). These cultures were maintained in HJJ medium. Inoculum was prepared by mixing an equal volume of 2- to 3-day old sub-cultures of each of 12 cultures ($\text{G1 } 10^{-1}$; $\text{G1 } 10^{-5}$; $\text{G2 } 10^{-1}$; $\text{G2 } 10^{-6}$; $\text{G3 } 10^{-1}$; $\text{G4 } 10^{-1}$; $\text{G4 } 10^{-6}$; $\text{H1 } 10^{-1}$; $\text{H1 } 10^{-7}$; $\text{H2 } 10^{-1}$; $\text{H3 } 10^{-1}$; H3 unspecified dilution). Flasks of HJJ medium were inoculated with a 10% (v/v) inoculum of the mixed bacterial suspension. Sodium lauryl sulphate, at concentrations of 0, 2,0 4,0 6,0 8,0 10,0 and 20,0 mg/l, was added at the start of the experiment or during the exponential phase of culture growth. Culture conditions were as for the ATCC 19859 and WLR pure cultures.

Inhibition of *T. ferrooxidans* strains and mine dump iron-oxidizing bacteria by SLS in cultures containing mine dump sand was studied in experiments similar to those without sand, but with each 100 ml culture supplemented with 50 g air-dried G5 sand from the base of the Nourse Mine sand dump (see section 4.2). In addition, cultures of *T. ferrooxidans* ATCC 19859 containing 0, 5, 10, 20, 40, 60, 80 and 100 g G5 sand per 100 ml were treated in duplicate with 20,0 mg/l SLS at the time of inoculation.

5.2.2. Inhibitor: Linear alkylbenzenesulphonate (LAS)

The LAS, NANSA HS 80/S (Polycor Chemicals, Observatory, Cape Town), which contains 80% active sodium dodecylbenzenesulphonate, was tested at concentrations of 0, 1,0, 2,0, 4,0 and 8,0 or 0, 5,0, 10,0, 20,0 and 30,0 mg/l against *T. ferrooxidans* ATCC 19859 in, respectively, the absence and presence of 50 g G5 mine dump sand per 100 ml culture. The inhibitor was added at the beginning of the experiment. The LAS was also tested, using the same concentrations, against a mixed population of iron-oxidizing bacteria from acid seepage enrichment cultures (see section 5.2.1) in the presence and absence of G5 sand, with the inhibitor added at the beginning of the experiment or during the exponential phase of culture growth.

5.3. Results

5.3.1. Inhibitor: SLS

The results of the inhibition studies with SLS are shown in full in Appendix Fig. 1 to 13. The main findings are summarized in the following paragraphs.

Very low concentrations of SLS were inhibitory to *T. ferrooxidans* WLR and ATCC 19859 in HJJ medium in the absence of mine dump sand (Appendix Fig. 1 to 4). With the SLS in the medium from the beginning of the experiment, strain WLR was inhibited by 2 mg/l (Appendix Fig. 1), while ATCC 19859 was inhibited by 4 mg/l (Appendix Fig. 2). When SLS was added during the exponential phase of culture growth, both strain WLR and ATCC 19859 were inhibited by 2 mg/l (Appendix Fig. 3 and 4). The lowest concentration of SLS giving complete inhibition of the mixed iron-oxidizing bacterial population in HJJ medium without sand was 6 mg/l when the inhibitor was added at the time of inoculation (Appendix Fig. 5) and 8 mg/l when it was added during the exponential growth phase of the cultures (Appendix Fig. 6).

The addition of 500 g/l mine dump sand G5 to the cultures of both test organisms slowed their growth, in agreement with the results of section 4.3 for *T. ferrooxidans* 19859. Thus, in the case of the cultures without SLS, the *T. ferrooxidans* WLR cultures lacking sand oxidized Fe^{2+} completely in 55 to 80 h (Appendix Fig. 1 and 3) whereas in the corresponding cultures with sand the time was 340 to 370 h (Appendix Fig. 7 and 9). The same effect was found with the ATCC 19859 cultures (Appendix Fig. 4, 8 and 10) and mixed iron-oxidizing bacterial cultures (Appendix Fig. 5, 6, 11 and 12). Higher concentrations of SLS were required for inhibition of the WLR and ATCC 19859 cultures containing sand (Appendix Fig. 7 to 10) than for those without sand (Appendix Fig. 1 to 4). In the cultures with mine dump sand G5, strain WLR was inhibited by 4

mg/l SLS added at the beginning of the experiment (Appendix Fig. 7), whereas 8 mg/l was needed for inhibition of ATCC 19859 (Appendix Fig. 8). When the SLS was added during the exponential phase, both WLR and ATCC 19858 were inhibited by 4 mg/l (Appendix Fig. 9 and 10). The mixed population of iron-oxidizing bacteria was much more resistant to SLS in the presence of the mine dump sand than the WLR and ATCC 19859 cultures, requiring greater than 10 mg/l for complete inhibition (Appendix Fig. 11 and 12). Whether the inhibitor was added initially or during the exponential phase of culture growth, 20 mg/l was completely inhibitory, but concentrations between 10 and 20 mg/l were not tested.

Appendix Fig. 13 shows how the inhibitory effect of 20 mg/l SLS for *T. ferrooxidans* ATCC 19859 in HJJ medium was overcome after 400 h in cultures containing 1000 g/l mine dump sand. Cultures containing 0 to 800 g/l sand failed to initiate bacterial Fe^{2+} oxidation by 800 h, when the experiment was ended.

5.3.2. Inhibitor LAS

The effect of the LAS, NANSA HS 80/S, added at the beginning of incubation, on *T. ferrooxidans* ATCC 19859 cultures with and without G5 sand, is shown in Appendix Fig. 14 and 15. In the absence of sand, complete inhibition was obtained with 8 but not 4 mg/l LAS. The addition of 500 g/l G5 sand to the cultures prevented complete inhibition of *T. ferrooxidans* ATCC 19859 by 20 mg/l LAS, but 30 mg/l was completely inhibitory.

The inhibitory effect of the LAS on growth of the mixed iron-oxidizing bacterial population enriched from acid mine dump seepage samples, is illustrated in Appendix Fig. 16-19. In the absence of mine dump sand, 8 but not 4 mg/l LAS was completely inhibitory, whether the inhibitor was added initially (Appendix Fig. 16) or during the exponential growth phase (Appendix Fig. 17) of the cultures. In the presence of 500 g/l G5 mine dump sand, 30 mg/l SLS added initially only temporarily inhibited growth of the mixed iron-oxidizing bacterial population (Appendix Fig. 18), but 20 mg/l added during the exponential phase of growth (Appendix Fig. 19) was completely inhibitory.

5.4. Discussion

5.4.1. Inhibitor: SLS

According to Appendix Fig. 1 to 4, SLS was inhibitory to the pure cultures of *T. ferrooxidans* at the low concentrations of 2 to 4 mg/l. This is in accordance with the findings of Dugan (1975) and Kleinmann (1980).

When SLS was added during the exponential phase of growth of the ATCC 19859 strain, only 2 mg/l was necessary for inhibition, whereas double that amount was necessary when it was added at the beginning of the experiment. It therefore appeared that the ATCC 19859 strain was more sensitive to SLS in the exponential phase when the cells were growing actively. No such effect was observed with the pure culture of the WLR strain. Sherman and Albus, according to Porter (1946), studied the effect of temperature and weak disinfectants on bacteria at different ages. They found that bacterial cells during the early part of the lag phase were less sensitive to slightly toxic salts and other inimical agents than were the organisms of the late lag and early exponential phases of growth. Stanier et al. (1976) have noted that cells in the stationary phase are more resistant to adverse physical and chemical agents.

The mixed populations of iron-oxidizing bacteria enriched from acid seepage samples required 6 to 8 mg/l SLS for their inhibition (Appendix Fig. 5 and 6), i.e. higher concentrations than the ATCC 19859 and WLR pure cultures. However, these concentrations of SLS inhibitory to the mixed population of local strains were low enough not to detract from the potential of SLS as an inhibitor for use in gold mine sand dumps on the Witwatersrand. Unexpectedly, the minimum inhibitory concentration when the SLS was added to the mixed cultures at the beginning of the experiment (6 mg/l) was lower than when it was added during the exponential phase of growth (8 mg/l).

The mine dump sand had two effects on the *T. ferrooxidans* cultures. It partly protected the bacteria against the action of SLS, so that higher concentrations of SLS were necessary to inhibit their activity. In most cultures 4 mg/l SLS was required to inhibit the bacteria in the presence of the sand (Appendix Fig. 7, 9, 10) compared to 2 mg/l in the absence of sand (Appendix Fig. 1, 3, 4), but in the case of the ATCC 19859 cultures where the SLS was added at the beginning of the experiment, the concentrations were 8 and 4 mg/l, respectively (Appendix Fig. 2 and 8). On the other hand the sand retarded the growth of *T. ferrooxidans*, as indicated by the cultures with sand taking

much longer to oxidize the ferrous iron. Thus, cultures with sand but without SLS, or with SLS at non-inhibitory concentrations, took 3 to 7 times longer than non-inhibited cultures without sand to oxidize ferrous iron completely. The mechanisms resulting in these effects are unknown. Differences in adsorption of the ferrous iron, bacteria and SLS onto the sand particles, resulting in reduced contact of the bacteria with the substrate or inhibitor, may have been involved. On the other hand, chemical components that could affect the system may have been introduced into the culture solution from the sand. As in the cultures without sand, increased susceptibility of exponential phase cells of ATCC 19859, but not WLR, to SLS was observed in the cultures to which mine dump sand was added.

In agreement with the pure culture results, the mixed iron-oxidizing bacterial cultures in the presence of the mine dump sand took much longer to completely oxidize the ferrous iron of the HJJ medium. They also required a higher concentration of SLS for their inhibition (between 10 and 20 mg/l) than in the absence of sand (6 to 8 mg/l).

The experiment in which 20,0 mg/l SLS was tested against the ATCC 19859 strain in the presence of varying concentrations of sand, showed that this concentration of inhibitor was not effective when the sand concentration reached 1000 g/l. This sand concentration represents a much lower sand:water ratio than that of mine dumps, suggesting that loss of SLS inhibitory activity may be even greater in the mine dump environment and concentrations of SLS greater than 20,0 mg/l required to inhibit *T. ferrooxidans*.

5.4.2. Inhibitor : LAS

During the treatment of *T. ferrooxidans* ATCC 19859 at the beginning of the experiment with LAS at 2 and 4 mg/l, one of the duplicate treatments in each case showed complete ferrous iron oxidation within 500 hours while the other showed complete or almost complete inhibition (Appendix Fig. 14). The difference between duplicates may have been caused by differences in the concentration of LAS resulting from its poor solubility. However, care was taken in the preparation of the inhibitor solution and the variability may reflect differences, possibly as a result of mutation, in bacterial susceptibility to LAS at the threshold (which may be quite broad) between inhibitory and non-inhibitory concentrations. The lowest completely inhibitory LAS concentration for *T. ferrooxidans* ATCC 19859 in the absence of sand was 8 mg/l. This is also the concentration that was required for inhibition of the mixed iron-oxidizing bacterial population, enriched from acid seepage samples, in the cultures without sand

(Appendix Fig. 16 and 17). The inhibitory LAS concentration of 8 mg/l or less in the absence of mine dump sand is not much greater than the 2 to 5 mg/l reported by Dugan and Lundgren (1964) and the 5 mg/l reported by Dugan (1975) as the inhibitory concentrations of branched alkylbenzenesulphonates. A branched alkylbenzenesulphonate was tested by Kleinmann (1980) and at the concentrations tested, which were not given, this compound inhibited acid production, but apparently not as well as SLS. Kleinmann and Erickson (1983) found that LAS was less expensive and less sensitive to acid than SLS, but was required in higher concentrations for comparable inhibition.

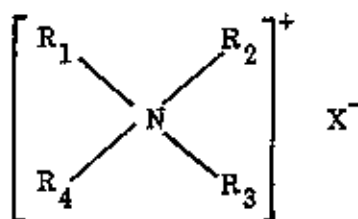
With mine dump sand in the cultures, the pure ATCC 19859 culture required 30 mg/l LAS for complete inhibition (Appendix Fig. 15) and the mixed population a concentration of greater than 30 mg/l when the inhibitor was added at the start of incubation (Appendix Fig. 18). The lower concentration (20 mg/l) required when the LAS was added during the exponential phase (Appendix Fig. 19) is a further example of greater susceptibility of exponential phase cells than resting cells to antibacterial chemicals (see section 5.4.1.). These inhibitory concentrations and the low solubility of LAS in water indicate that, under gold sand dump conditions, LAS would be a less efficient inhibitor of *T. ferrooxidans* than SLS. Comparison of the pure *T. ferrooxidans* ATCC 19859 culture and the mixed population of this species shows that, in the presence of sand, the mixed population was marginally more resistant to LAS inhibition than the laboratory cultures. This effect was also noted with SLS (section 5.4.1.).

Dugan and Apel (1983) recommended that branched alkylbenzenesulphonates be more extensively studied for potential application in mine drainage problems. These potential inhibitors have a resistance to biodegradation which might be advantageous in mine refuse treatment. However, their relatively slow biodegradability in the environment lead to their replacement in commercial detergents by the more readily degradable LAS (Atlas and Bartha, 1981) and they are not available for testing or use in South Africa.

6. INHIBITION OF *T. FERROOXIDANS* AND IRON-OXIDIZING BACTERIA FROM
MINE DUMP SEEPAGE BY SYNTHETIC CHEMICALS
B. CATIONIC SURFACE-ACTIVE AGENTS

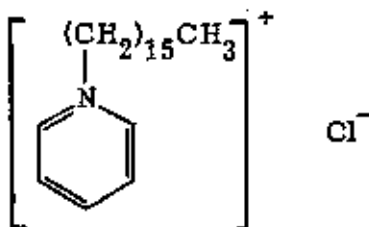
6.1. Introduction

Quaternary ammonium and pyridinium compounds are the most important cationic surface-active agents with antimicrobial activity (Sykes, 1965). The quaternary ammonium compounds are organically substituted ammonium compounds in which the nitrogen atom has a valency of 5 (Hamilton, 1971). Their general formula is:



where R_1 to R_4 are organic radicals and X is usually a halogen. For marked antibacterial activity, one of the four organic radicals must have a chain length of between 8 and 18 carbons. A widely used compound of this type is the C_{16} compound, cetyltrimethylammonium bromide (Cetrimide, CTAB or Cetavlon) (Sykes, 1965). The formulation used in our experiments was a mixture of three benzyldimethyl(*n*-alkyl) ammonium chlorides with differing *n*-alkyl side chains.

The important quaternary pyridinium compound with antimicrobial activity is cetylpyridinium chloride (Ceepryn), the quaternary salt formed from pyridine and cetyl chloride. It has the structure:



Gram-negative bacteria are somewhat less sensitive than Gram-positive bacteria to the action of the quaternaries (Hamilton, 1971). Bactericidal concentrations of Cetrimide for several Gram-negative bacteria ranged from 33 to 333 mg/l, depending on the method of testing, whereas the bactericidal concentrations of Ceepryn ranged from 15 to 67 mg/l (Sykes, 1965). Adsorption has an important influence on the activity of quaternaries, for example, glass and metal surfaces adsorb quaternaries so that even the containers in

because bacteria are particulate, they also act as adsorbants, and adsorption must be the first stage in the process of inhibition or killing. The high surface activities of quaternaries often cause clumping in a bacterial population, thus leading to protection of some of the cells from the antibacterial action of the quaternary as well as to an apparent reduction in numbers of survivors if estimated by clonal development. Cetrimide has destructive effects on the cell membrane, resulting in the leakage of cell constituents, which in *Escherichia coli* paralleled loss of cell viability (Franklin and Snow, 1975). However, an effect on bacterial growth was evident at concentrations that affected neither viability nor permeability.

Quaternaries are normally most active at alkaline pH, and acidity decreases the efficiencies of many quaternaries to such an extent that at pH 3 their bactericidal activities almost disappear (Hamilton, 1971). Thus, the concentration of Roccal (benzyltrimethylammonium chloride) for killing *E. coli* in 5 min was 100 mg/l at pH 3, 15 mg/l at pH 7 and 8 mg/l at pH 10. However, Ceepryn is exceptional in resisting inactivation at low pH. This property suggested that it could be a potentially useful inhibitor for the control of acid mine drainage.

Quaternary ammonium compounds have the problem of incompatibilities with certain other groups of compounds (Sykes, 1965). They are completely inactivated by anionic compounds, including SLS and soaps, as well as by several non-ionic compounds such as Lubrol-W and Tween 80. Metal ions, such as Ca^{2+} , Mg^{2+} , Fe^{3+} and some forms of aluminium, as well as organic matter, detrimentally affect the antimicrobial activity of quaternaries.

Apart from the antimicrobial activity of Ceepryn at low pH, activity of trialkylamines and the quaternary ammonium compound, triethylmethylammonium chloride (Aliquat 336), as inhibitors of oxygen uptake by *T. ferrooxidans* in a chalcopyrite medium was reported by Torma and Itzkovitch (1976). These observations and a total lack of knowledge of how quaternary ammonium and pyridinium compounds would be affected by mine dump sand, encouraged our study of a benzyltrimethyl-n-alkylammonium chlorides formulation (Hyamine 3500) and Ceepryn as inhibitors of iron-oxidizing mine dump bacteria, including *T. ferrooxidans*, in HJJ medium with and without mine dump sand.

6.2. Materials and Methods

6.2.1. Inhibitor: Benzyldimethyl-n-alkylammonium chlorides (Hyamine 3500)

The quaternary ammonium formulation of three benzyldimethyl-n-alkyl ammonium chlorides, known as Hyamine 3500, was supplied by Rohm and Haas South Africa (Pty) Ltd., Bellville, Cape Province. The three active ingredients had the formula $[(C_6H_5-CH_2)(CH_3)_2(C_nH_{2n-1})N]^+Cl^-$, with $n = 12, 14$ or 16 . The respective concentrations of the C_{12} , C_{14} and C_{16} -alkyl compounds in the formulation were 40, 50 and 10% (m/m).

Hyamine 3500 was tested against *T. ferrooxidans* ATCC 19859 and WLR in HJJ medium in the same way as SLS (section 5.2.1). In addition, it was tested against iron-oxidizing enrichment cultures derived from soil G4 10^{-1} MPN cultures (see section 3.3.2.). The various Hyamine 3500 concentrations from 0 to 128.0 mg/l were added, in duplicate, either at the beginning of the experiment or during the exponential phase, to cultures without or with mine dump sand G5 (50 g/100 ml culture), except that the G4 10^{-1} enrichment cultures were tested only with sand in the medium.

6.2.2. Inhibitor: Cetylpyridinium chloride (Ceepryn)

Ceepryn (BDH Chemicals Ltd., Poole, England) was tested against *T. ferrooxidans* ATCC 19859 in HJJ medium at concentrations of 0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/l in cultures without mine dump sand, or 0, 10.0, 20.0, 40.0 and 60.0 mg/l in the presence of G5 sand at 50 g/100 ml culture. The inhibitor was added at the beginning of the experiment or during the exponential phase of culture growth. The cultures were incubated and monitored as in the SLS experiments (section 5.2.1).

6.3. Results

6.3.1. Inhibitor: Hyamine 3500

Hyamine 3500 slowed the growth of *T. ferrooxidans* ATCC 19859 and WLR in HJJ medium, but at concentrations to 128 mg/l failed to inhibit it completely (Appendix Fig. 20 to 23). When Hyamine 3500 was added to WLR cultures at the beginning of the experiment (Appendix Fig. 20), the time for complete oxidation of the Fe^{2+} in the medium was 405 and 450 h at inhibitor concentrations of 64 and 128 mg/l, respectively, compared with 92 h in non-inhibited cultures (to 16 mg/l Hyamine 3500). When Hyamine 3500 was added at 64 and 128 mg/l to WLR cultures during the exponential

phase of growth (Appendix Fig. 21), the cultures required 265 and 350 h, respectively, for complete Fe^{2+} oxidation. The addition of Hyamine 3500 at concentrations of 16, 32, 64 and 128 mg/l to cultures of ATCC 19859 at the beginning of the experiment slowed culture growth so that the respective times for complete Fe^{2+} oxidation were 170, 170, 250 and 360 h (Appendix Fig. 22), compared with 72 h in non-inhibited cultures (to 8 mg/l Hyamine 3500). Addition of the two highest concentrations of Hyamine 3500 during the exponential phase of growth slowed the time for complete Fe^{2+} oxidation to 500 h compared with 67 to 92 h for 0 to 32 mg/l (Appendix Fig. 23).

The addition of 50 g of mine dump sand G5 to 100-ml cultures of *T. ferrooxidans* WLR and ATCC 19859 retarded considerably the oxidation of Fe^{2+} in cultures without Hyamine 3500 or which were not inhibited by low or even high concentrations of the inhibitor (Appendix Fig. 24 to 27). In the WLR cultures where the Hyamine 3500 was added at the time of inoculation and in the ATCC 19859 cultures where it was added during the exponential phase of growth, the highest concentration (128 mg/l) caused no or only slight inhibition of the bacterial growth. However, concentrations of 4 to 128 mg/l of inhibitor added during the exponential phase caused some inhibition of the WLR cultures, whereas 2 to 128 mg/l, added at the time of inoculation, caused a marked lag in the development of the ATCC 19859 cultures.

When Hyamine 3500 was added to iron-oxidizing enrichment cultures from the G4 10^{-1} MPN cultures, containing mine dump sand G5, it had little inhibitory effect on the growth of the iron-oxidizing bacteria (Appendix Fig. 28 and 29). However, growth in all cultures was slow. In the experiment where the Hyamine 3500 was added at the time of inoculation, Fe^{2+} oxidation was complete only after 305 to 425 h of incubation (all treatments), and in the experiment where it was added during the exponential phase, the times for complete Fe^{2+} oxidation (all treatments) were 500 to 570 h.

6.3.2. Inhibitor: Ceepryn

The inhibitory effect of Ceepryn on *T. ferrooxidans* ATCC 19859 is shown in Appendix Fig. 30 to 33. In the absence of sand, whether the inhibitor was added at the start of the experiment or during the exponential phase of growth (Appendix Fig. 30 and 31), 2 mg/l Ceepryn was the lowest completely inhibitory concentration. In the presence of 500 g/l G5 sand, 20 but not 10 mg/l Ceepryn was completely inhibitory when inhibitor was added at the start of the experiment (Appendix Fig. 32), whereas 40 but not 20 mg/l was completely inhibitory when the Ceepryn was added during the exponential phase of growth (Appendix Fig. 33).

6.4. Discussion

6.4.1 Inhibitor: Hyamine 3500

Hyamine 3500, to the highest concentration tested (128 mg/l), had only a partial or temporary inhibitory effect on the growth of pure cultures of *T. ferrooxidans* (Appendix Fig. 20 to 23). In the corresponding cultures to which mine dump sand was added, the growth was retarded further (Appendix Fig. 24 to 27), confirming the previously observed detrimental effect of the sand on the activity of the bacteria, but also there was no clear evidence that the sand protected the bacteria against Hyamine 3500. When Hyamine 3500 was added to enrichment cultures of iron-oxidizing bacteria, derived from soil G4 10^{-1} MPN cultures and containing mine dump sand G5, growth in all cultures was slow and there was no or very little inhibition of Fe^{2+} oxidation by even the highest concentration (128 mg/l) of Hyamine (Appendix Fig. 28 to 29). These inhibition studies with Hyamine 3500 indicate that this formulation of three quaternary ammonium salts is not suitable for the inhibition of iron-oxidizing bacteria, such as *T. ferrooxidans* in gold mine sand dumps. The results are in agreement with the general observations that quaternary ammonium salts have poor activity in acid media and lose much activity through high levels of adsorption to different surfaces (Sykes, 1965).

6.4.2. Inhibitor: Ceepryn

The quaternary pyridinium compound, Ceepryn, was tested because it should be better suited to acid environments (Sykes, 1965) than the quaternary ammonium compounds, exemplified by Hyamine 3500. It is extensively used as a bactericidal agent and is readily available. In the absence of mine dump sand (Appendix Fig. 30 and 31), the inhibiting potential of Ceepryn against *T. ferrooxidans* ATCC 19859 compared favourably with that of SLS (section 5.3.1). However, the effect of 500 g/l sand in reducing the effectiveness of the quaternary pyridinium inhibitor (Appendix Fig. 32 and 33) was far greater than in the case of SLS. Complete inhibition of *T. ferrooxidans* ATCC 19859 in the presence of G5 sand (500 g/l) required 20 mg/l Ceepryn when the inhibitor was added at the start of the experiment and 40 mg/l when it was added during the exponential phase of bacterial growth. The corresponding inhibitory concentrations of SLS were 8 mg/l and 4 mg/l, respectively, or 20 mg/l with the mixed culture of iron-oxidizing bacteria from acid seepage samples.

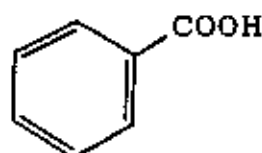
The activity of quaternary pyridinium compounds is reduced in the presence of organic matter, certain metal ions (e.g. Ca^{2+} , Mg^{2+} and Fe^{2+}) and anionic compounds such as SLS (Sykes, 1965). Organic matter in grassed dumps and metal ions would adversely affect the efficiency of Ceepryn as an inhibitor on mine dumps. The effects of organic matter and metal ions, coupled with the demonstrated relatively poor performance of Ceepryn in cultures containing mine dump sand, suggest that cetylpyridinium chloride is unsuitable as an inhibitor for application to sand dumps.

7. INHIBITION OF *T. FERROOXIDANS* BY SYNTHETIC CHEMICALS.

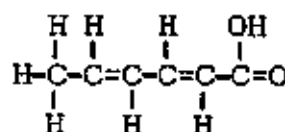
C. FOOD PRESERVATIVE ORGANIC ACIDS

7.1 Introduction

Benzoic and sorbic acid are commercially important antimicrobial agents, active especially against yeasts and moulds. They are widely used as food preservatives, particularly in low acid foods (Jay, 1978). Both are normally applied as either their sodium or potassium salt, as the salts are more soluble in water than the acids. The antimicrobial activity of benzoate and sorbate is related to pH, with the greatest activity below pH 4 and pH 6, respectively. The antimicrobial activity of benzoate and sorbate resides in both the undissociated molecule and dissociated anion (Eklund, 1980, 1983). The structures of the acids are:



Benzoic acid



Sorbic acid

Onysko et al. (1984b) showed sodium benzoate and potassium sorbate at 10 mg/l to be inhibitory to iron oxidation by *T. ferrooxidans* in pure culture. According to Jay (1978), benzoate and sorbate act against heterotrophic microorganisms by inhibiting cellular uptake of substrate molecules such as amino acids, phosphate, organic acids and the like. However, Tuttle and Dugan (1976) noted that relative electronegativity and solubility in the cell envelope and possibly the cell membrane, seemed to be major factors contributing to the inhibitory action of various mono- and dicarboxylic organic acids on *T. ferrooxidans* (although benzoic acid and sorbic acid were not tested). The action of the inhibitory acids could be due to the following:

- (i) Direct inhibition of the iron-oxidizing enzyme system;
- (ii) Interference with the role of sulphate or phosphate in iron oxidation;
- (iii) Non-selective disruption of the cell envelope or cell membrane, blocking iron transport through the envelope to the site of oxidation or electron transport in the membrane.

In support of mechanism (iii), Tuttle et al. (1977) showed by electron microscopy disruption of the cell envelope or membrane of *T. ferrooxidans* cells exposed to inhibitory organic acids. The organic acids also caused the cells to release RNA, DNA and other materials, providing further evidence of disruption of the cell envelope and membrane. Tuttle et al. (1977) suggested that inhibitory organic acids might disrupt the cell envelope and membrane by reacting with cations that contribute to their structural integrity. Disruption of the permeability barriers could result in an influx of hydrogen ions from the environment, which could inhibit the cell. This theory was supported by the results of Alexander et al. (1987), who showed that the toxicity of several organic acids followed approximately the efficacy of the acids in lowering the internal cell pH. However, the toxicity might also be related to the accumulation of a high concentration of anions in the cytoplasm. Respiration of Fe^{2+} appeared to be blocked by the organic acids at the level of the respiratory chain cytochrome oxidase.

In flask experiments of Dugan (1987a), sodium benzoate at 75 and 100 mg/l completely inhibited pyrite oxidation in a 30% coal slurry for 22 and 18 days, respectively. Thereafter, the 75 mg/l concentration permitted slower pyrite oxidation than the 100 mg/l concentration. This unexpected trend towards the end of the experiment was ascribed to a variation in the coal refuse material (Dugan, 1987b).

Pilot scale studies with sodium benzoate and potassium sorbate as inhibitors of iron-oxidation in coal refuse were conducted by Onysko et al. (1984a). The inhibitors were applied in solution as they were shown to react with ferric iron in synthetic mine water to form sparingly soluble precipitates which dissolved in an acid environment. Such precipitates should therefore have served as sources of inhibitor in the coal refuse, with liberation of benzoic or sorbic acid as acidity was generated. Coal refuse (200 kg) with a pyrite sulphur content of ca. 5% was placed in barrels to a depth of 60 cm. Solutions (24 l) containing 500 and 5000 mg/l of sodium benzoate and potassium sorbate were applied to the coal refuse and allowed to drain into the bottoms of the barrels. After 24 hours the leachate was removed and analysed. The coal refuse was subsequently leached in the same way with 24 l of water weekly. When applied at the low dose (60 mg/kg coal refuse), sodium benzoate and potassium sorbate delayed acid formation for, respectively, 2 and 5 weeks more than the 7 weeks required for acidification of the control. The high dose (600 mg/kg coal refuse) of sodium benzoate and potassium sorbate delayed acid formation for 8 and 10 weeks, respectively. Costs for the treatments calculated by Onysko et al. (1984a) showed sodium benzoate to be the most cost-effective treatment for the control of acid mine drainage in heavily leached coal refuse.

These promising results with benzoate- and sorbate-treated coal refuse indicated that the two acids should be included in our tests of potential inhibitors of *T. ferrooxidans* in gold mine dump sand. They were therefore tested against *T. ferrooxidans* ATCC 19859 and WLR in HJJ medium without or containing G5 sand.

7.2. Materials and Methods

7.2.1. Inhibitor: Sodium benzoate

Sodium benzoate (99% pure, supplied by Saarchem, Muldersdrift, Transvaal), was added in duplicate at concentrations of 0, 5,0, 10,0, 15,0, 20,0 and 25,0 mg/l to cultures of *T. ferrooxidans* ATCC 19859 and WLR in HJJ medium not containing mine dump sand at the start of the experiment, or during the exponential growth phase at concentrations of 0, 10,0, 20,0, 30,0, 40,0, 50,0 and 60,0 mg/l. It was also added to cultures containing G5 sand at 50 g/100 ml culture, at the beginning and during the exponential phase of culture growth, to give concentrations of 0, 15,0, 20,0, 25,0 and 30,0 mg/l or 0, 20,0, 25,0, 30,0 and 35,0 mg/l. The cultures were incubated and monitored as in the experiments with SLS (section 5.2.1).

7.2.2. Inhibitor: Sorbic acid

Sorbic acid (Analytical Reagent grade, supplied by E. Merck, Darmstadt, West Germany) was tested as an inhibitor of *T. ferrooxidans* ATCC 19859 and WLR in the same way as sodium benzoate, except for minor differences in the concentrations tested.

7.3 Results

7.3.1. Inhibitor: Sodium benzoate

The results of the sodium benzoate inhibition experiments are shown in Appendix Fig. 34 to 41. The concentrations of sodium benzoate needed to inhibit *T. ferrooxidans* WLR and ATCC 19859 in HJJ medium in the absence of mine dump sand were 15 and 30 mg/l for inhibitor added at the beginning of the experiment and during the exponential phase of culture growth, respectively (Appendix Fig. 34 to 37).

With 500 g/l mine dump sand G5 in the cultures, sodium benzoate totally inhibited *T. ferrooxidans* WLR at 25 mg/l and probably totally inhibited strain ATCC 19859 at 30

mg/l, when added at the beginning of the experiment (Appendix Fig. 38 and 40). When added after 23 hours, during the exponential phase of culture growth, 30 mg/l sodium benzoate totally inhibited the growth of *T. ferrooxidans* WLR (Appendix Fig. 39) and 35 mg/l totally inhibited *T. ferrooxidans* ATCC 19859 (Appendix Fig. 41).

7.3.2. Inhibitor: Sorbic acid

The results of the sorbic acid inhibition experiments are shown in Appendix Fig. 42 to 49. The concentrations of sorbic acid needed to inhibit *T. ferrooxidans* WLR and ATCC 19859 in HJJ medium in the absence of mine dump sand were 15 and 20 mg/l, respectively, when the inhibitor was added at the beginning of the experiment (Appendix Fig. 42 and 44), and 20 mg/l for both strains when it was added during the exponential phase of growth (Appendix Fig. 43 and 45).

With the mine dump G5 sand in the cultures, sorbic acid totally inhibited *T. ferrooxidans* WLR and ATCC 19859 at 20 and 25 mg/l, respectively, when added at the beginning of the experiment (Appendix Fig. 46 and 48). When added after 23 hours, during the exponential phase of growth, 30 mg/l sorbic acid was required for complete inhibition of both the WLR and ATCC 19859 strains (Appendix Fig. 47 and 49).

7.4. Discussion

7.4.1. Inhibitor: Sodium benzoate

The antimicrobial activity of sodium benzoate is related to pH, with the greatest activity at low pH (Jay, 1978). Inhibitor studies in the U.S.A. with cultures of *T. ferrooxidans* supplemented with benzoic acid indicated that 10 mg/l benzoic acid effectively inhibited bacterial ferrous iron oxidation (Onysko *et al.*, 1984b). In the present studies, greater concentrations of sodium benzoate than 10 mg/l were necessary for the complete inhibition of *T. ferrooxidans* ATCC 19859 or WLR in HJJ medium, namely, 15 mg/l when the inhibitor was added at the beginning of the experiment and 30 mg/l when it was added during the exponential phase of growth (Appendix Fig. 34 to 37). The higher concentrations of sodium benzoate required for complete inhibition of *T. ferrooxidans* ATCC 19859 and WLR when the addition was made during the exponential phase of growth, may be related to the formation of a sparingly soluble precipitate with ferric iron (Onysko *et al.*, 1984b), which would have been formed by the *T. ferrooxidans* cultures in the exponential phase by the time the inhibitor was added. However, such a

precipitate may serve as a slow-release source of benzoic acid for the control of *T. ferrooxidans* in mine wastes (Onysko *et al.*, 1984a,b).

Differences of experimental technique are possibly responsible for the differences in the results of Onysko *et al.* (1984b) and those of our experiments. The inhibitory 10 mg/l benzoic acid of Onysko *et al.* (1984b) equals 11,8 mg/l sodium benzoate, thus this was not the reason. Differences in the *T. ferrooxidans* inocula are possibly the main reasons for the inhibitory concentrations of benzoate, added at the beginning of the experiment, being higher in our experiments than in that of Onysko *et al.* (1984b). They used a different *T. ferrooxidans* strain at an inoculation rate of 0,1% (v/v). Furthermore, the initial pH of their medium was pH 1,16 compared with our pH 2,0, and the compositions of the media were not identical.

In the presence of gold mine dump sand (Appendix Fig. 38 to 41), the inhibitory concentrations of 25 to 35 mg/l sodium benzoate were mainly higher than the 15 to 30 mg/l which were inhibitory in the corresponding cultures containing no gold mine dump sand. Thus there appeared to be a protective effect of the mine dump sand. Adsorption of the inhibitor could perhaps play a role, but this has not yet been researched. The formation of a precipitate, as postulated by Onysko *et al.* (1984b), between ferric iron possibly present in the gold mine dump sand and benzoate, would increase the concentration of benzoate needed for inhibition of the bacteria in the presence of gold mine dump sand. As in the case of the cultures without sand and possibly for the same reason, both *T. ferrooxidans* strains appeared more resistant to sodium benzoate when the inhibitor was added during the exponential phase of growth than when it was added at the start of the experiment.

In comparison with SLS, sodium benzoate is not a very effective inhibitor. In the present study SLS was inhibitory to *T. ferrooxidans* ATCC 19859 and WLR at concentrations of 4 and 2 mg/l in the absence of gold mine dump sand and 8 and 4 mg/l in the presence of the sand. However, Onysko *et al.* (1984b) found benzoic acid to be as inhibitory and more cost effective than SLS on coal refuse.

7.4.2. Inhibitor: Sorbic acid

Sorbic acid is most effective as an antimicrobial agent below pH 3, when more than 80% of the compound is undissociated (Jay, 1978). Undissociated molecules are essential for antimicrobial activity as they prevent growth by inhibiting cellular uptake of substrate molecules. Onysko *et al.* (1984b) reported that 10 mg/l sorbic acid effectively inhibited

the oxidation of ferrous iron by *T. ferrooxidans*. In the present experiments with *T. ferrooxidans* WLR and ATCC 19859, 15 and 20 mg/l, respectively, were necessary for complete inhibition when the sorbic acid was added at the beginning of the experiment. When the sorbic acid was added during the exponential phase of growth, 20 mg/l completely inhibited the ferrous iron oxidation by both the WLR and the ATCC 19859 strains.

As in the case of sodium benzoate, the presence of mine dump sand increased the concentrations of sorbic acid needed for inhibition of the bacteria (Appendix Fig. 46 to 49) from the 15 to 20 mg/l that was inhibitory in the absence of sand to 20 to 30 mg/l. The increase is about as great as in the case of sodium benzoate. A protective effect of the sand is therefore indicated, possibly involving adsorption of the sorbic acid or the formation of a sparingly soluble precipitate as discussed for sodium benzoate.

Concentrations of sorbic acid required for inhibition of *T. ferrooxidans* ATCC 19859 and WLR were mainly lower when the compound was added at the beginning of the experiment than when it was added during the exponential phase of growth (when ferric iron would be available to precipitate some of it). The results also indicate that *T. ferrooxidans* ATCC 19859 was more resistant to sorbic acid than *T. ferrooxidans* WLR.

When minimum inhibitory concentrations are compared, sorbic acid (Appendix Fig. 46 to 49) was less effective against *T. ferrooxidans* ATCC 19859 and WLR than SLS (Conradie, 1984) in the absence or presence of gold mine dump sand. In the presence of the mine dump sand, the overall performance of sorbic acid (Appendix Fig. 46 to 49) was better than that of sodium benzoate (Appendix Fig. 38 to 41). Onysko *et al.* (1984b) found sorbic acid to be the most effective inhibitor when compared to both SLS and sodium benzoate in coal refuse. The likely cause of the different SLS result in the two studies is different adsorption behaviour of the SLS on the coal refuse and the gold mine dump sand. However, on the basis of cost (Onysko *et al.*, 1984b), sodium benzoate would be a better alternative to SLS than sorbic acid for prevention of *T. ferrooxidans* growth in gold mine sand dumps.

8. INHIBITION OF *T. FERROOXIDANS* BY SYNTHETIC CHEMICALS.

D. SODIUM LIGNOSULPHONATE AND POLYACRYLIC ACID

8.1 Introduction

Sodium lignosulphonate is produced in large volumes as a by-product of the paper industry and polyacrylic acid is a polymer that could be made available to the mining industry at reasonable cost. None of these materials has been tested for its inhibitory activity against cultures of *T. ferrooxidans*, although Dugan (1987a) reported little activity of lignin sulphonates against acid-producing microorganisms cultured in a coal refuse slurry. It was hoped that the polymers might show some of the activity of the low molecular mass aromatic sulphonate and organic acid inhibitors considered in sections 5 and 7.

8.2 Materials and Methods

8.2.1. Inhibitor: Sodium lignosulphonate

Sodium lignosulphonate was supplied by Sappi Fine Paper (Pty) Ltd., Enstra, Transvaal, as a dark-brown, smelly liquid product from their wood-pulping process. Initially the effect of 0, 1,0, 2,0, 4,0, 8,0, 16,0, 32,0, 64,0 and 128,0 mg sodium lignosulphonate solution/l on *T. ferrooxidans* WLR and ATCC 19859 in HJJ medium without mine dump sand was investigated as described for SLS (section 5.2.1.), with the inhibitor added either at the beginning of the experiment or during the exponential phase of growth. Subsequently, concentrations of 0, 100, 200, 300, 400 and 500 mg sodium lignosulphonate solution/l were tested against *T. ferrooxidans* WLR in HJJ medium, with addition of the lignosulphonate at the beginning of the experiment.

8.2.2. Inhibitor: Polyacrylic acid

A 33,15% aqueous solution of polyacrylic acid (Primal A3) was supplied by Dr. R.D. Sanderson, Institute for Polymer Science, University of Stellenbosch. Inhibition of duplicate cultures of WLR and ATCC 19859 mixed with 50 g mine dump sand G5 in 100 ml HJJ medium, by 0, 0,33, 0,66, 1,32, 2,65, 5,30, 10,61, 21,22 and 42,43 mg/l polyacrylic acid (0, 1, 2, 4, 8, 16, 32, 64, and 128 ml of the 33,15% solution/l) was tested. The inhibitor was added at the beginning of the experiment as a $256 \times 33,15/100 = 85$ mg/l stock solution or during the exponential phase as a $1280 \times 33,15/100 = 425$ mg/l stock solution, after 20 ml of culture had been removed. Appropriate volumes of

stock solution and fresh HJJ medium were added. The cultures were incubated and monitored as in the SLS experiments (section 5.2.1).

8.3. Results

8.3.1. Inhibitor: Sodium lignosulphonate

No or only slight inhibition was observed when sodium lignosulphonate solution was added to *T. ferrooxidans* WLR and ATCC 19859 cultures at concentrations to 128 mg/l, either at the beginning of the experiment or during the exponential phase of culture growth (Appendix Fig. 50 to 52 show the results of three of the four experiments). When higher concentrations of sodium lignosulphonate were added to WLR cultures at the beginning of the experiment, growth of the bacteria was not inhibited by 400 mg/l and was only slightly inhibited by 500 mg/l (Appendix Fig. 53). No further tests were conducted with these higher sodium lignosulphonate concentrations.

8.3.2. Inhibitor: Polyacrylic acid

The results of the experiments to test the inhibition of *T. ferrooxidans* WLR and ATCC 19859 by polyacrylic acid are shown in Appendix Fig. 54 to 58. Only slight inhibition of the WLR cultures was indicated with polyacrylic acid concentrations to 42,43 mg/l (Appendix Fig. 54 and 55). However, 21,22 and 42,33 mg/l had an inhibitory effect on the growth of *T. ferrooxidans* ATCC 19859 in the absence of mine dump sand (Appendix Fig. 56 and 57). The effect was most clearly evident when the polyacrylic acid was added to cultures in the exponential phase of growth, but although the growth was retarded, it was not completely inhibited (Appendix Fig. 57). When polyacrylic acid was added at the beginning of the experiment to ATCC 19859 cultures supplemented with 50 g air-dried mine dump sand G5, it had negligible or no effect on the Fe^{2+} oxidation (Appendix Fig. 58).

8.4. Discussion

8.4.1. Inhibitor: Sodium lignosulphonate

Concentrations of sodium lignosulphonate to 128 mg/l caused no or only slight inhibition of *T. ferrooxidans* ATCC 19859 and WLR (Appendix Fig. 50 to 52). At 500 mg/l, but not at 400 mg/l, sodium lignosulphonate had a slight retarding effect on the oxidation of Fe^{2+} by the WLR strain (Fig. 53). It therefore seems not to have potential as an

inhibitor of *T. ferrooxidans* in gold mine sand dumps, which is unfortunate in view of its availability as a by-product of the paper industry. Dugan (1987a) likewise found that four lignin sulphonate formulations at 1 g/l were ineffective or had only low inhibitory activity against shaken cultures of acidifying microorganisms in 20% slurries of a high sulphur coal refuse.

8.4.2. Polyacrylic acid

Polyacrylic acid at concentrations of 21,22 and 42,43 mg/l only partially inhibited the growth of *T. ferrooxidans* ATCC 19859 and WLR. It had a greater effect on the ATCC 19859 strain (Appendix Fig. 56 and 57) than on the WLR strain (Appendix Fig. 54 and 55). It seemed to affect the ATCC 19859 strain only late in the exponential phase of growth. In the corresponding ATCC 19859 cultures to which mine dump sand G5 was added at the beginning of the experiment, polyacrylic acid had a negligible effect on the Fe^{2+} oxidation, providing evidence that the mine dump sand protected the bacteria against polyacrylic acid. The previously observed detrimental effect of sand G5 on the activity of *T. ferrooxidans* was evident but not pronounced. Polyacrylic acid seems to have no potential for use as an inhibitor of *T. ferrooxidans* in gold mine sand dumps.

9. DISTRIBUTION OF IRON-OXIDIZING BACTERIA IN GOLD MINE SAND DUMPS

9.1 Introduction

The demonstration that SLS and less effectively, LAS, Ceepryn, benzoic and sorbic acids, could inhibit *T. ferrooxidans* in the presence of gold mine dump sand (sections 5 to 7) raised the question of whether these inhibitors could be used in the field to control the bacteria and the formation of acid mine drainage in sand dumps. As the sand reduced the effectiveness of the inhibitors, possibly by adsorption, it became necessary to locate the target bacteria in the dumps, to determine to what depth in the sand the inhibitors would have to move, against retaining forces of adsorption, in sufficient concentrations to achieve the desired control.

No data on the distribution of iron-oxidizing bacteria in South African sand dumps could be obtained, but Matic and Mrost (1964) and Mrost and Lloyd (1970) investigated their distribution in slimes dams. The bacteria occurred in high concentrations only in the upper 2 to 3 m of the slimes dams, where acidification had taken place and pyrite levels had been greatly reduced. The iron-oxidizing bacterial populations peaked at depths of about 1 m, in the vicinity of rusty layers that developed in the grey slimes (Matic and Mrost, 1964). Both the presence of the bacteria in high numbers and pyrite oxidation were therefore associated with the aerated outer zones of slimes dams. If the same principles applied to sand dumps, high iron-oxidizing bacterial populations would be expected in the outer 10 m of dump material, this being the approximate depth to which oxidation of pyrite in sand dumps has proceeded (Marsden, 1986). The unexpected cited result of oxidation to ca. 30 m depth in the 3A8 dump is a misinterpretation of our results (Whillier, 1987), as the ca. 30 m refers to horizontal distance into the dump. The 3A8 dump had a slope angle of 27° (Jones et al., 1988), hence the distance of the sampling point from the surface was only about 13,5 m.

Our studies of the distribution of iron-oxidizing bacteria in sand dumps involved sampling both the outer yellow or orange oxidized zone and the grey unoxidized inner zone (exposed on a dump being excavated for extraction of residual gold), as well as the boundary zone where orange streaks occurred in the otherwise grey sand. Samples were also taken from seepage at the base of dumps, including samples from within a dump behind the seepage outfall, along the line of flow of seepage water from inside the dump. Samples were taken during both wet summer and dry winter periods.

9.2. Materials and Methods

9.2.1. Sampling of dumps

Sand sample A3 was collected in April 1984 from the acid seepage area at the base of the southwestern corner of the Nourse Mine 3A19 sand dump (from approximately the same site as samples G1 to 4 and H1 to 3 of section 3.2). At the same time, samples A4 to A10 were taken from the grassed top and sides of the 3A19 sand dump near its western (A4) and eastern (A5 to 10) ends. Sampled sand was loosened by spade to <30 cm depth and transferred by means of sterilized spatulas to sterile glass jars for transport to Stellenbosch.

Sand samples M1 to 9 and L1 to 5 were collected by auger from different depths to almost 4 m on the eastern slope of the City Deep 3A17 sand dump at different distances into the dump from the edge of an acid seepage pool. Depths and distances are indicated under Results (section 9.3.1). The M1 to 9 samples were taken in March 1985 and the L1 to 5 samples in June 1985.

Sand samples J1 to 8 were taken by sterilized spatula from the foot of the excavated face on the western side of the Crown Mines (Rand Mines Proprietary) 3A8 sand dump. Sample J1 was grey sand from the centre of the face; J2 to 4 grey sand, without or with orange streaks, from near the orange-coloured oxidation front; J5, 7 and 8 orange-coloured sand within 30 m (horizontal distance) of the dump surface, and J5 a light grey sand forming a streak in the orange-coloured outer zone. The sampling time was July 1984.

The 3A8 dump was sampled further by sterilized spatula on several occasions. Sample M10, an orange sand, was collected in March 1985 from the drainage area on the south side of the dump and samples L6 and 7 in June 1985 from the northeastern side of the dump. Sample L6 was a white and orange slimes sample taken from an exposed area of a slimes dam on which the 3A8 dump was situated and L7 a yellow sand from immediately above the slimes-sand interface. Samples FY1 to 11 were sand sampled in February 1978 from the excavated face on the west side of the 3A8 sand dump and from accumulations of material from collapsed parts of the face. Samples FY1 to 7 were from the core of the dump where the sand was grey or where it was grey-orange, either because its pyrite was partly oxidized or because it had become mixed with oxidized orange sand that had slid from the upper part of the face. Samples FY8 to 11 were grey- to orange-coloured sand from the boundary between the inner grey and outer

orange zones of the dump. The FYI to 11 samples were collected on 4 February 1987, 2 days after a heavy rain storm during which 100 mm rain fell in 2 h; however, they were from a face that was also kept moist by spray irrigation to control wind-blown dust. The last samples from the 3A8 dump, MH1 to 10, were collected on 30 March 1987 after excavation had proceeded 50 to 100 m farther into the dump since the February sampling. Samples MH1 and 2 were collected high on the face just below the vegetation on the outside of the dump; they consisted of orange sand. Sample MH3 was a red fine-textured sample from an old slimes deposit underlying the sand dump; it was located about 200 m into the area where the excavated dump had been and was underlain by grey slimes residues. Run-off water from the sand dump had formed many erosion gullies across this basal slimes deposit. Samples MH4 to 6 were grey to grey-orange sand from the inner zone of the excavated face and samples MH7 to 10 grey to orange sand from the boundary between the inner grey and the outer orange zones.

9.2.2. Analyses

Samples were transported by air to Stellenbosch on the day of sampling and analysed as soon as possible, usually on the following day. However, samples FYI to 7 were stored at room temperature for 10 days before they could be analysed.

Moisture and pH of the sand samples were determined as described in section 3.2.1. Iron-oxidizing bacterial populations in the A3 to A10 and J1 to J8 samples were estimated by the MPN method described in section 3.2.2 with five MPN tubes per dilution, but with the incubation period extended to 93 days to permit the detection of slow ferrous-oxidizing bacteria. Iron-oxidizing bacteria in all other samples were estimated by an alternative MPN method, in which 50-ml wide-necked conical flasks were substituted for test tubes to ensure better aeration, the volume of medium was increased to 15 ml and later (1987 samples) 20 ml to allow for greater evaporation from the flasks, three flasks per dilution were used and the duration of the experiment was decreased to 21 days after which no change in the results occurred in the initial studies with this modified method.

9.3 Results

Figures 18 to 22 show the location of sampling sites on sand dumps 3A19, 3A17 and 3A8. Tables 5 to 9 show the moisture content, pH and iron-oxidizing bacterial populations of the respective samples.

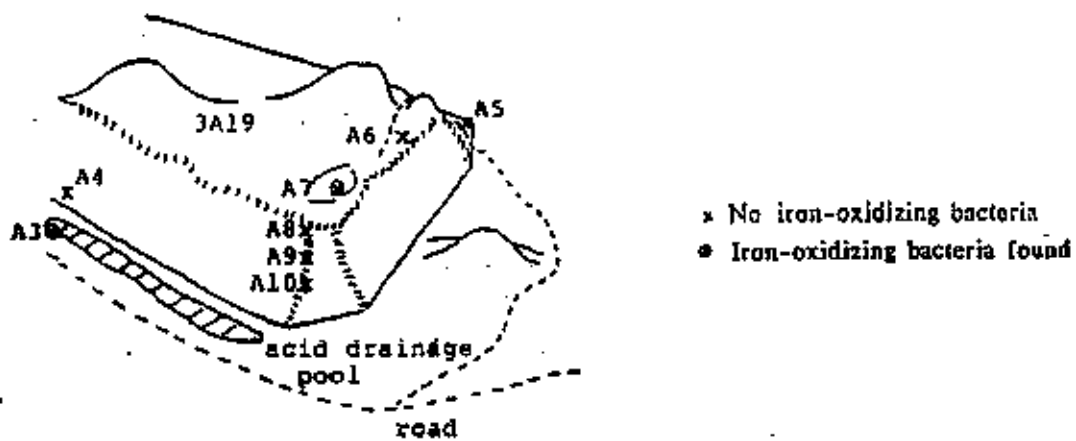


FIG. 18. Topography of the Nourse Mine 3A19 sand dump showing positions of the sampling sites and where iron-oxidizing bacteria were found.

TABLE 5. Moisture, pH and counts of iron-oxidizing bacteria for samples collected in April 1984 from the Nourse Mine 3A19 sand dump

Sample	Moisture g/100 g wet sand ^a	pH in water ^b	Iron-oxidizing bacteria MPN/g dry sand ^c
A3	22,93	2,34	$5,6 \times 10^5$
A4	5,19	4,12	0
A5	4,20	4,57	0
A6	2,63	6,12	0
A7	3,89	3,61	$2,1 \times 10^{-1}$
A8	3,00	4,27	0
A9	2,74	6,70	0
A10	4,52	4,67	0

^aMean of duplicate determinations.

^bpH of 1:2,5 sand:water suspensions; mean of duplicate determinations.

^cDetermined from de Man (1983) MPN tables. See Whittier (1987) for statistical categorization of results and theoretical 95% confidence limits. 0 = no iron-oxidizing bacteria detected in tubes containing up to 1 g wet sand.

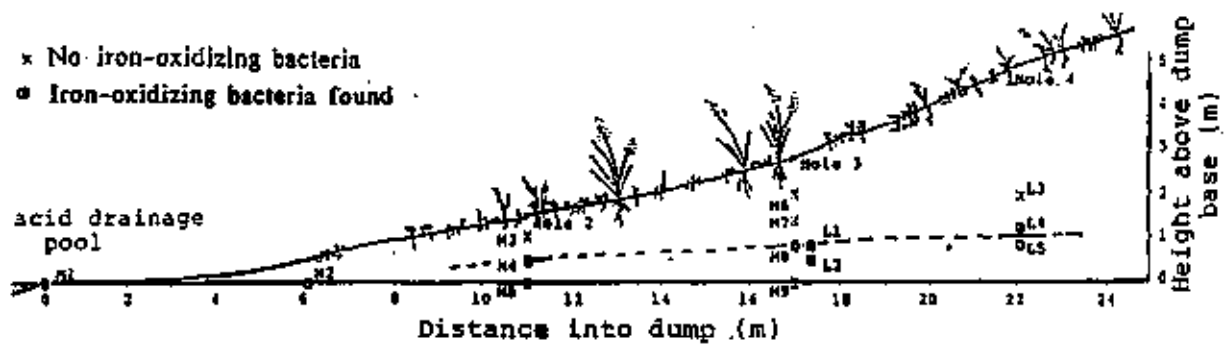


FIG. 19. Section through the City Deep 3A17 sand dump showing positions of the sampling sites and where iron-oxidizing bacteria were found.

TABLE 6. Moisture, pH and counts of iron-oxidizing bacteria for samples collected in March (M1 to 9 samples) and June (L1 to 5 samples) 1985 from the City Deep 3A17 sand dump

Sample	Moisture g/100 g wet sand ^a	pH in water ^b	Iron-oxidizing bacteria MPN/g dry sand ^c
M1	30,16	2,44	$2,2 \times 10^5$
M2	25,84	2,38	$8,2 \times 10^5$
M3	5,30	3,72	0
M4	21,96	2,49	$1,2 \times 10^2$
M5	27,19	2,66	$3,3 \times 10^3$
M6	4,46	3,57	0
M7	3,42	3,83	0
M8	16,00	2,56	$1,8 \times 10^4$
M9	26,27	2,94	0
L1	21,37	2,76	$3,7 \times 10^1$
L2	21,24	2,81	$1,4 \times 10^3$
L3	3,60	4,01	0
L4	8,28	2,79	$4,7 \times 10^2$
L5	14,13	2,69	$8,6 \times 10^3$

^aMean of duplicate determinations.

^bpH of 1:2,5 sand:water suspensions; mean of duplicate determinations.

^cDetermined from de Man (1983) MPN tables. See Whillier (1987) for statistical categorization of results and the theoretical 95% confidence limits. 0 = no iron-oxidizing bacteria detected in flasks containing up to 1 g wet sand.

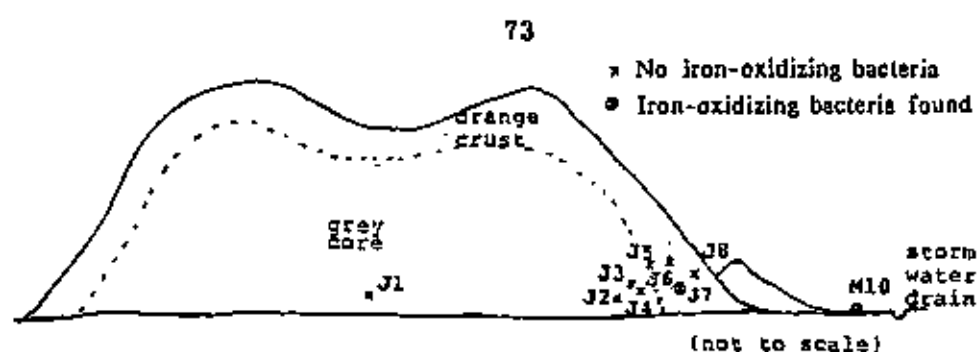


FIG. 20. Excavated western end of the Rand Mines Proprietary 3A8 sand dump showing positions of the 1984 and 1985 sampling sites and where iron-oxidizing bacteria were found.

TABLE 7. Moisture, pH and counts of iron-oxidizing bacteria for samples collected from the Rand Mines Proprietary 3A8 sand dump in July 1984 (samples J1 to 8), March 1985 (sample M10) and June 1985 (samples L6 and 7)

Sample	Sample position ^a and sand or slimes colour	Moisture g/100 g wet sand or slimes ^b	pH in water ^c	Iron-oxidizing bacteria MPN/g dry sand or slimes ^d
J1	120 m into dump; grey sand	7.34	3.59	0
J2	40 m into dump; grey sand	5.27	3.04	0
J3	30 m into dump; grey sand	5.73	2.92	0 ^e
J4	30 m into dump; grey sand, orange streak	5.57	2.83	0
J5	30 m into dump; orange sand	4.03	3.62	0
J6	25 m into dump; light grey sand	7.46	4.09	0
J7	20 m into dump; orange sand	5.76	3.69	1.2
J8	4 m below grassed surface; orange sand	4.84	3.57	0
M10	South drainage area, 20 cm deep; orange sand	6.56	3.15	2.6×10^2
L6	North-east slope; 20 cm deep; white and orange slimes	13.43	7.27	0
L7	North-east slope; 50 cm deep; yellow sand	3.01	4.66	0

^aApproximate horizontal distance from south edge indicated for samples J1 to 8.

^bMean of duplicate determinations.

^cpH of 1:2.5 sand or slimes:water suspensions; mean of duplicate determinations.

^dDetermined from de Man (1983) MPN tables. See Whillier (1987) for statistical categorization of results and the theoretical 95% confidence limits. 0 = no iron-oxidizing bacteria detected in tubes containing up to 1 g wet sand or slimes.

^eSome fungal growth in MPN tubes.

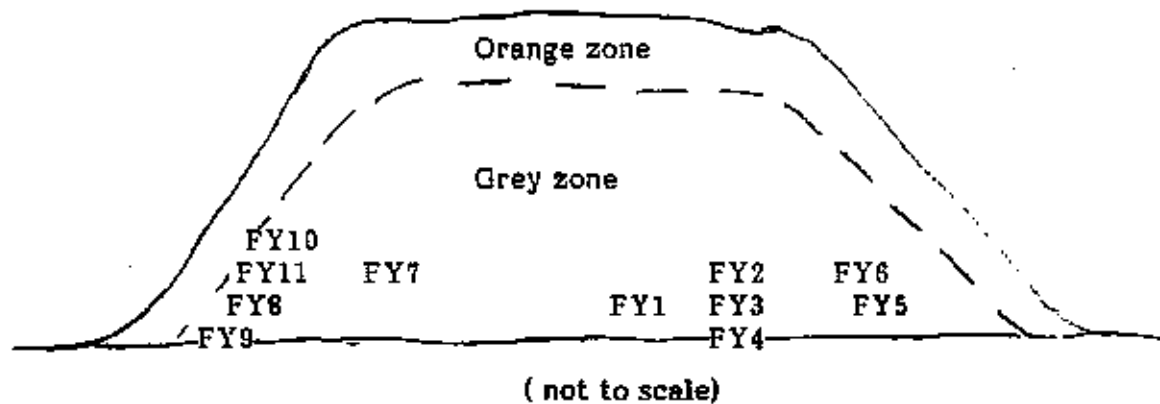


FIG. 21. Excavated western end of the Rand Mines Proprietary 3A8 sand dump showing positions of the February 1987 sampling sites.

TABLE 8. Source, description, moisture, pH and counts of iron-oxidizing bacteria for samples collected from the Rand Mines Proprietary 3A8 sand dump in February 1987

Sample	Source of sample	Colour and nature of sample	Moisture g/100 g wet sand ^a	pH in water ^b	Iron-oxidizing bacteria MPN/g dry sand ^c
FY1	Core of dump	grey sand	9,00	3,04	4.73×10^7
FY2	"	grey sand	9,00	2,75	2.64×10^7
FY3	"	grey sand	12,00	2,57	0
FY4	"	grey-orange sand	5,00	2,66	9.79×10^6
FY5	"	grey sand	10,40	2,33	2.68×10^7
FY6	"	grey-orange sand	5,40	2,67	9.83×10^6
FY7	"	grey-orange sand	7,60	2,58	2.27×10^7
FY8	Boundary between	grey-orange sand	12,00	3,91	4.89
FY9	grey core and	grey-orange sand	5,80	4,02	2.44
FY10	outer orange	grey-orange sand	9,40	4,43	1.03×10^2
FY11	zone of dumps	grey-orange sand	5,00	4,07	9.79×10^2

^aMean of duplicate determinations.

^bpH of 1:2.5 sand:water suspensions; mean of duplicate determinations.

^cDetermined from de Man (1983) MPN tables. See Bosch (1990) for statistical categorization of results and theoretical 95% confidence limits. 0 = no iron-oxidizing bacteria detected in flasks containing up to 1 g wet sand.

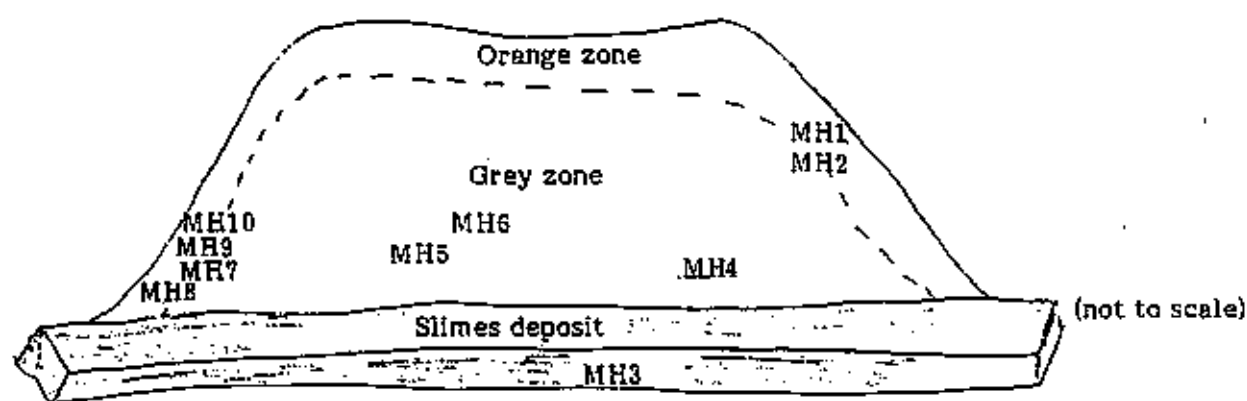


FIG. 22. Excavated western end of the Rand Mines Proprietary 3A8 sand dump showing positions of the March 1987 sampling sites.

TABLE 9. Source, description, moisture, pH and counts of iron-oxidizing bacteria for samples collected from the Rand Mines Proprietary 3A8 sand dump in March 1987

Sample	Source of sample	Colour and nature of sample	Moisture g/100 g wet sand or slimes ^a	pH in water ^b	Iron-oxidizing bacteria MPN/g dry sand ^c
MH1	Below vegetation on	orange sand	2,70	4,06	0
MH2	outside of dump	orange sand	8,40	3,23	2,51
MH3	Surface of slimes deposit, previously below excavated dump	red slimes	5,20	2,29	2,43
MH4	Core of dump	grey sand	6,60	3,00	2.25×10^3
MH5	"	grey sand	5,60	2,48	1.17×10^5
MH6	"	grey sand	7,40	3,00	4.64×10^5
MH7	Boundary between	orange sand	6,30	2,67	7.90×10^3
MH8	grey core and	grey-orange sand	6,50	2,27	7,91
MH9	orange outer	grey-orange sand	5,50	3,80	2,43
MH10	zone of dump	grey-orange sand	7,60	4,00	2,49

^aMean of duplicate determinations

^b1:2.5 sand or slimes:water suspensions; mean of duplicate determinations.

^cDetermined from de Man (1983) MPN tables. See Bosch (1990) for statistical categorization of results and theoretical 95% confidence limits. 0 = no iron-oxidizing bacteria detected in flasks containing up to 1 g wet sand or slimes.

The study on the 3A19 dump (Fig. 18, Table 5) showed a high population of iron-oxidizing bacteria only in sand sample A3 from the acid seepage area where high populations had been recorded previously in soil and water samples (section 3.2). This sample was saturated or almost saturated with acid drainage water, as indicated by the high moisture content (22,93% m/m) and low pH (2,34). The other samples showed no or (sample A7) almost no iron-oxidizing bacteria. These samples were all orange or yellow sand from the vegetated surface layer (0-30 cm deep) of the dump (non-rhizosphere samples); they were well-drained, rather dry (moisture 2,63 to 5,19% m/m) and with pH ranging from pH 3,61 to 6,70, much less acid than sample A3. Sample A7, which showed a very low level of iron-oxidizing bacteria, was taken from a small hollow which collected rain water and possibly retained moisture for longer periods than the other sampling sites on the dump.

Sample M1, from the acid seepage area of dump 3A17 (Fig. 19), also showed (Table 6) a high moisture content (30,16% m/m), low pH (2,44) and high population of iron-oxidizing bacteria ($2,2 \times 10^5$ /g). Most of the lower samples from the auger holes in the dump contained high populations of iron-oxidizing bacteria ranging from 2×10^3 to 1×10^8 cells/g sand. Samples M4, M8, L1, L4 and L5, with relatively high moisture contents, were from a distinct coarse layer of rust-coloured sand containing detectable clay. Samples M3, M6, M7 and L3, from the upper regions of auger holes in the 3A17 dump, showed no detectable iron-oxidizing bacteria. These samples also showed lower moisture levels and higher pH than the other 3A17 samples. Sample M9, from the bottom of an auger hole, had a low pH and high moisture content, but no iron-oxidizing bacteria were detected. However, the general pattern was that the presence of iron-oxidizing bacteria in large numbers corresponded to high moisture and low pH in the samples.

Among the samples collected in July 1984 from the excavated face of sand dump 3A8 (Fig. 20, Table 7), only one sample (J7) close to the grey-orange transition zone contained iron-oxidizing bacteria, but the count was low (1,2 viable cells/g dry sand). Both the moisture contents (4,03 to 7,46 % m/m) and pH (2,83 to 4,09) of these samples were low. Sample M10, collected alongside dump 3A8 soon after a rain storm in March 1985, showed $2,6 \times 10^2$ iron-oxidizing bacteria per g dry sand, but samples L6 and L7, collected in June 1985 from and just above the interface with the old slimes dam on which the 3A8 dump was constructed, showed no iron-oxidizers.

The results for the February and March 1987 samples from the 3A8 sand dump are recorded in Tables 8 and 9. Ten of the 11 February (FY) samples and five of the 10 March (MH) samples showed populations of iron-oxidizing bacteria. The highest populations ($>10^6$ /g) in the FY samples were in the highly acid (pH <3.04) grey or grey-orange samples from the inner regions of the dump, while the less acid (pH >3.91) orange samples from the boundary between the inner grey and outer orange regions contained lower populations. The MH samples with iron-oxidizing bacteria were all highly acid (pH <3.0), grey or grey-orange samples from the inner region (MH4 to 6) or orange samples from the boundary between the grey and orange regions (MH7 and 8). However, in the MH samples also, the MPN counts of iron-oxidizing bacteria were higher for the samples from the inner unoxidized core of the dump than for those from the boundary between the core and the outer oxidized zone of the dump. Samples FY3, MH1 to 3, MH9 and 10 showed no detectable iron-oxidizing bacteria, even though FY3 and MH3 were highly acid. There was no obvious relationship between the moisture content of the sand and the presence or absence of iron-oxidizing bacteria; however, all the sand samples with these bacteria were moderately moist (at least 5.0% m/m moisture). The FY1 to 7 samples were kept at room temperature for 10 days between sampling and analysis, providing considerable opportunity for the multiplication of iron-oxidizing bacteria in the samples during that time. By contrast, the FY8 to 11 and all the MH samples were at room temperature for only 1 day before they were analysed.

9.4 Discussion

The high MPN count of iron-oxidizing bacteria in sample A3 from near the drainage pool below the Nourse Mine 3A19 sand dump confirmed the results of section 3.3.2. The presence of oxidized iron was evident from the orange-brown colour of the water and the sand sample, which was also highly acidic (pH 2.34). The observed high moisture content and low pH of sample A3, which probably also had a high initial ferrous iron substrate content (Kleinmann et al., 1981; Thompson, 1980), would have provided good growth conditions for iron-oxidizing bacteria such as *T. ferrooxidans* (Conradie, 1984). However, conditions were quite different in the shallow samples (A4 to 10) collected from the top and sides of the 3A19 dump. The moisture content of all samples was low ($<5.19\%$ m/m) and the pH variable (pH 3.61–6.70), probably as a result of the extensive liming that preceded vegetation of the dump. Low moisture content during periodic drying, the relatively high pH and especially lack of ferrous iron substrate (from oxidation of the pyrite shortly after establishment of the dump) in the surface sand, coupled with the demonstrated growth-retarding properties of the sand (section 4), would have been unfavourable conditions for *T. ferrooxidans* and could

explain the very low population of iron-oxidizing bacteria in the A7 sample and its absence from the others.

Among the samples from the City Deep 3A17 sand dump, samples M1, M2 and M5, with high iron-oxidizing bacterial populations, were from a saturated layer at the base of the dump. As these March samples were collected within 12 hours of a large rain storm, this saturated layer may have represented an elevated water table, or the normal water table. Lack of iron-oxidizing bacteria in sample M9 from the base of the dump but farther from the outer edge, may have been the result of limited gas exchange nearly 3 m below the surface in a saturated environment. Samples M4, M8, L1, L4 and L5, with relatively high moisture contents and iron-oxidizing bacterial populations, were from a higher level within the dump in a distinct coarse layer of rust-coloured sand containing detectable clay. This layer may have been the uppermost level of the water table in the dump, or a water channel carrying subsurface drainage water through the dump, or a layer of deposited material leached from the sand above, similar to the compacted sediment which may form an "oxygen barrier" below the surface layer of coal piles (Dugan, 1975). Studies on desert sand have shown that clay compacts below the surface in a B horizon (Zaslavsky and Sinai, 1981). This suggests that there could be "B horizons" within mine dumps, exemplified by the rust-coloured layer of clay-containing sand sampled at the 3A17 dump. Between this layer and the saturated sand at the base of the dump lay yellow sand from which samples M5, L2 and L5, also with a high moisture content, were taken. Moderate populations of iron-oxidizing bacteria in these samples suggest that the rust-coloured layer was not impermeable to oxygen diffusion, i.e. that it did not form an oxygen barrier. Samples M3, M6, M7 and L3, which contained no iron-oxidizing bacteria, were yellow sand from above the rust-coloured layer. They were drier and tended to have a higher pH than the deeper samples from the rust-coloured layer and the base of the dump. Overall our observations suggest that pyrite oxidation was no longer proceeding in the portion of the 3A17 dump which was sampled, but that the iron-oxidizing bacterial populations in the rust-coloured and deeper samples were growing in acid effluent water containing ferrous iron formed deeper in the dump.

Samples were collected along the freshly exposed face of the excavated Rand Mines Proprietary 3A8 dump in an attempt to study acidification and the presence of iron-oxidizing bacterial populations deep within the dump. Surprisingly, iron-oxidizing bacteria were detected in only very low numbers in just one of the J1 to 8 samples from the face. The pH of these samples was acid (pH 2.92 to 4.09), but the moisture contents were less than 7.5% (m/m). The pH was thus suitable for *T. ferrooxidans*, but moisture

and oxygen may have been limiting (the latter before exposure of the face). July, in the middle of the dry season, may have been a completely unsuitable time to have taken the samples.

The outer zone of the 3A8 mine dump was orange in contrast to the grey sand of the central core region (Figure 20). The orange colour was shown, during the course of this study (see section 10.3), to be correlated with loss of sulphur, which can be ascribed to pyrite oxidation and the loss of oxidation products by leaching. The presence of orange, oxidized iron does not necessarily imply the presence of iron-oxidizing bacteria, as they may long since have disappeared. It may also be possible for ferric ions produced near the dump surface to leach into the dump with acid and to chemically oxidize pyrite in regions where bacteria are not present (Dugan, 1975). The J1 to 8 samples were taken mainly from the orange to grey transition zone in the dump where the possibility of finding iron-oxidizing bacteria should have been greatest. Notwithstanding the almost complete absence of these bacteria from the J1 to 8 samples and the absence of acid drainage pools around the 3A8 dump, acid drainage from the dump seemed to be occurring, as indicated by the high MPN count of iron-oxidizing bacteria in sample M10 of March 1985. The close proximity of a storm water drain to the M10 sampling point was probably responsible for the lack of drainage pools in the area and the low moisture content of the M10 sample. Acid drainage from the 3A8 dump was apparent on the north-east slope of the dump where vegetation below a sand-slimes interface showed signs of acid burn. However, the L6 and L7 samples taken in June 1985 at this interface contained no detectable iron-oxidizing bacteria. The slimes sample, L6, had a relatively high moisture content (13.43% m/m) and small pore spaces which affect gas diffusion, as well as a pH of 7.27. This high pH was probably responsible for the absence of iron-oxidizing bacteria and appears to contradict the suggestion that the sand-slimes interface was an area of acid seepage. However, water might seep from the sand and run down the slimes slope with minimal infiltration, because of the low permeability of the slimes. Sample L7 was sandy with a not too unfavourable pH for *T. ferrooxidans*, but with a low moisture content (3.01% m/m) that may have influenced the bacteria detrimentally. A further slimes sample (MH3) taken in March 1987 from the surface of an old slimes dump on which the 3A8 dump had lain before being excavated, also showed no iron-oxidizing bacteria in spite of having a pH of 2.29.

The suggestion that the taking of samples J1 to 8 in the middle of the dry season may have been responsible for their general lack of iron-oxidizing bacteria is supported by the finding of considerable populations of these bacteria in many of the February (FY) and March (MH) 1987 samples (Tables 8 and 9), particularly in the grey and grey-

orange samples where unoxidized pyrite substrate would have been available as an oxidizable energy source. The six populations of $>10^6$ /g among the FY1 to 7 samples were considerably higher than the populations detected in the FY8 to 11 and MH4 to 8 samples, possibly because the 10-day delay with their analyses would have given the bacteria the opportunity to multiply in the sample bottles. The size of these FY populations at the time of sampling is thus uncertain, as is the size of all the iron-oxidizing bacterial populations in the dump before excavation of the face, which would have improved the supply of oxygen and created favourable conditions for growth of the aerobic *T. ferrooxidans*. Nonetheless, the ability of the bacteria to develop to high populations in dump sand containing unoxidized pyrite if the necessary moisture and oxygen are present, has been established. This finding, and the absence of iron-oxidizing bacteria from seven of the eight J1 to 8 samples collected in July 1984, suggest that the bacterial oxidation of pyrite in gold mine sand dumps is seasonal, occurring when moist warm conditions prevail in summer but not during the dry cold winters when these bacterial populations almost completely disappear. However, it is surprising that the samples from the boundary between the core and outer oxidized zone of the dump, i.e. from the 'oxidation front', seemed to contain lower and more sporadic populations of iron-oxidizing bacteria than samples of the grey core material. Possibly in the former samples the more readily oxidizable pyrite had been oxidized and only more resistant pyrite material was available as a substrate for bacterially catalysed oxidation.

The requirement of the iron-oxidizing bacteria for unoxidized pyrite, indicated in the present study, makes chemical inhibition of pyrite oxidation catalysed by these bacteria in gold mine sand dumps a daunting prospect. The pyrite in the old sand dumps has been oxidized to depths of ca. 10 m (Marsden, 1986) and to treat the sand dumps at these depths with adequate concentrations of inhibitor in the face of adsorption by the sand requires the development of a suitable delivery system which is unlikely to be cheap. Possible delivery strategies in relation to SLS adsorption receive attention in section 12.4. The fact that the sand dumps are being reprocessed for extraction of residual gold and converted to slimes renders questionable the spending of large amounts of money to alleviate a pollution problem which in any case will be solved by the dump reprocessing.

10. ANALYSIS OF TOTAL SULPHUR IN MINE DUMP 3A8 SAND SAMPLES

10.1 Introduction

In the studies of section 9 on the distribution of iron-oxidizing bacteria in gold mine sand dumps, we assumed that the pyrite in the outer orange- to yellow-coloured sand had been oxidized, but remained unoxidized in the grey sand in the cores of the dumps. This concept was based on the analyses of many borehole samples from sand dumps (Marsden, 1986) showing sand through much of the outer ca. 10 m of dumps with sulphur contents much lower than the 0,85 to 1,60% (m/m) recorded for a range of gold ores. Deeper in the dumps the sulphur contents were mainly within this range. The data indicate that with the oxidation of pyrite in the outer regions of dumps, the pyrite sulphur is lost, presumably as leached sulphate. To confirm the assumed relationship between sand colour and residual pyrite, our range of J1 to 8 samples, comprising sand from the grey core and orange outer zone of the excavated 3A8 sand dump, plus a further core sample (J9) and the G5 sand used in the inhibitor studies (sections 5 to 8), were subjected to total sulphur analysis.

10.2. Materials and Methods

The J1 to J8 sand samples from the Rand Mines Proprietary 3A8 sand dump, a further grey sand sample (J9) from the excavated face near the J1 site in the core of the dump and the yellow G5 sand from the base of the Nourse Mine 3A19 dump (section 4) were analysed for total sulphur using the LECO sulphur determination apparatus and method (LECO Corp., St. Joseph, MI 49085, U.S.A.). The sand samples were pulverized for 6 min and weighed in the combustion crucibles. The sample had a mass calculated to require 8 to 9 ml of titrant and did not exceed the recommended maximum mass of 0,5 g. An ignitor, vanadium pentoxide (V_2O_5 , LECO), was added to each crucible; approximately three-quarters of a pellet was used for each sample. The crucible was covered with a porous lid and placed in the LECO induction furnace. A solution consisting of 50 ml 2% (m/v) HCl and 15 ml 0,1 N KI-1% (m/v) sodium glycollate-starch solution (LECO) was tapped into the titration vessel. The titrant was 1,1125 g/l potassium iodate (KIO_3). Approximately 50% of the expected titrant volume was added to the titration vessel. The oxygen flow rate was adjusted to 0,4 l/min. Combustion was initiated after the previous steps had been carried out; its duration was set at 6 min. When the titration end point was reached and the furnace had switched off, the sample was removed from the furnace and the titrant volume recorded. One ml of KIO_3 titrant = 0,0005 g sulphur.

10.3. Results

Table 10 records the total sulphur values for sand samples J1 to 9 from the excavated face of the 3A8 sand dump, and G5 from the east side of the 3A19 dump. The grey-coloured sands (samples J1 to 4 and J9) had sulphur contents ranging from 1,11 to 1,87% (m/m). The orange and yellow sands (samples J5, J7, J8 and G5) had sulphur contents from 0,08 to 0,19% (m/m). The light grey sand (J6) had a sulphur content of 0,13% (m/m), falling within the range of the orange sands. Thus, the outer, oxidized orange layers of the 3A8 dump were characterized by sand with a very low total sulphur content, while the deeper, unoxidized or largely unoxidised grey zone contained sand with a much higher sulphur content.

10.4. Discussion

South African gold bearing ores contain ca. 1 to 3% sulphur (van Staden, 1979), with values often between 0,85 and 1,60% (Marsden, 1986). The sulphur contents of the grey sand samples from the 3A8 dump corresponded with these values. In view of the observation of Atkins (1978) that the pyritic sulphur content of seven pyritic ores from the British Isles and Spain averaged 95,6% of the total sulphur, it is not unreasonable to assume that most of the sulphur in the gold ores and dumps was pyrite sulphur. Oxidation of pyrite produces, with ferric iron, sulphuric acid which leaches out of the sand dumps as acid drainage water. The result is a decrease in the amount of sulphur in the dumps. Ferrous sulphate oxidation by *T. ferrooxidans* in the laboratory can be monitored by the development of jarosite deposits and a deep orange colour in the medium. The change of colour in the sand from grey to orange, that correlated with a decrease in total sulphur, is therefore ascribed to pyrite oxidation.

Earlier analyses of material from a mine dump in South Africa by Hahne et al. (1976) produced results in conflict with these observations. Three sand samples were analysed, namely, a yellow and a grey sample from the dump and a brown sample from the drainage area at the base of the dump. Although the highest iron concentration was found in the grey sand, in keeping with the results reported here, the sulphate concentration in this sample was found to be tenfold greater than in the yellow and brown samples. If the analysis value for total sulphur had been used as an indicator of pyrite sulphur, this sulphate would have been registered as high unoxidised pyrite. The high sulphate concentration was no indication of a high degree of pyrite oxidation in the grey sample, as sulphates may have collected in this area through incomplete leaching.

TABLE 10. Colour and total sulphur analysis of ten mine dump sand samples

Sample	Colour	S in SO ₂ (%)	Total S (%) ^a
J1	grey	1,35	1,39
J2	grey	1,07	1,11
J3	grey	1,79	1,87
J4	grey	1,59	1,66
J5	orange	0,16	0,16
J6	light grey	0,12	0,13
J7	orange	0,19	0,19
J8	orange	0,13	0,14
J9	grey	1,18	1,22
G5	yellow	0,08	0,08

^aConversion from SO₂-S to total S required compensation for SO₃. This calibration factor was determined at the start of the experiment using a MINTEK 16/81 Sulphur Standard containing 1,15% S (SD 0,01). The calibration factor was 1,044.

However, this study raises the question: How much of the total sulphur in Table 10 was pyritic and how much was in the oxidised sulphate form?

Sample J6, a light grey sand, did not fit the pattern of high sulphur in the grey samples. Its sulphur content was in the range shown by the orange sands, but its colour was closer to that of the grey sands. This result becomes interesting when the samples are related to their positions in the mine dump. Samples J2 to J7 were close to each other in the transition zone between the grey core and the orange outer zone. Samples J2 to J4 were grey, or grey with orange streaks, J5 was orange, J6 light grey and J7 orange. The presence of a light grey area in the orange region may be explained in two ways. The light grey sand had a very low sulphur content, so it may be the tailings of an ore seam that was originally low in pyrite. Run-of-mine ore is processed in a continuous flow system so it is reasonable to suggest that tailings from successive seams were deposited with a degree of order. Alternatively, it might be a zone where pyrite was oxidized with the resulting orange deposits being subsequently leached from the sand. Why deposits would leach so completely from one zone and not another is not clear, but this light grey area may have formed a waterway through which rainwater was channelled, thereby enhancing leaching. This idea is supported by the moisture content of this sample (7.56%), which was marginally higher than that of the other samples from the exposed face.

The determinations of total sulphur in the J1 to 9 samples show that the interface between oxidized and unoxidized pyrite was about 30 m (horizontal distance) from the side of the dump, where the transition from orange to grey sand occurred. According to Mrost (1970), air in sand dumps is believed to be stagnant, i.e. diffusion is severely limited, at depths greater than 3 to 5 m. No data were presented in support of this suggestion, but if it is correct, the oxidation of pyrite would not be expected below this depth. However, core samples from sand dumps on the Witwatersrand have shown varying depths of oxidation down to 18 m (Marsden, 1986), which suggests that air is not always stagnant below 5 m. As air diffusion is a function of a number of factors, including pore size and atmospheric pressure, it may not be unusual for pyrite oxidation to occur below 5 m in sand dumps. Calculations recorded in section 9 suggest that the samples taken 30 m (horizontal distance) into the dump at the oxidation front were within ca. 13.5 m of the dump surface, hence within the range of previously recorded oxidation zone depths.

11. EFFECT OF MINE DUMP SAND DRYING ON THE VIABILITY OF *T. FERROOXIDANS* AND IRON-OXIDIZING BACTERIA FROM MINE DUMP SEEPAGE

11.1. Introduction

The observations reported in section 9 on the occurrence and distribution of iron-oxidizing bacteria in gold mine sand dumps, suggested that these bacteria developed only when moisture was adequate (in seepage zones or during the wet season) and where reduced iron was available as a substrate (dissolved ferrous iron in effluent water in seepage zones or iron pyrite in the unoxidized grey sand in the centre of a dump). Our studies of section 4 indicated a detrimental influence of oxidized mine dump sand on *T. ferrooxidans*, even where it was growing under favourable moisture and substrate conditions in HJJ medium.

In analogous studies with coal wastes, Olson et al. (1981) reported the disappearance of iron-oxidizing bacteria from acid coal spoils in Montana with change of season from wet spring to dry summer, while Brock (1975) showed partial inhibition of iron oxidation by *T. ferrooxidans* at a water potential of -23 bars and complete inhibition at -32 bars. In the study of Belly and Brock (1974), declines in iron-oxidizing bacterial numbers and autotrophic fixation of CO₂ in 40-year-old coal waste from maximum levels in 3-year-old coals waste possibly reflected exhaustion of energy-yielding substrate or the accumulation of toxic materials over the years.

With such indications that dry oxidized mine dump sand might be a highly stressed environment for chemolithotrophic iron-oxidizing bacteria such as *T. ferrooxidans*, we investigated their viability in an oxidized and an unoxidized sand on drying.

11.2. Materials and Methods

A 20% (v/m) inoculum of a 3-day-old culture of *T. ferrooxidans* ATCC 19859 in HJJ medium was inoculated into 150-ml PVC breakers containing 100 g J9 (5.4% moisture; mainly unoxidised) or G5 (0.7% moisture; highly oxidised) sand (see sections 4 and 10). The beakers were incubated at 26°C. After 0, 10 and 34 days, MPN counts of *T. ferrooxidans*, moisture and pH determinations were performed on the contents of duplicate beakers. An identical experiment was conducted using an inoculum consisting of a mixed population of iron-oxidizing bacteria from acid seepage, prepared as for the

experiment on the effect of SLS on these organisms (section 5.2.1), but readings were taken at 0, 4, 9 and 17 days, to obtain data at shorter time intervals.

In order to compare the effect of the two sands, J9 and G5, with high and low pyrite contents, respectively, on a *T. ferrooxidans* population in the presence of adequate moisture, the experiment using *T. ferrooxidans* ATCC 19859 was repeated with the moisture in the beakers replenished after 4, 7, 11 and 14 days by adding 10 ml sterile distilled water. Duplicate samples were analysed at regular intervals as for the previous experiments.

11.3. Results

The effect of drying at 26°C on the viability of *T. ferrooxidans* ATCC 19859 in the two mine dump sands is illustrated in Fig. 23. Similar total losses of moisture were observed from both sand types during the first 10 days. At this stage the G5 oxidised sand was dry and the J9 unoxidised sand almost dry; however, moisture loss from the J9 sand was negligible during the next 24 days. No viable *T. ferrooxidans* cells were detected in the G5 sand on day 10, but only a small decrease in the MPN count in the J9 sand was observed by that time. At day 34, no viable *T. ferrooxidans* cells were detected in the J9 sand.

The effect of the drying of the sands on the viability of a mixture of iron-oxidizing bacterial populations enriched from acid mine seepage is illustrated in Fig. 24. By using shorter intervals between sampling times, no decrease or even an increase in the iron-oxidizing bacterial count after 4 days was shown. On day 9 the moisture in the G5 oxidised sand had decreased to 1,16% and the iron-oxidizing bacteria count to less than 0,1% of the initial count, while the count in the J9 unoxidised sand, which still contained 6,08% moisture, was only slightly lower than the initial count. By day 17 both sand types contained less than 1% moisture and no detectable viable iron-oxidizing bacteria.

That the viability of *T. ferrooxidans* ATCC 19859 was maintained when the sands were not subjected to drying, was shown in the experiment in which the moisture in the beakers was replenished for the first 14 days. The bacterial counts remained high during this period (Fig. 25), decreasing only after the moisture was allowed to evaporate. The decrease in viable bacteria was faster in the G5 oxidised sand than in the J9 sand containing residual pyrite; there was a slightly faster moisture loss from sand G5, which

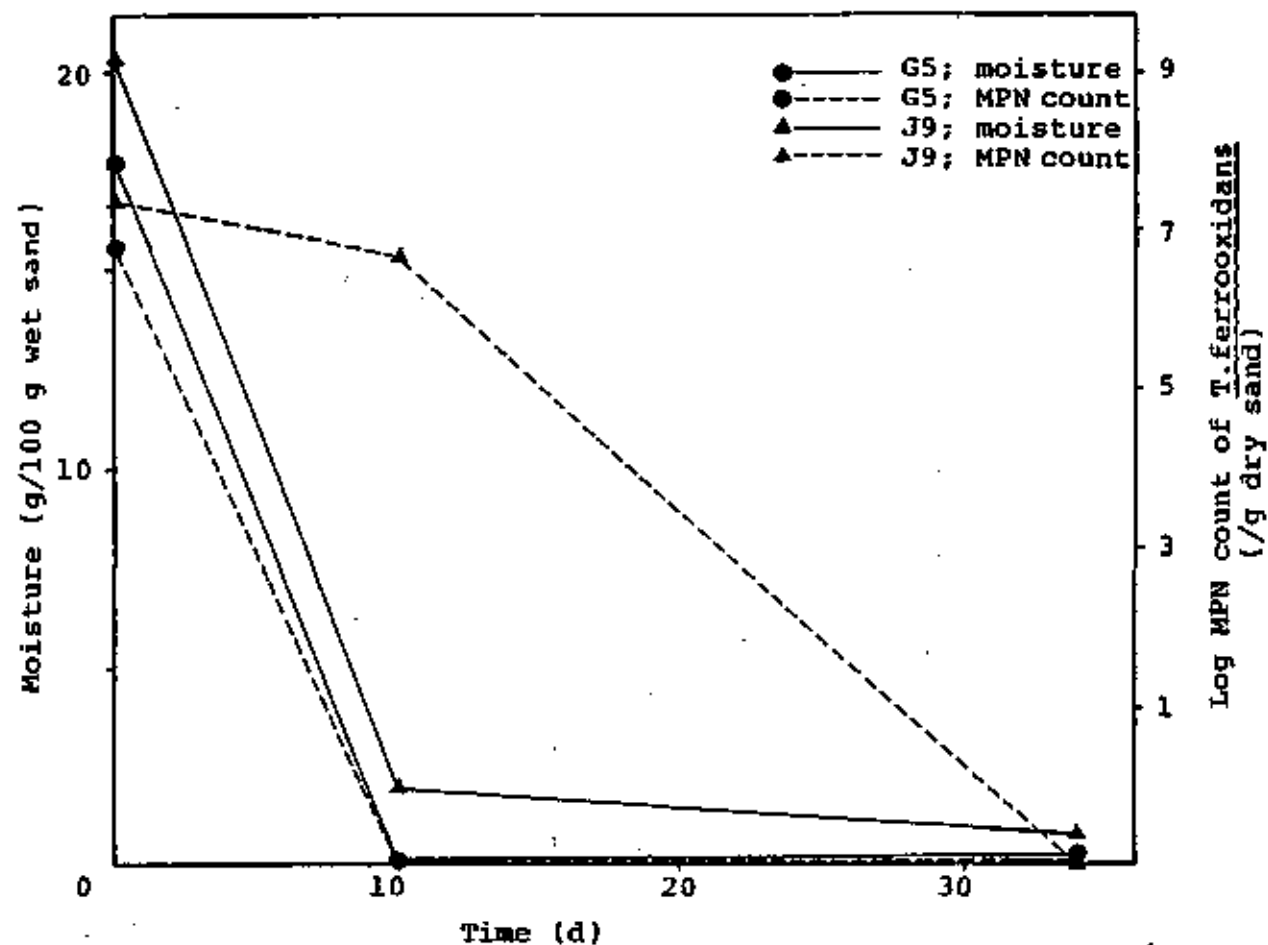


FIG. 23. Effect of sand drying at 26°C on moisture and log MPN of *T. ferrooxidans* ATCC 19859 in G5 oxidized and J9 unoxidized mine dump sand.

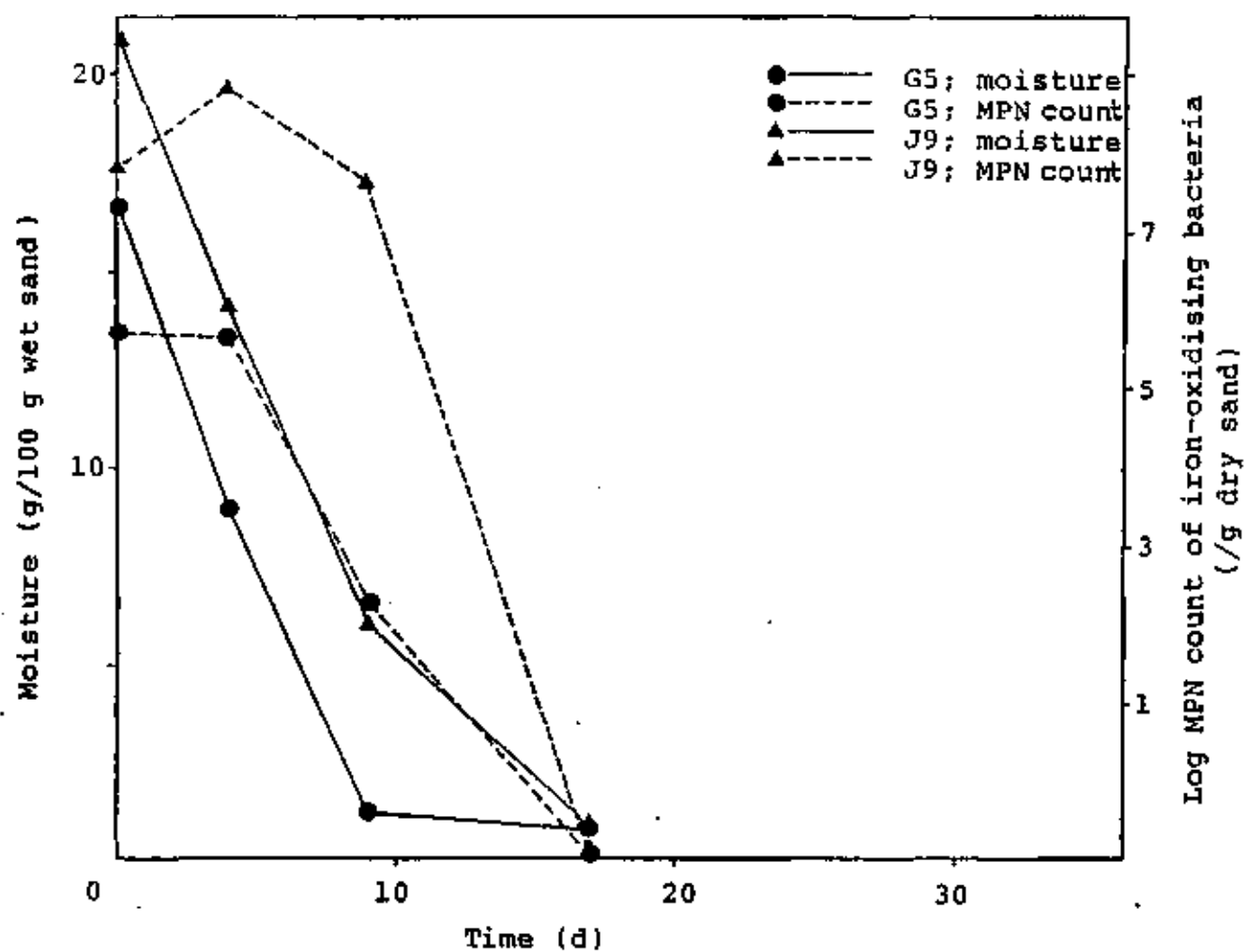


FIG. 24. Effect of sand drying at 26°C on moisture and log MPN of iron-oxidizing bacteria, from a mixture of enrichment culture populations from sand dump seepage, in G5 oxidized and J9 unoxidized mine dump sand.

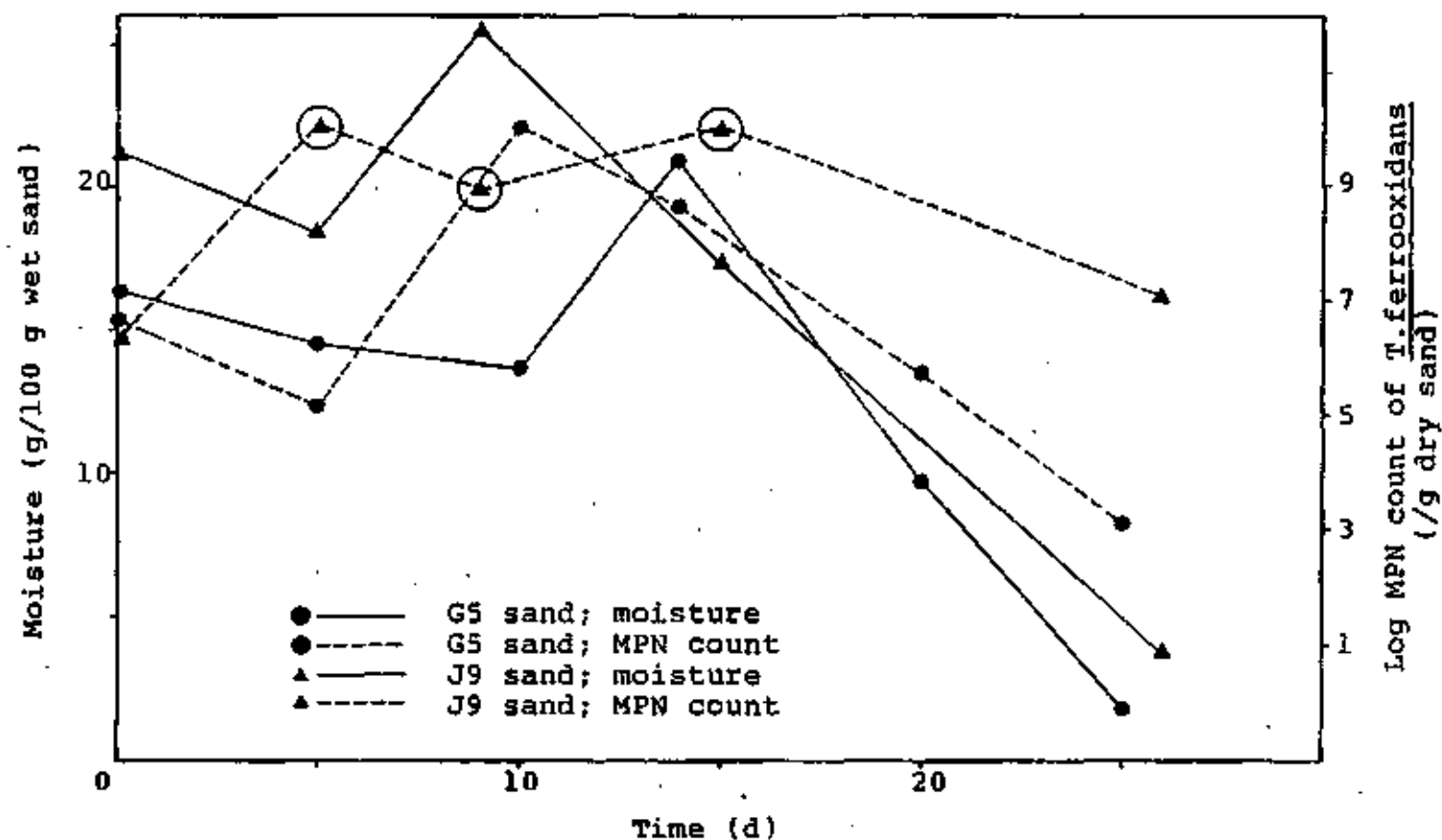


FIG. 25. Effect on moisture and log MPN of *T. ferrooxidans* ATCC 19859 in G5 oxidized and J9 unoxidized sand of periodic re-moistening of the sand for 14 days before allowing it to dry at 26°C. Encircled MPN points indicate where growth occurred in all flasks throughout the dilution series; the counts would be equal to or greater than the values plotted.

had a coarser texture than the J9 sand. The pH of the sands remained fairly constant during the experiments, within the range pH 2,2 to 2,9.

11.4. Discussion

The distribution of iron-oxidizing bacteria in samples from sand dumps (section 9, Tables 5 to 9) indicated that a low moisture content of the mine dump sand might adversely affect iron-oxidizing bacterial concentrations. This was suggested by the consistently low moisture levels (less than 6% m/m) of sand samples that showed no iron-oxidizing bacteria.

Rapid dying of *T. ferrooxidans* ATCC 19859 and the mixed iron-oxidizing bacterial population was observed in drying sand by day 10 (Fig. 23 and 24). Bacteria in J9 sand, containing unoxidized pyrite, showed a slower death rate than those in the oxidised G5 sand. This result suggested that die-off may be related to a lack of energy source as well as to loss of moisture. When moisture was maintained in the sand, the viability of the bacteria in the oxidised sand remained high up to day 14, after which the moisture level was allowed to drop (Fig. 25). As the moisture evaporated, the viability of the bacteria in the oxidised G5 sand also fell rapidly, confirming that moisture is important in determining the presence of *T. ferrooxidans* in mine dump environments. In the J9 sand, where pyrite substrate was apparently available, the viability of the bacteria was still high at day 26, when the moisture in the sand had dropped to 3,64%. This observation is a further indication that substrate availability may be an important factor determining iron-oxidizing bacterial viability in the mine dump sand, but as the two sands may have differed in other characteristics, this suggestion requires further investigation.

12. ADSORPTION OF SLS BY GOLD MINE DUMP SAND

12.1. Introduction

Our studies on the distribution of iron-oxidizing bacteria in gold mine sand dumps (section 9) indicated that they required unoxidized pyrite and moisture for their development, hence would multiply and produce acid at or deeper than the pyrite oxidation front at ca. 10 m depth (Marsden, 1986). Their development in seepage water, involving the oxidation of ferrous iron, would be secondary and dependent on the production of these ions during pyrite oxidation deeper in the dump. Inhibition of the bacteria causing acid drainage in sand dumps by application of an antibacterial chemical, such as SLS, will require its movement through at least the outer 10 m of dump sand to the sites of pyrite oxidation. Adsorption of the inhibitor to the sand is an important factor determining whether an adequate concentration of the chemical can be delivered to these sites.

Kleinmann and Erickson (1983) found considerable adsorption of SLS to coal waste (45 mg SLS/kg waste). They also noted that about 10 times more surfactant was required to kill *T. ferrooxidans* in coal refuse than in the 9K medium of Silverman and Lundgren (1959a). In our inhibition experiments (sections 5 to 7), higher concentrations of inhibitors were required to prevent iron oxidation by *T. ferrooxidans* in HJJ medium containing gold mine dump sand than in the medium without sand. In the case of SLS, the minimum inhibitory concentration was doubled when sand was included in the medium (section 5). The analogy with the coal waste results suggested that adsorption of the SLS by the gold mine dump sand might lower the effective concentration of the inhibitor in the medium, thus raising the apparent minimum inhibitory concentration. Adsorption of SLS by the mine dump sand was studied quantitatively to provide data for assessing the effect of the sand on the SLS dose required for the inhibition of iron-oxidizing bacteria in gold mine sand dumps.

12.2. Materials and Methods

12.2.1. Adsorption experiments

Adsorption of SLS by 100 g G5 sand from the Nourse Mine was investigated by the solution deletion procedure described by Parfitt and Rochester (1983). The SLS was dissolved in distilled water to concentrations of 100, 500, 1000, 2000 and 3000 mg/l. The gold mine dump sand was shaken with 100 ml of the SLS solutions in 250 ml

Erlenmeyer flasks for 3 hours at 120 r.p.m. at 26°C. The flasks were then allowed to stand until the supernatants were clear, when 1 ml samples were removed for analysis of the SLS in solution as described in section 12.2.2. The adsorbed SLS was calculated. In further adsorption experiments, 50 g gold mine dump sand was tested with a similar range of SLS concentrations, as well as concentrations of 40, 60, 80, 200, 400, 600 and 800 mg/l.

12.2.2. Analysis of SLS in solution

The SLS in the samples was determined by the colorimetric measurement (648 nm) of methylene blue-SLS extracted into chloroform (Abbott, 1962) according to a modification of a procedure developed by the Chamber of Mines (D.D. Marsden, Pollution Control Division, Chamber of Mines, Johannesburg, personal communication). In this modified procedure, a stock solution of methylene blue was prepared by dissolving 100 mg methylene blue (May & Baker Ltd., Dagenham, England) in 100 ml distilled water. A working methylene blue solution was prepared by adding 50 g sodium dihydrogen orthophosphate dihydrate and 6.8 ml concentrated sulphuric acid to 500 ml distilled water in a 1-l flask; 30 ml methylene blue stock solution were then added and the flask filled to the 1-l mark with distilled water. A washing solution was prepared in a similar way but without the methylene blue stock solution. A phenolphthalein solution was prepared by dissolving 80 mg in 100 ml absolute methanol. A separating funnel was rinsed three times with distilled water and a 1-ml sample added, followed by 99 ml distilled water. A drop of phenolphthalein was added and the pH adjusted with 1N sodium hydroxide to pH 10. The solution was decolourized with 1N sulphuric acid until it was just colourless, when 25 ml of methylene blue working solution and 10 ml chloroform were added. After shaking for 30 seconds, the chloroform layer was separated into a clean separating funnel; the aqueous layer was then extracted twice more with chloroform which was added to the first extract. The pooled chloroform extract was washed with 50 ml of washing solution and drained into a 100 ml flask. The washing solution was extracted again with 10 ml chloroform, which was added to the pooled extract. The volume of the extract was adjusted to 100 ml with chloroform and the absorbance read at 648 nm on a Varian 634 spectrophotometer (Varian Techtron (Pty) Ltd., Perth, Australia). For the preparation of a standard curve, 100 ml volumes of aqueous solutions containing 10 to 400 µg SLS (corresponding to a 1 ml sample diluted to 100 ml) were treated with methylene blue and extracted in the same way.

12.3. Results

Figures 26 to 28 show the adsorption of SLS from 100 ml water in shaken flasks supplied with 100 or 50 g gold mine dump sand. If the SLS concentrations supplied and adsorbed are expressed as mg SLS/g sand as in Fig. 26 to 28, the adsorption curves from the three experiments approximately coincide. There was a high level of adsorption (62% to 94%) of the SLS supplied at concentrations of 1,0 to 6,0 mg/g sand, but at concentrations below ca. 0,1 to 0,6 mg/g sand, more SLS remained in solution than was adsorbed. However, although the latter trend was evident, the variation in analysed SLS concentrations in solution, at equivalent SLS doses, was high from one experiment to another at these low SLS levels.

12.4. Discussion

Adsorption of SLS to gold mine dump sand is clearly an important factor influencing the possible usefulness of this inhibitor for the control of acid mine drainage in sand dumps. In respect of the inhibition experiments of section 5, it can explain the increased concentrations of SLS needed to inhibit *T. ferrooxidans* ATCC 19859 and WLR in the cultures containing 500 g/l sand. The doubling of the concentrations of SLS needed to inhibit the bacteria in the presence of the sand suggests that not more than 50% of the SLS was adsorbed from the 100-ml cultures; in fact, considerably less than 50% was probably adsorbed, as the inhibitory SLS doses of 4 and 8 mg/l are equivalent to only 0,008 and 0,016 mg/g sand, thus within the concentration range at which more SLS remains in solution than is adsorbed. Even the SLS concentrations of 10-20 mg/l required to inhibit iron-oxidizing bacteria from sand dump acid seepage in 100-ml cultures with 50 g sand (0,02 to 0,04 mg SLS/g sand) fall well within that concentration range.

On account of the wide variation among experiments in the percentages of SLS adsorbed and in solution at the low SLS concentrations, it is not possible to relate apparent minimum inhibitory concentrations in cultures containing sand (section 5) to a calculated SLS concentration in solution, nor to predict accurately the dose of SLS required for dump treatment to inhibit iron-oxidizing bacteria. However, the experiment shown in Appendix Fig. 13 demonstrated that more than 20 mg SLS/l were required to inhibit *T. ferrooxidans* in 100 ml liquid cultures containing 100 g mine dump sand. This result can be used to calculate a dose of SLS which, assuming the SLS to be distributed evenly through the outer zone of the sand dump, will have to be exceeded for inhibition of iron-oxidizing bacteria 10 m deep in the dump. The volume of sand beneath 1 ha of

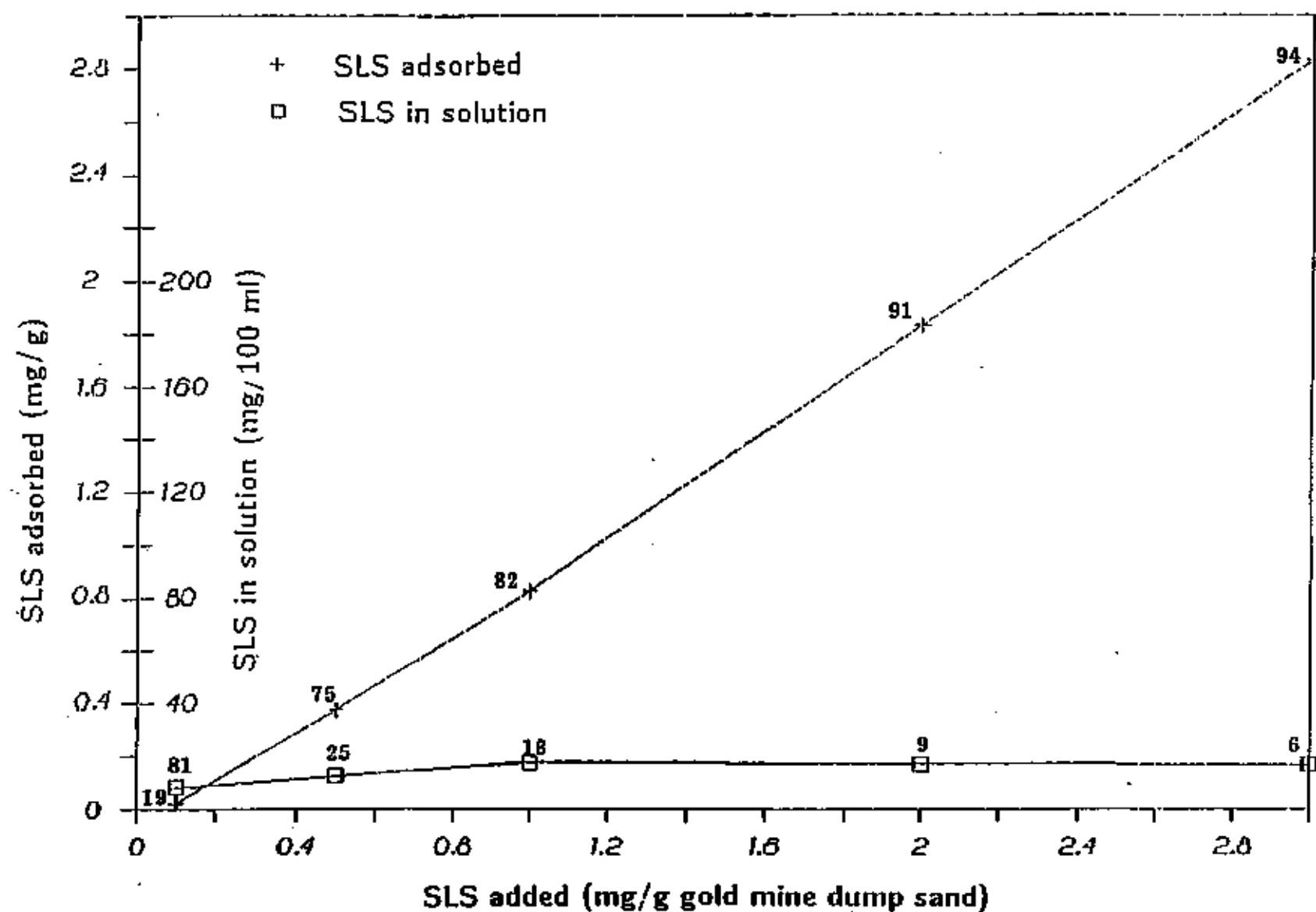


FIG. 26. Adsorption of SLS, supplied at 100 to 3000 mg/l in 100 ml aqueous solution, to 100 g mine dump sand G5 and residual SLS in solution. Figures alongside plotted points indicate percentage of supplied SLS that was adsorbed or remained in solution.

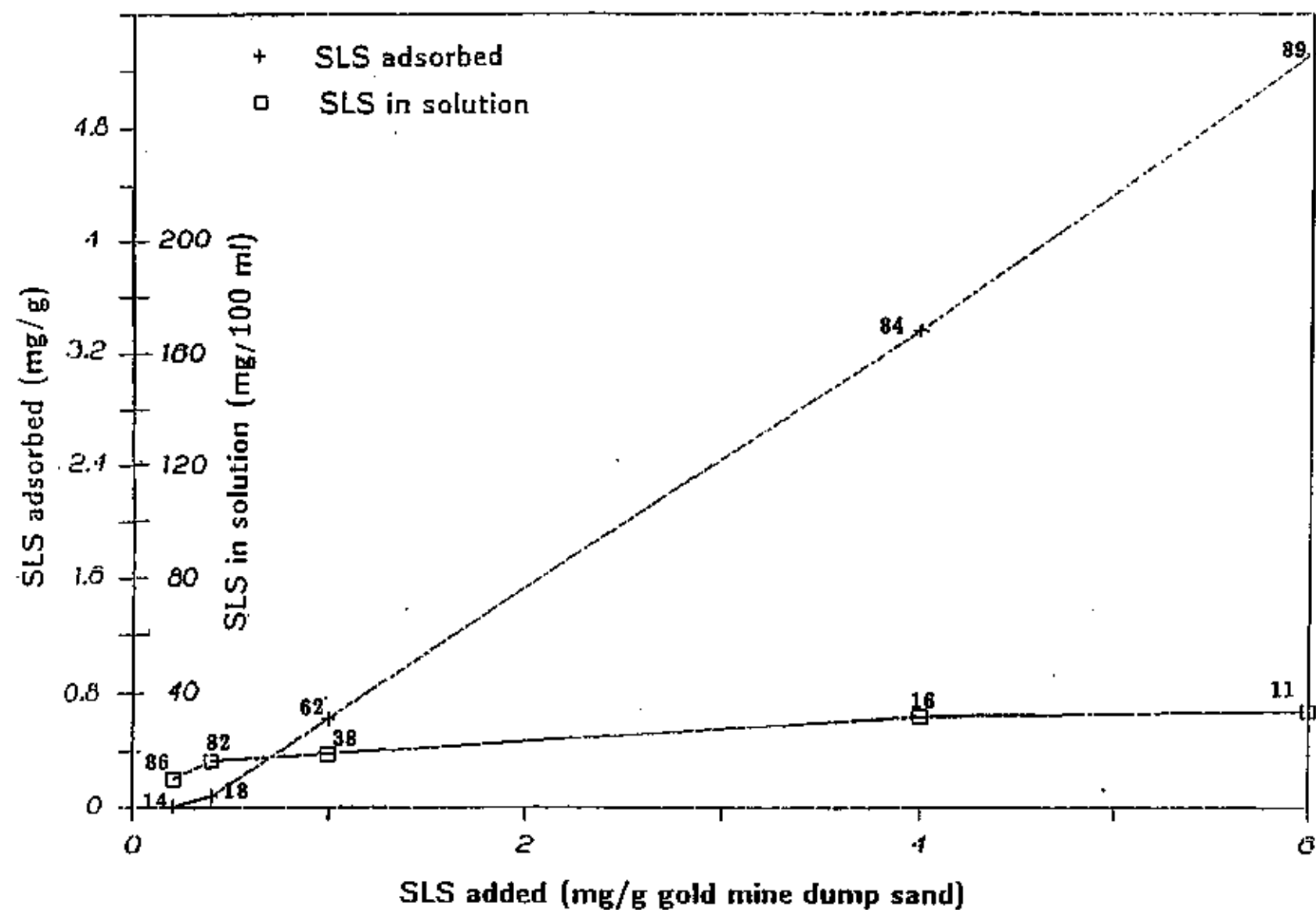


FIG. 27. Adsorption of SLS, supplied at 100 to 3000 mg/l in 100 ml aqueous solution, to 50 g mine dump sand G5 and residual SLS in solution. Figures alongside plotted points indicate percentage of supplied SLS that was adsorbed or remained in solution.

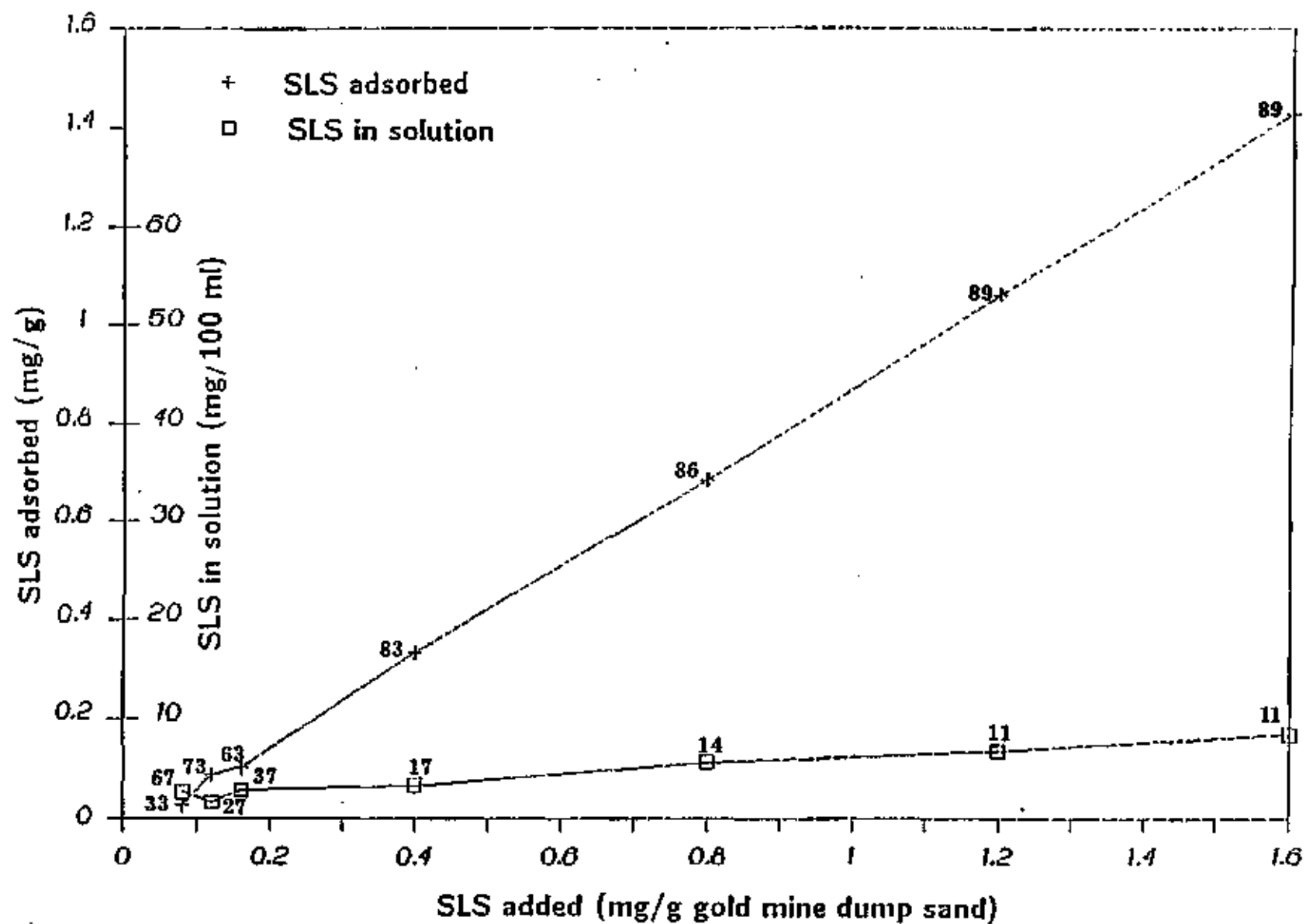


FIG. 28. Adsorption of SLS, supplied at 40 to 800 mg/l in 100 ml aqueous solution, to 50 g mine dump sand G5 and residual SLS in solution. Figures alongside plotted points indicate percentage of supplied SLS that was adsorbed or remained in solution.

dump surface to a depth of 10 m is 100 000 m³. The mass of this sand is about 115 000 t. Under dryish conditions the moisture content of the sand is about 5%, while under saturated or near saturated conditions along a seep, it is about 20% (section 9). At these moisture contents, 115 000 t sand hold 5 750 000 and 23 000 000 l water, respectively. If this water in the sand contains 20 mg/l SLS, 115 000 t sand will contain 115 and 460 kg SLS, respectively. Dosages of SLS to provide more than 20 mg/l to a depth of 10 m would therefore have to be greater than 115 and 460 kg/ha, respectively, under the two sets of conditions. Most sand in the dump does not contain as much as 20% moisture (section 9), so 460 kg/ha SLS will provide more than 20 mg/ml, but whether this will be adequate to inhibit the target bacteria requires further experimental evaluation.

The calculation in the previous paragraph assumes a delivery system which gives an even distribution of SLS through the dump profile. Whether such a distribution can be achieved, for example, by continued input from a slow release system, such as SLS-rubber pellets (Immelman, 1987; this report, part 3) and whether this approach would be the best method of treatment depends on the behaviour of the SLS as it leaches through the sand profile. Thus, if a dose of SLS should move through the sand as a band, treatment providing pulses of SLS at suitable intervals, giving waves of a bactericidal concentration of SLS at the site of pyrite oxidation, might be a better approach. Studies of SLS leaching behaviour in gold mine sand dumps, necessary for a full evaluation of SLS as a potential acid drainage inhibitor, have yet to be performed.

13. CONCLUSIONS

The seepage of acid water from Witwatersrand gold mine sand dumps, which released an estimated 50 000 t of salts into the catchment of the Vaal Barrage during 1985 (Jones et al., 1988), shows the characteristics of acid drainage from mine waste deposits elsewhere in the world. The pH of the water was below pH 3, sulphate comprised a large proportion of the tonnage of salts in the acid drainage (Jones et al., 1988) and the brown colour of the seepage water and precipitates in and around seepage pools indicated the presence of oxidized iron. That bacterially catalyzed iron oxidation was occurring in the seepage was indicated by the presence of populations of chemolithotrophic iron-oxidizing bacteria of ca. 10^6 to 10^7 /ml in drainage water and ca. 10^3 to 10^6 /g in the brown-coloured soil of a seepage area (section 3). It is presumed that the bacteria were *T. ferrooxidans* or contained a large proportion of *T. ferrooxidans* in a mixed population. These bacteria are the major iron-oxidizing bacteria of acid drainage elsewhere and the HJJ growth medium and conditions of incubation for the MPN estimates of the iron-oxidizing bacteria in sand dump seepage were selected for their suitability for *T. ferrooxidans* (section 2).

The gold mine sand dumps appear to be a not very favourable growth environment for *T. ferrooxidans* and other possible iron-oxidizing chemolithotrophic bacteria of acid seepage. Increasing concentrations of an oxidized sand (i.e. in which the pyrite had been oxidized) increasingly retarded the growth of *T. ferrooxidans* in HJJ medium (section 4). The drying of mine dump sand resulted in the rapid destruction of large inoculum populations of *T. ferrooxidans* and iron-oxidizing bacteria from acid seepage, the destruction being more rapid in an oxidized sand than in an unoxidized sand containing pyrite substrate (section 11). However, high populations of *T. ferrooxidans* were maintained when the sands were kept moist.

Of the compounds tested in laboratory cultures as possible inhibitors of *T. ferrooxidans* and mixed populations of chemolithotrophic iron-oxidizing bacteria from sand dump acid drainage, the anionic detergent SLS was the most effective. Inhibition of the most resistant cultures was achieved with 8 mg SLS/l in cultures containing no mine dump sand and with 20 mg SLS/l in cultures containing mine dump sand at 500 g/l (section 5). However, when the sand concentration was raised to 1000 g/l, 20 mg SLS/l only retarded ferrous iron oxidation by a less resistant *T. ferrooxidans* strain. Adsorption of the SLS by the sand (section 12) can explain, or partly explain, this result. The sand-inhibitor interaction must receive further attention as a major factor determining the inhibitor dose and method of application for the control of acid drainage from sand dumps.

The anionic detergent LAS was almost as effective as SLS as an inhibitor of *T. ferrooxidans* and iron-oxidizing acid drainage bacteria in cultures without mine dump sand (LAS at 8 mg/l inhibited all cultures), but with 500 g/l sand in the cultures was less effective than SLS in that not all cultures were inhibited by 30 mg/l LAS (section 5). This result provides no grounds for considering LAS as an alternative inhibitor to SLS for the control of acid drainage formation in sand dumps, but attention has not been given to economic considerations, which would also necessitate further comparison of the effectiveness of LAS and SLS.

Among the cationic surface-active compounds tested (section 6), the low pH antimicrobial quaternary pyridinium compound Ceepryn was more effective than SLS against *T. ferrooxidans* ATCC 19859 in cultures without sand (2 mg Ceepryn/l inhibited all cultures), but was considerably less effective in cultures containing 500 g/l sand, with 20 to 40 mg/l the minimum inhibitory concentration (in contrast to 8 mg SLS/l for the same *T. ferrooxidans* strain). The quaternary ammonium formulation Hyamine 3500 showed only partial or temporary inhibition of *T. ferrooxidans* and the acid drainage bacteria at the highest concentration tested (128 mg/l), even in cultures without mine dump sand. However, acidity is known to be highly detrimental to the activity of quaternary ammonium compounds (Hamilton, 1971). Adsorption to surfaces is a general characteristic of the quaternary pyridinium and ammonium compounds, reducing their antimicrobial activity (Sykes, 1965), thus the reduction of the effectiveness of Ceepryn shown in the cultures with 500 g/l sand would likely be greatly enhanced in a sand dump. Evidence available to date provides no grounds for considering Ceepryn as a possible alternative to SLS for the combatting of acid drainage in sand dumps.

The food preservative organic acid inhibitors, benzoic and sorbic acid, are inherently less effective inhibitors of *T. ferrooxidans* in respect of minimum inhibitory concentrations than the surface-active chemicals, SLS, LAS and Ceepryn. The minimum inhibitory concentrations of sodium benzoate and sorbic acid in cultures without mine dump sand were 15 to 30 and 15 to 20 mg/l, respectively; these concentrations increased to 25 to 35 and 20 to 30 mg/l when 500 g/l mine dump sand was included in the cultures (section 7). The limited reduction of their effectiveness in the presence of the sand indicates that they have the advantage of little adsorption to the sand. It appears that doses of benzoic or sorbic acid to inhibit *T. ferrooxidans* in sand dumps would need to be about four times that of SLS, making even the cheaper benzoic acid probably not an economical alternative inhibitor to SLS.

Sodium lignosulphonate and polyacrylic acid had such limited activity against *T. ferrooxidans* (section 8) that they cannot be regarded as possible inhibitors of acid drainage formation in sand dumps.

Investigations of the distribution of chemolithotrophic iron-oxidizing bacteria in sand dumps (section 9) showed large populations only in wet regions where acid drainage water was seeping through the sand at the base of the dump and emerging at the surface or in the interior grey-coloured sand exposed on excavated faces where the pyrite had not yet been oxidized to sulphate and yellow or orange oxidized forms of iron. The outer orange-coloured layer of sand dumps away from seepage zones contained almost no iron-oxidizing bacteria nor sulphur (section 10), both observations indicating that the pyrite substrate on which the bacteria grow had been lost from this part of the dumps during more than half a century of oxidation. As this process has proceeded to depths of ca. 10 m in sand dumps (Marsden, 1986), the iron-oxidizing bacteria must be catalysing pyrite oxidation at or beyond this depth. Bacterial inhibitors to control acid production in the dumps must therefore be delivered in inhibitory concentrations to at least this depth. The absence of iron-oxidizing bacteria from most winter samples from the exposed face of the excavated 3A8 dump, in contrast to their abundance in similar (but moister) grey-coloured samples in summer, suggests that pyrite oxidation is seasonal. It is possibly negligible in winter as drying of the dump sand results in rapid death of the iron-oxidizing bacteria (section 11).

The delivery of an inhibitor such as SLS to the sites of pyrite oxidation at least 10 m deep in sand dumps and the effectiveness of the inhibitor in the presence of the sand will be influenced by adsorption of the inhibitor to the sand. Although mine dump sand (50 or 100 g) adsorbed most of the SLS supplied in 100 ml water at concentrations of 1,0 to 6,0 mg/g sand, less than half the SLS supplied at below ca. 0,1 to 0,6 mg/g sand was adsorbed. Inhibitory SLS concentrations in laboratory cultures were below the latter concentrations, thus in the presence of sand at 500 g/l were only about double the inhibitory concentrations in the cultures without sand. Inhibitor delivery strategy will have to take into account adsorption and movement of the inhibitor in the sand dump. Continuous dosing of the dump from a slow-release formulation (Immelman, 1987; this report, part 3) is one possible strategy and dosing in repeated pulses another. Continuous dosing with SLS would require an estimated total application of more than 115 kg/ha mine dump surface and possibly more than 460 kg/ha (section 12). The cost of the SLS as priced in 1990 (locally produced formulation with 28% SLS, transport and application excluded) would be at least $R9,68 \times 115 = R1113/\text{ha}$ and possibly greater than $R9,68 \times 460 = R4453/\text{ha}$. Whether such applications are justified in view of the probable complete reprocessing of the sand dumps during the next 20 years, is questionable. However, more research is needed on the adsorption and movement of potential inhibitors in sand dumps for a reliable comparative assessment of the two strategies for any specific inhibitor.

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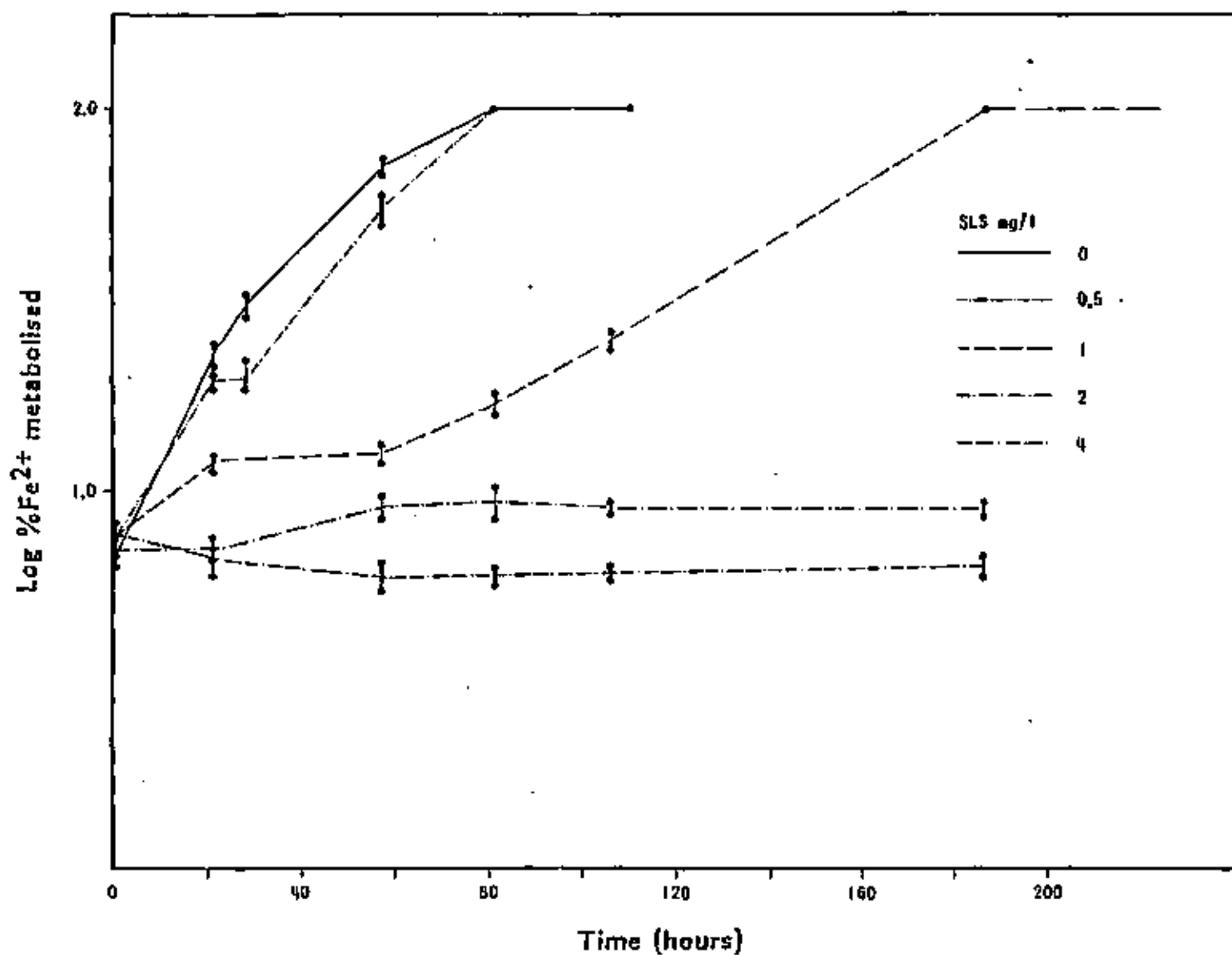
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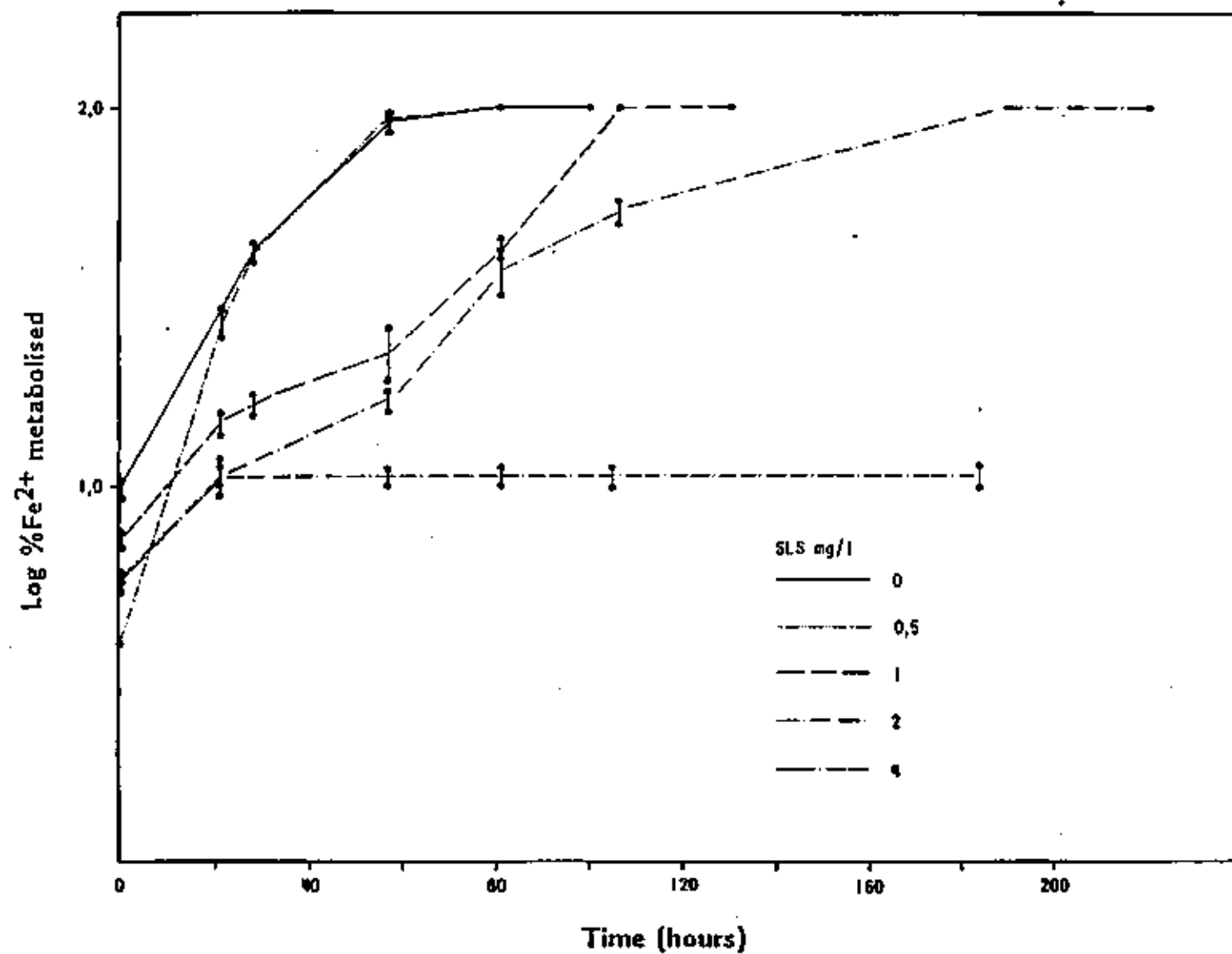
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**15. APPENDIX: COMPLETE RESULTS OF LABORATORY STUDIES OF CHEMICAL
INHIBITION OF FERROUS IRON OXIDATION BY *T. FERROOXIDANS*
OR CHEMOLITHOTROPHIC IRON-OXIDIZING BACTERIA FROM
ACID SAND DUMP SEEPAGE**

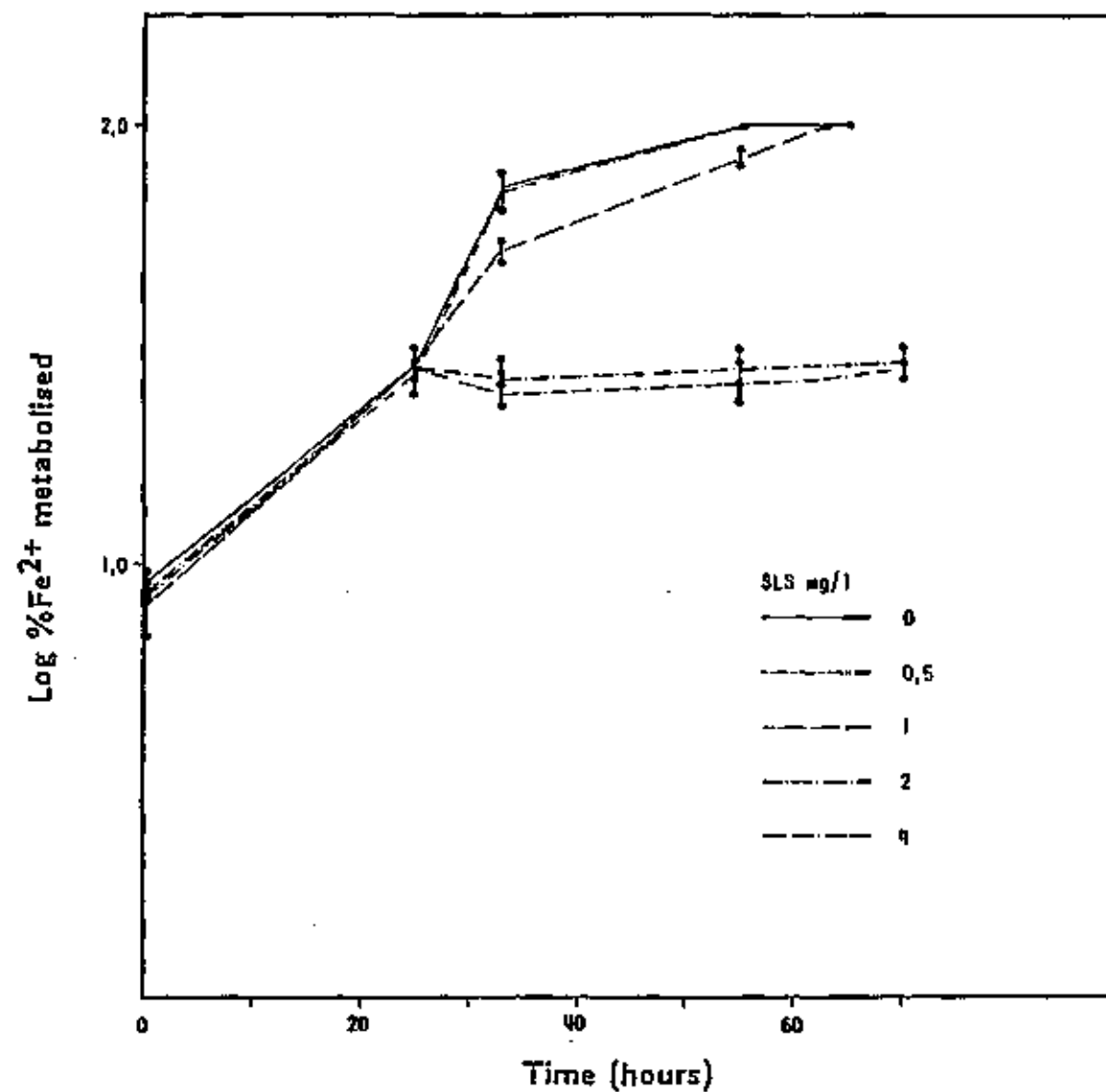
Appendix Fig. :	Inhibitor	Page
1 to 13	Sodium lauryl sulphate (SLS)	110
14 to 19	Linear alkylbenzenesulphonate (LAS)	123
20 to 29	Benzyldimethyl-n-alkylammonium chlorides (Hyamine 3500)	129
30 to 33	Cetylpyridinium chloride (Ceepryn)	139
34 to 41	Sodium benzoate	143
42 to 49	Sorbic acid	151
50 to 53	Sodium lignosulphonate	159
54 to 58	Polyacrylic acid	163



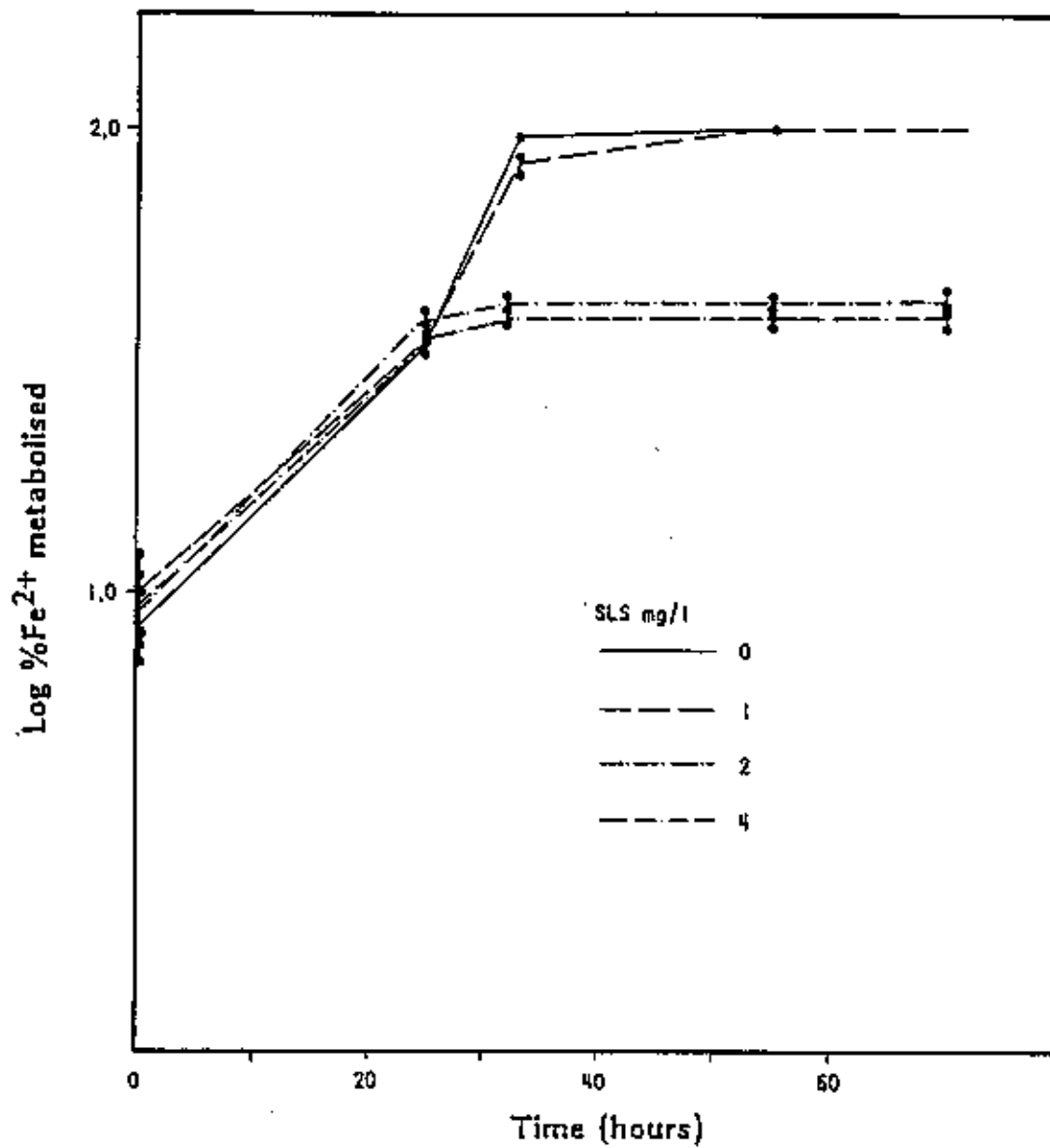
APPENDIX FIG. 1. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* WLR in HJJ medium. The SLS was added at the beginning of the experiment. Vertical bars indicate Fe²⁺ metabolism in duplicate flasks; graph lines connect the means.



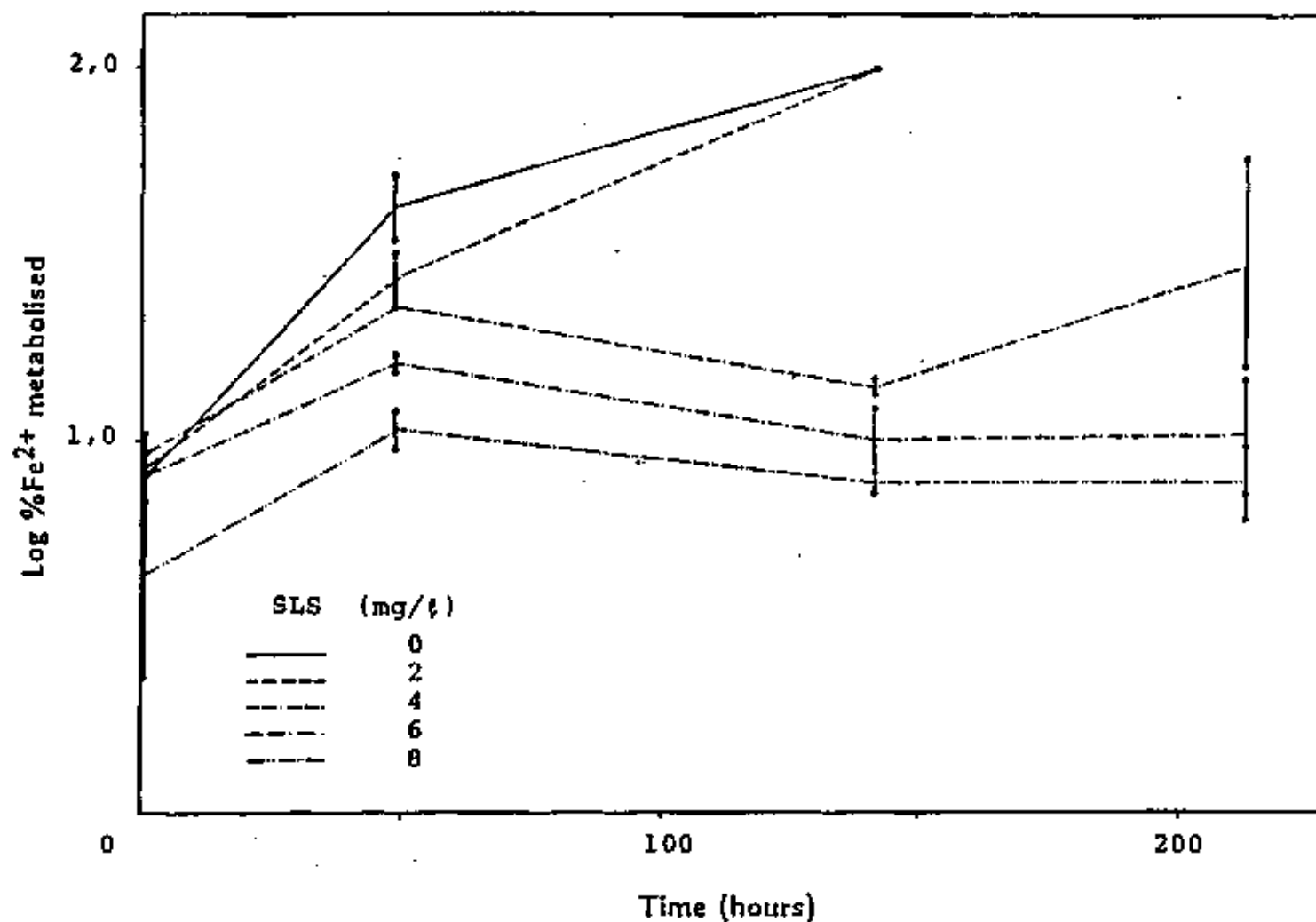
APPENDIX FIG. 2. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The SLS was added at the beginning of the experiment.



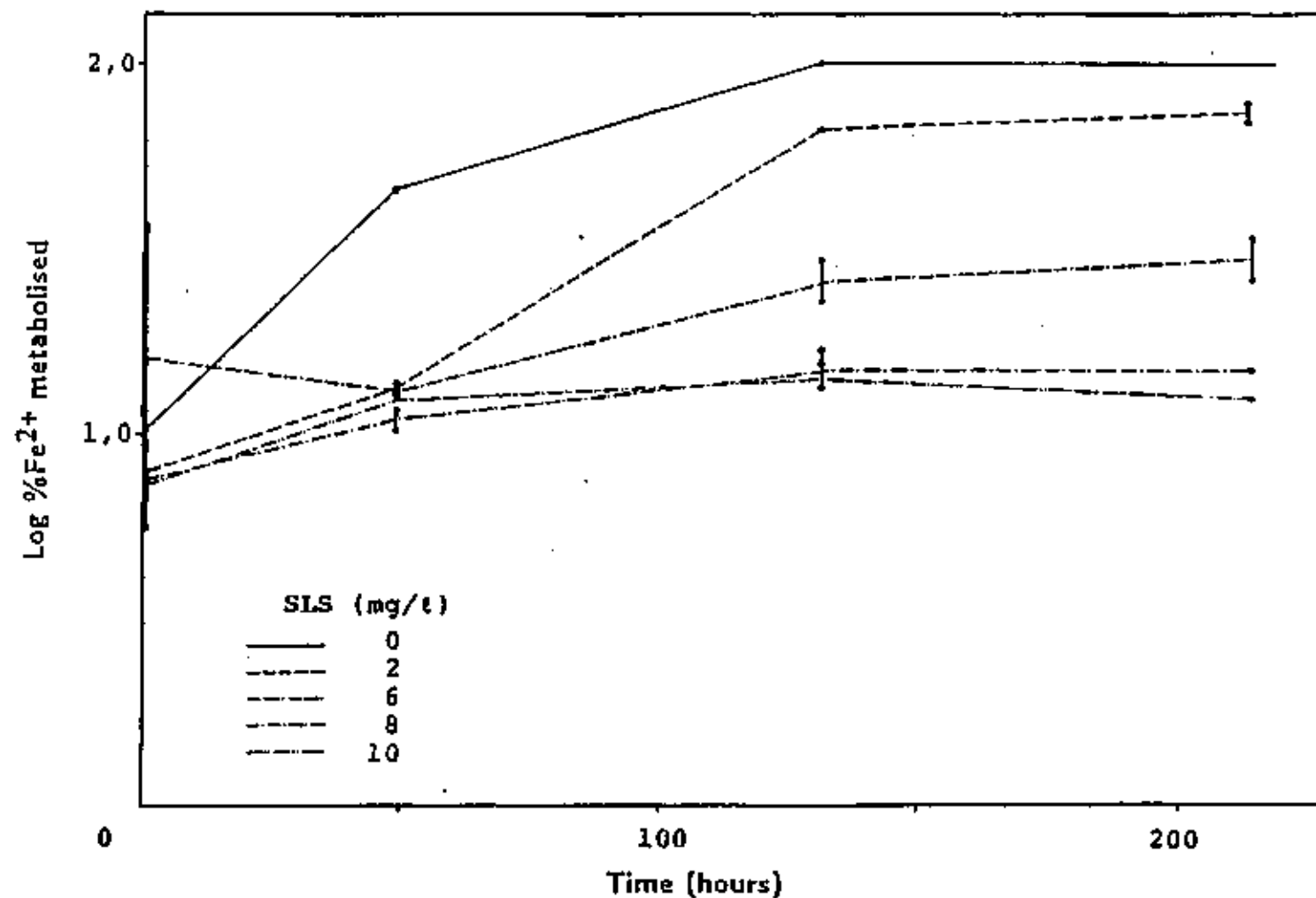
APPENDIX FIG. 3. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* WLR in HJJ medium. The SLS was added during the exponential phase of culture growth (25 h).



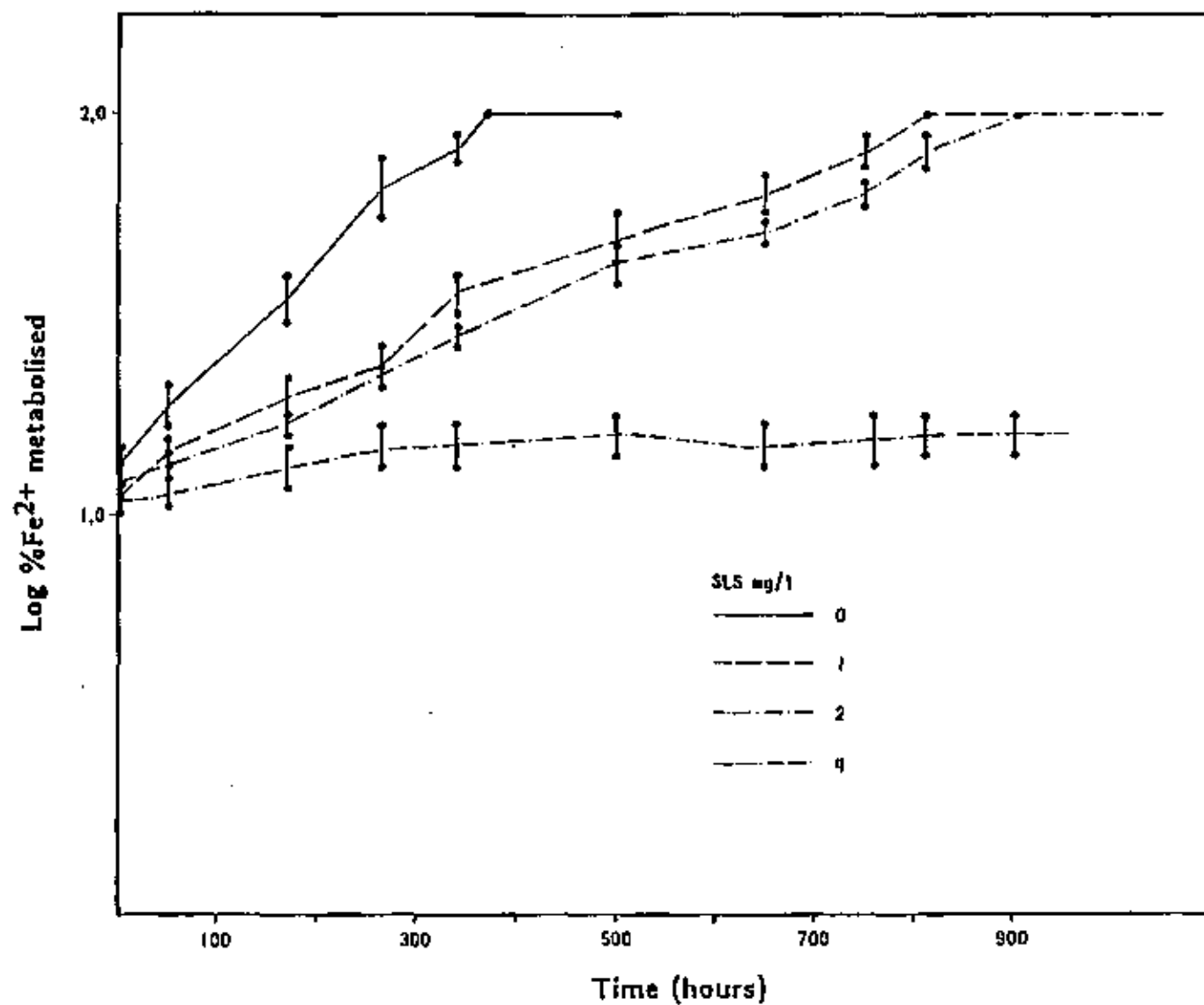
APPENDIX FIG. 4. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The SLS was added during the exponential phase of culture growth (25 h).



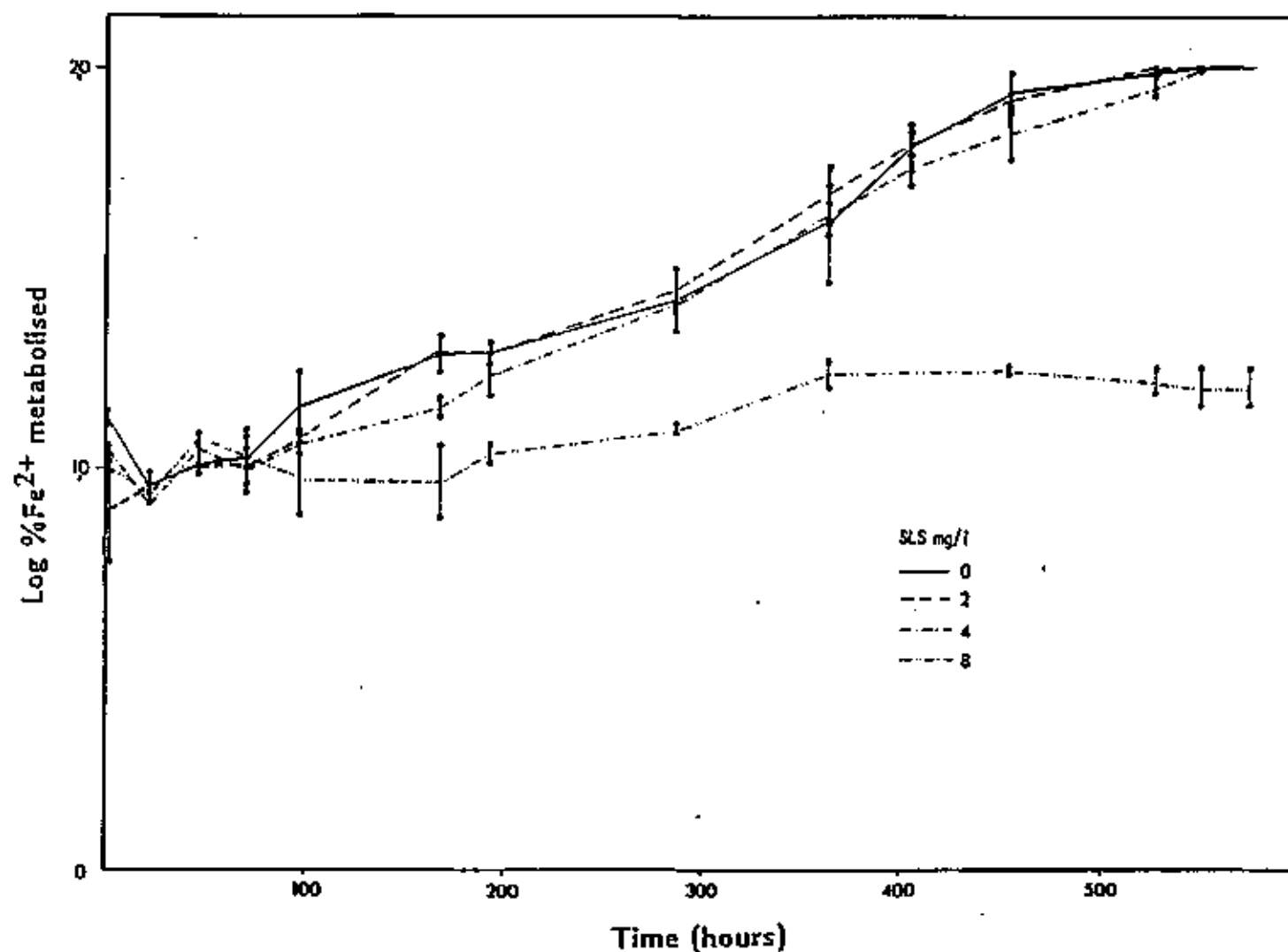
APPENDIX FIG. 5. Effect of increasing concentrations of SLS on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in HJJ medium when the inhibitor was added at the beginning of the experiment.



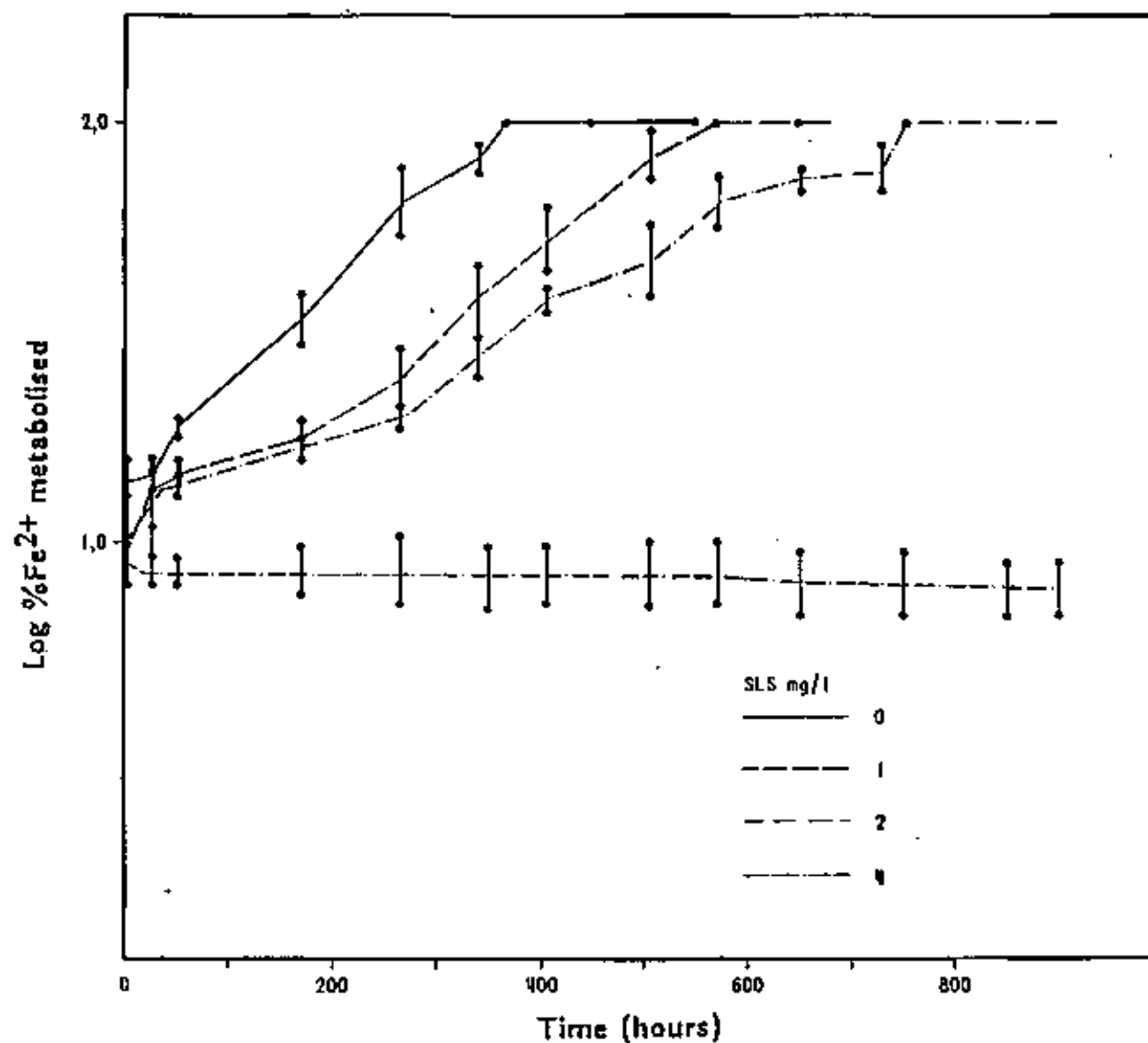
APPENDIX FIG. 6. Effect of increasing concentrations of SLS on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in HJJ medium when the inhibitor was added during the exponential phase of culture growth (23 h).



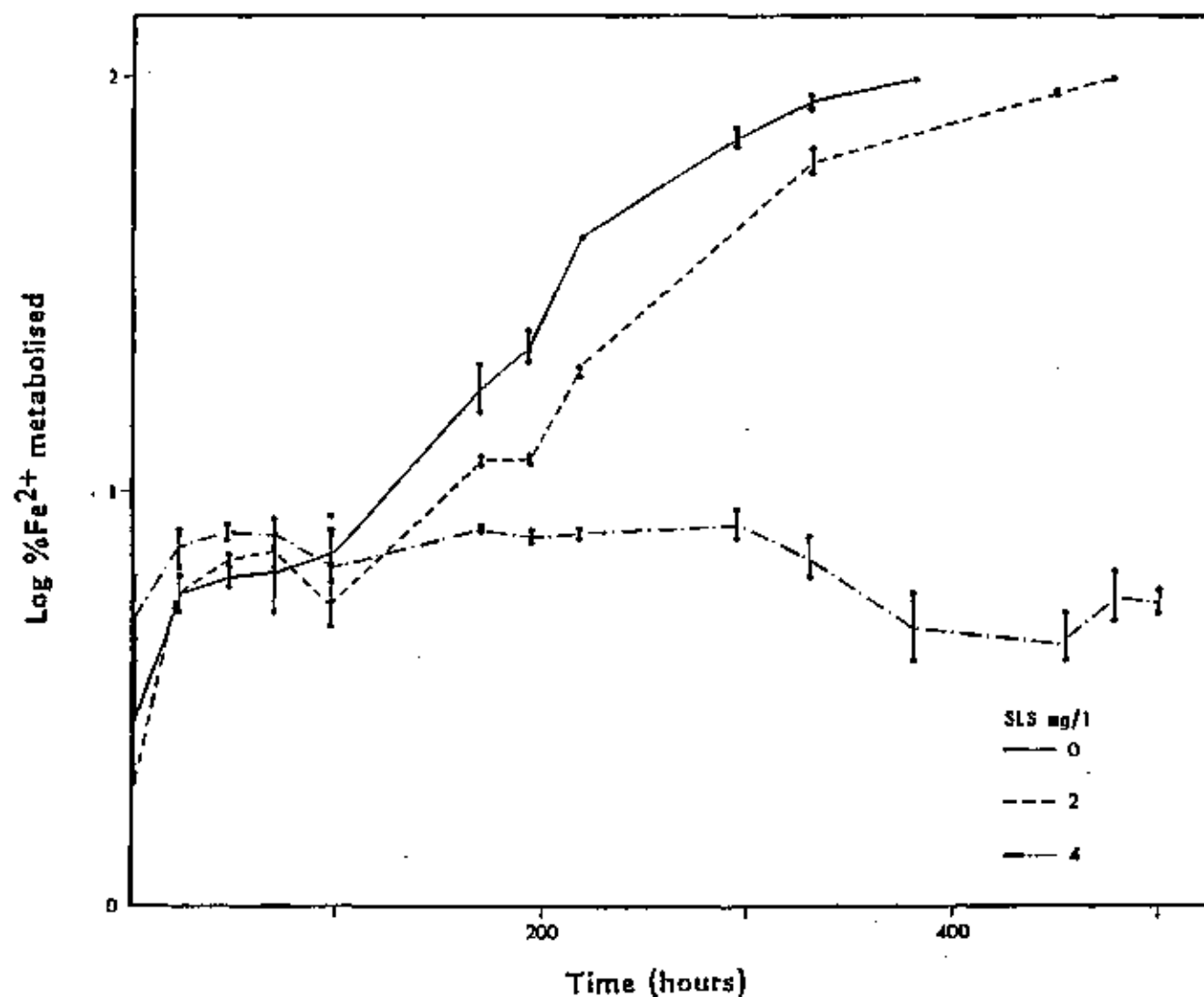
APPENDIX FIG. 7. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The SLS was added at the beginning of the experiment.



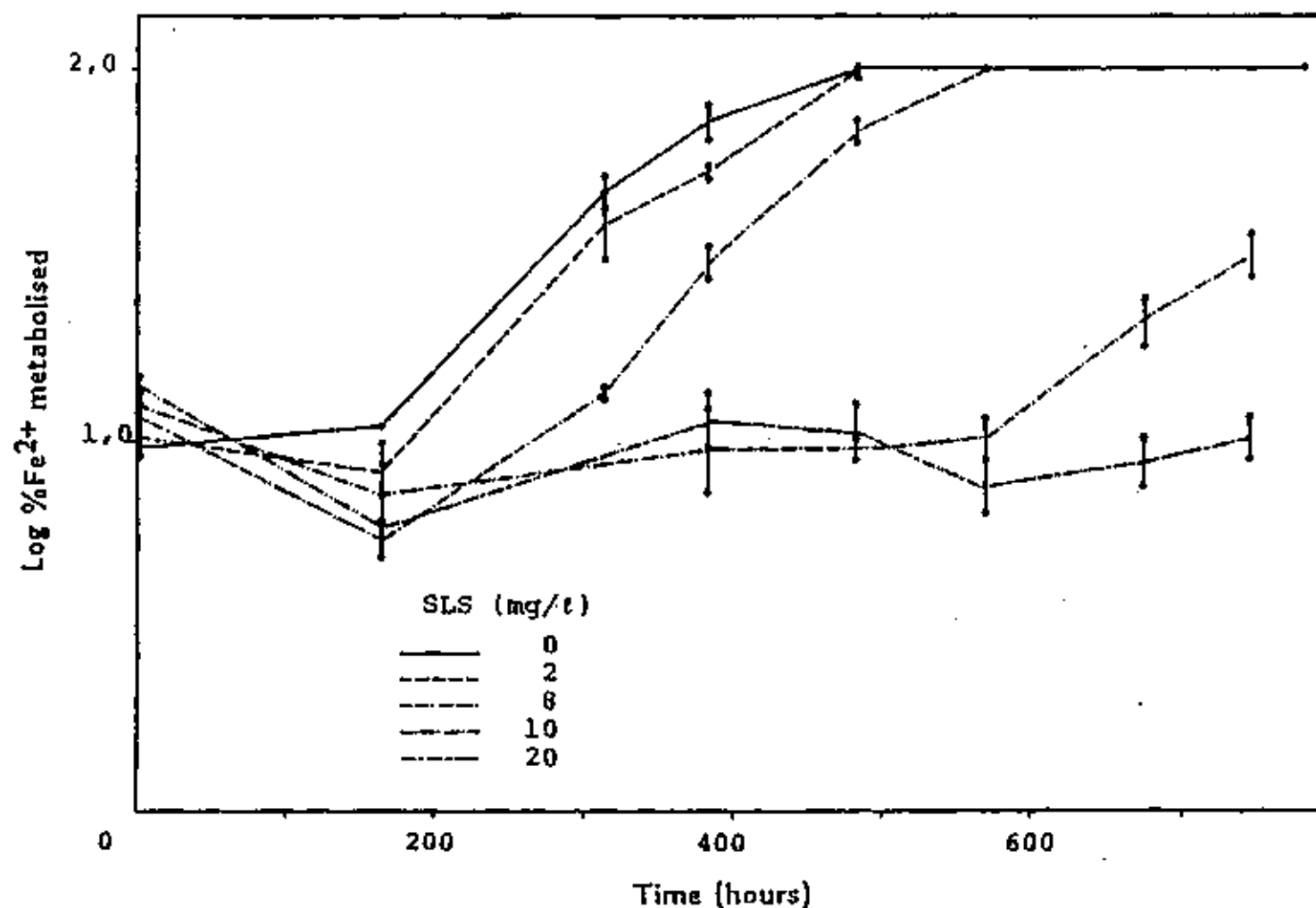
APPENDIX FIG. 8. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The SLS was added at the beginning of the experiment.



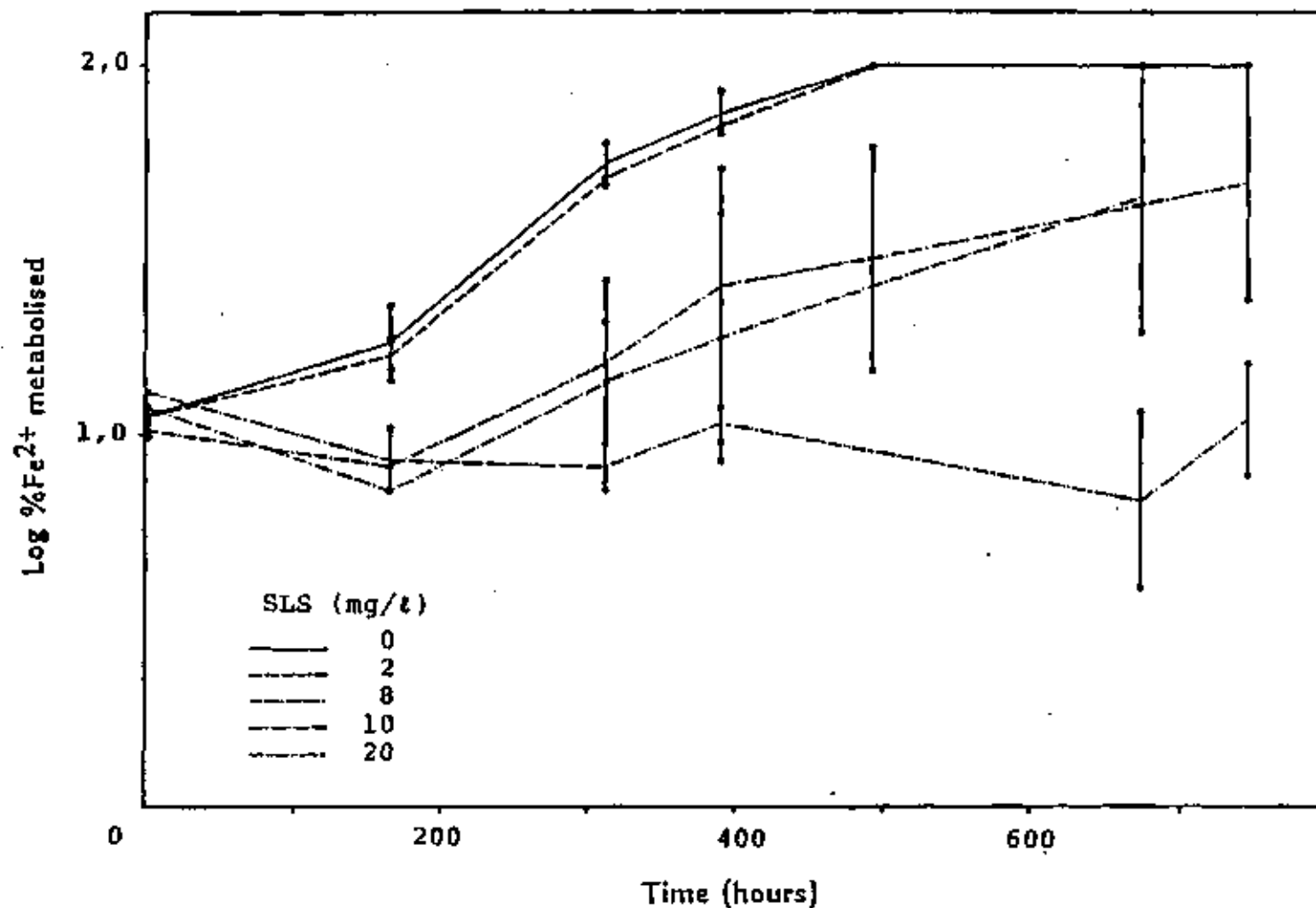
APPENDIX FIG. 9. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The SLS was added during the exponential phase of culture growth (25 h).



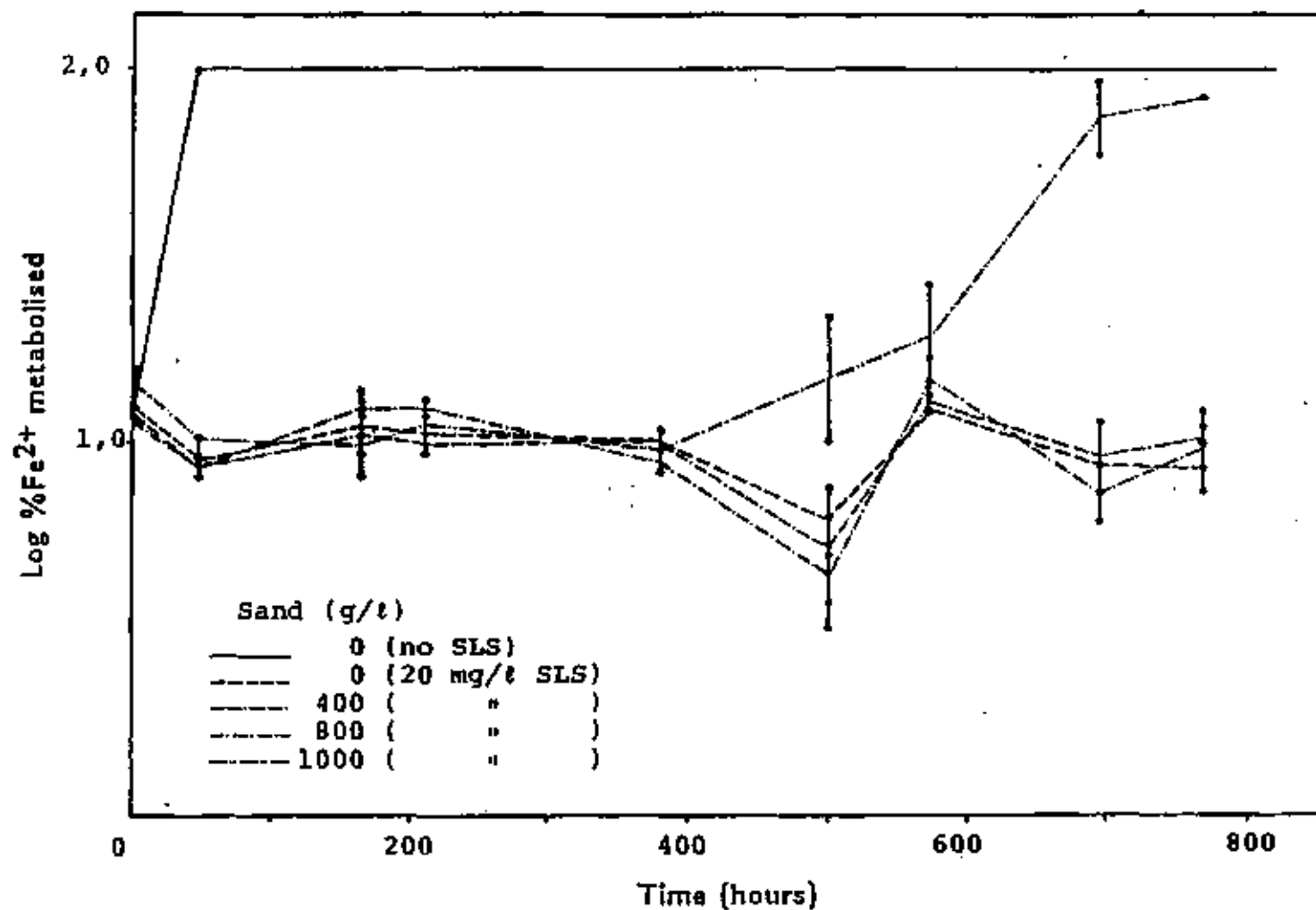
APPENDIX FIG. 10. Effect of increasing concentrations of SLS on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The SLS was added during the exponential phase of culture growth (24 h).



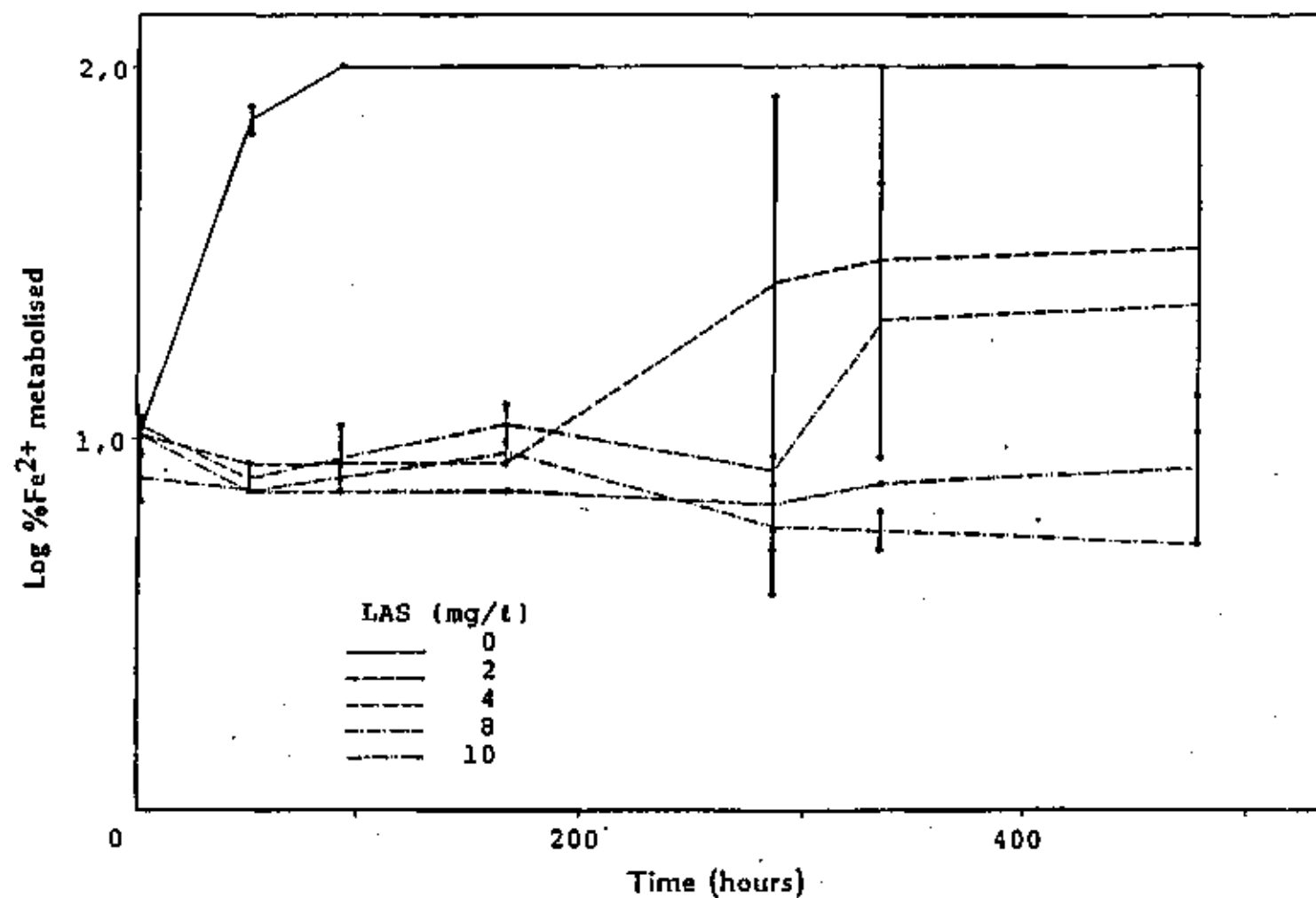
APPENDIX FIG. 11. Effect of increasing concentrations of SLS on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The SLS was added at the beginning of the experiment.



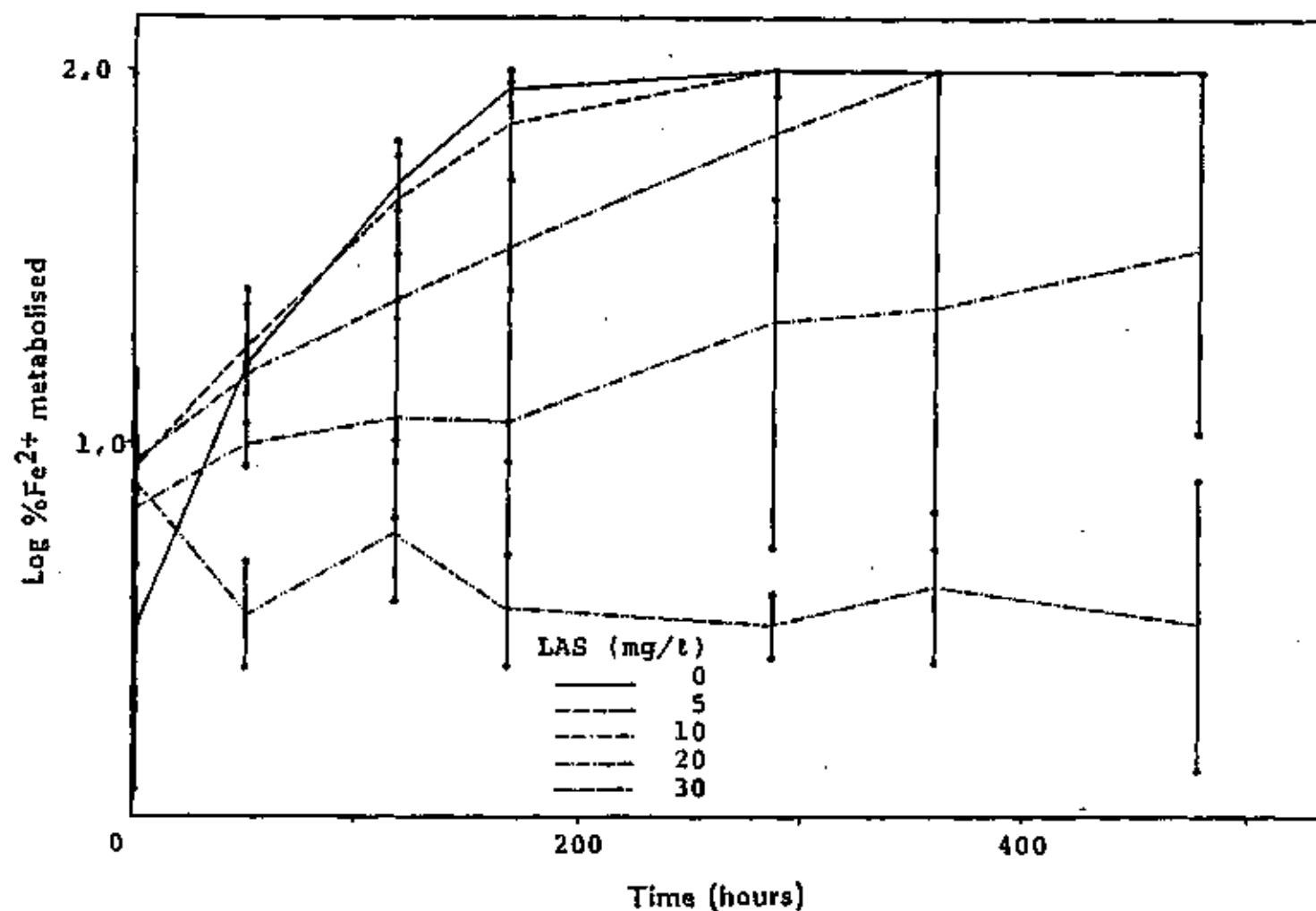
APPENDIX FIG. 12. Effect of increasing concentrations of SLS on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The SLS was added during the exponential phase of culture growth (26 h).



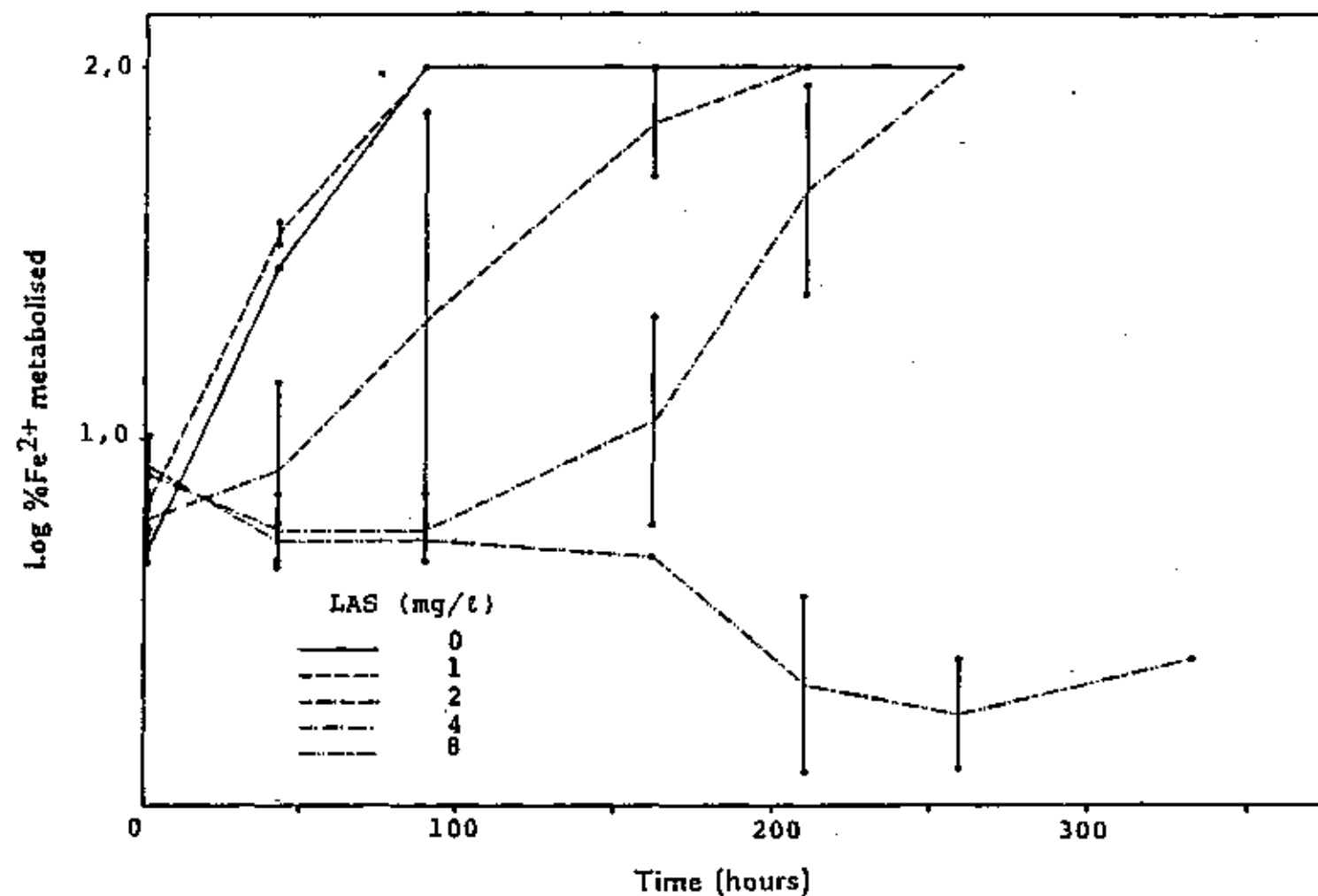
APPENDIX FIG. 13. Effect of increasing concentrations of mine dump sand G5 on the inhibitory effect of 20 mg/l SLS on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The SLS was added at the beginning of the experiment.



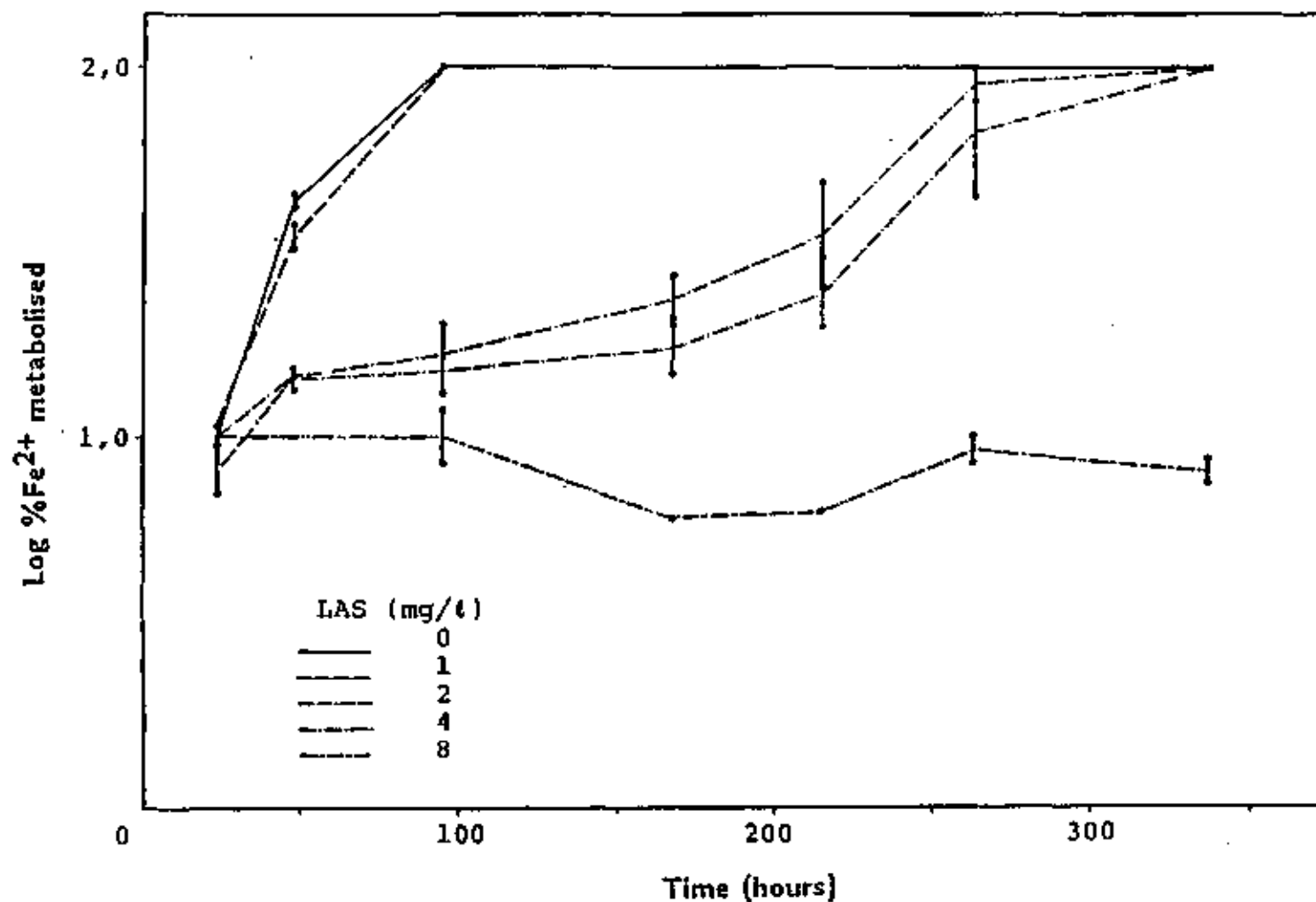
APPENDIX FIG. 14. Effect of increasing concentrations of the LAS, NANSA HS 80/S, on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The LAS was added at the beginning of the experiment.



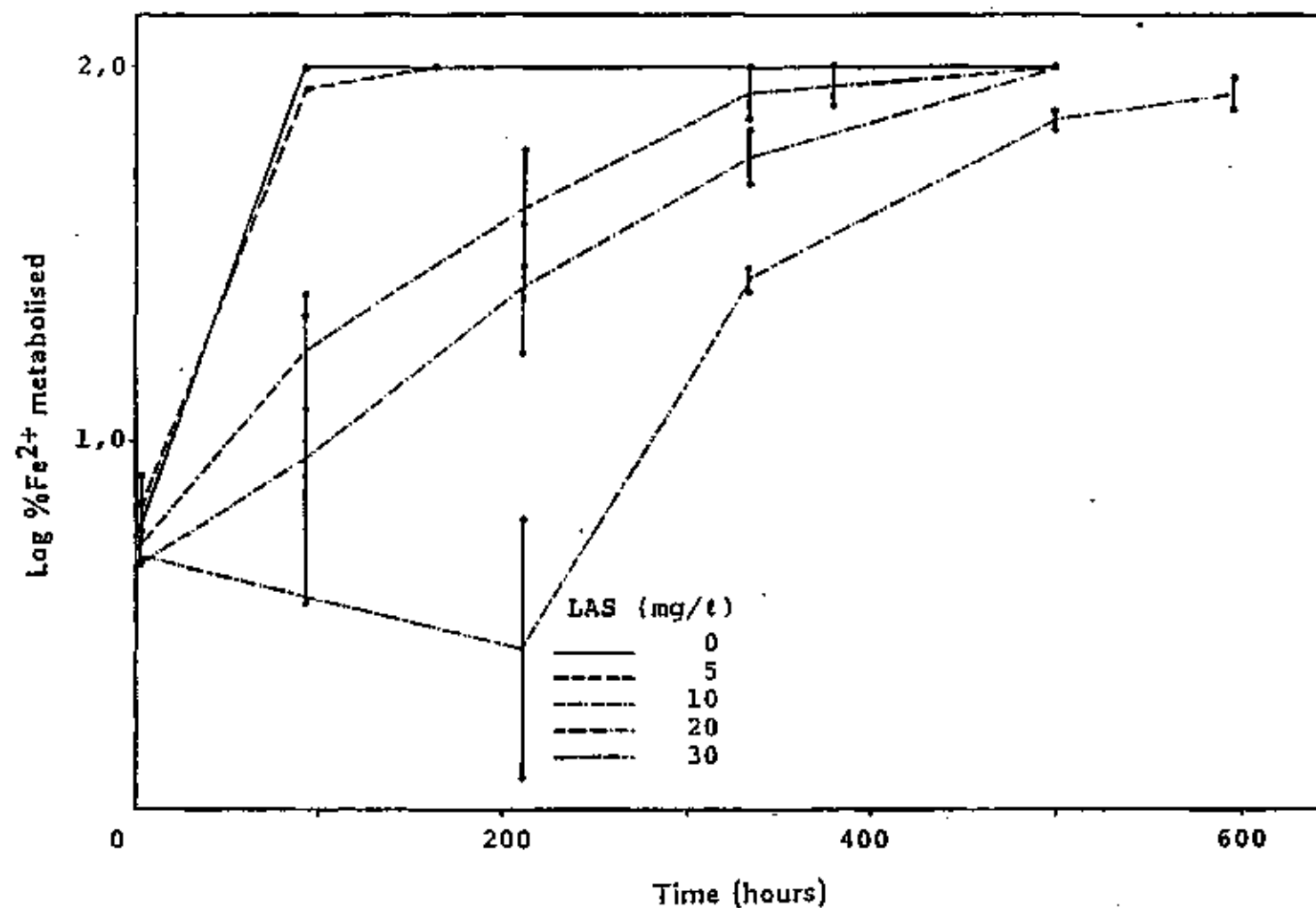
APPENDIX FIG. 15. Effect of increasing concentrations of the LAS, NANSA HS 80/S, on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The LAS was added at the beginning of the experiment.



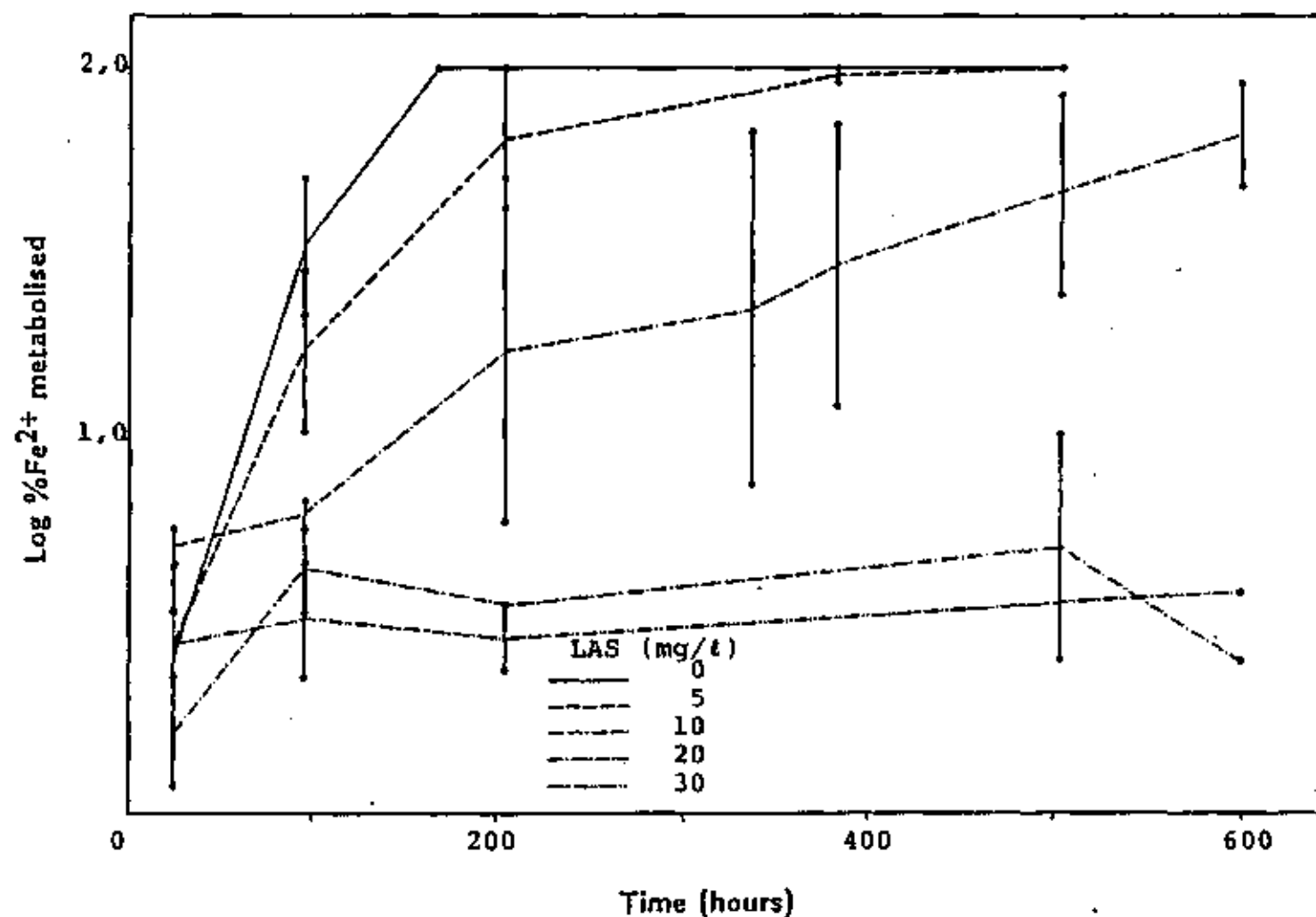
APPENDIX FIG. 16. Effect of increasing concentrations of the LAS, NANSA HS 80/S, on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in HJJ medium when the inhibitor was added at the beginning of the experiment.



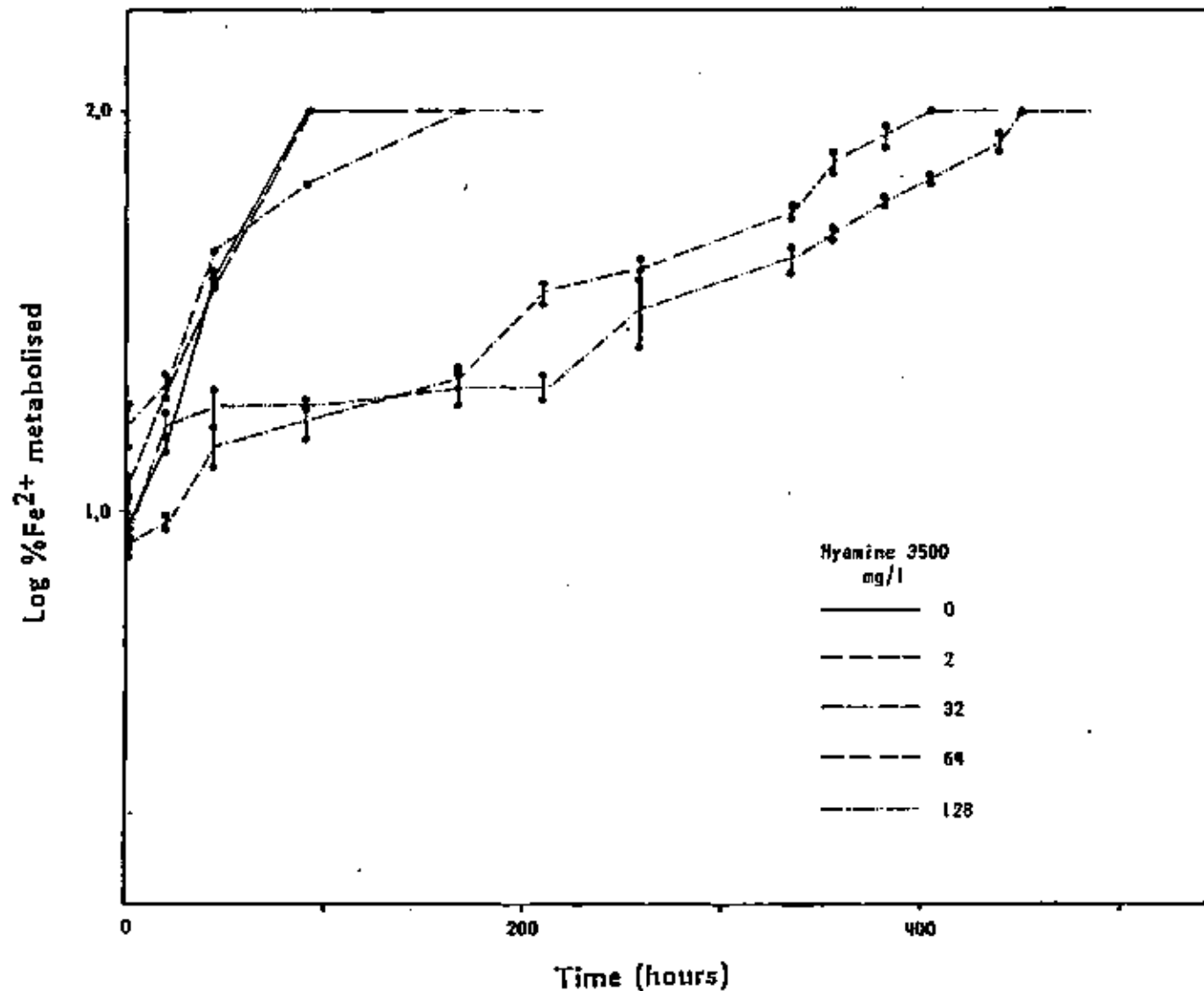
APPENDIX FIG. 17. Effect of increasing concentrations of the LAS, NANSA HS 80/S, on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in HJJ medium when the inhibitor was added during the exponential phase of culture growth (23 h).



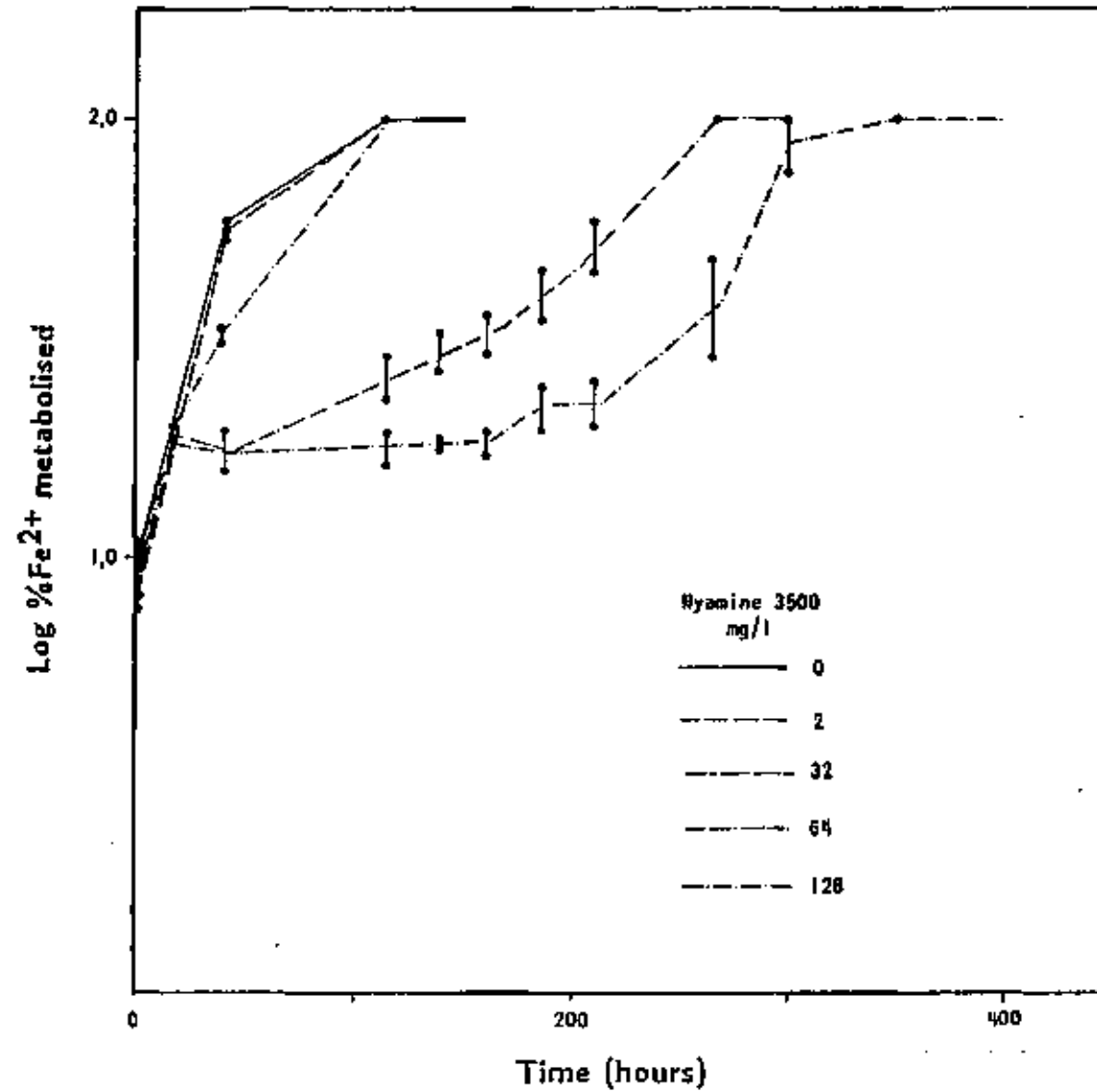
APPENDIX FIG. 18. Effect of increasing concentrations of the LAS, NANSA HS 80/S, on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The LAS was added at the beginning of the experiment.



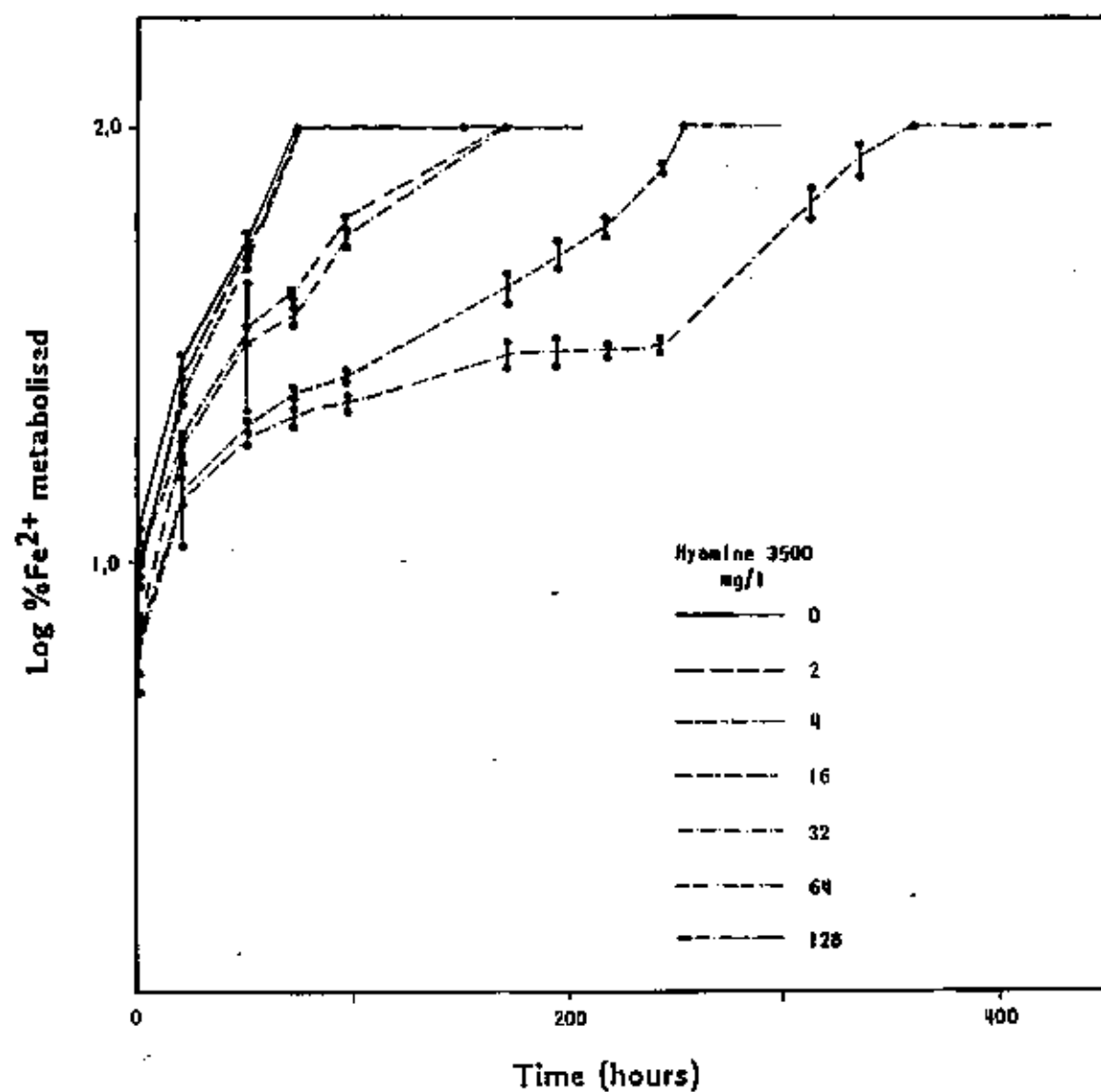
APPENDIX FIG. 19. Effect of increasing concentrations of the LAS, NANSA HS 80/ S, on the growth of a mixed *T. ferrooxidans* population, enriched from acid seepage samples, in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The LAS was added during the exponential phase of culture growth (24 h).



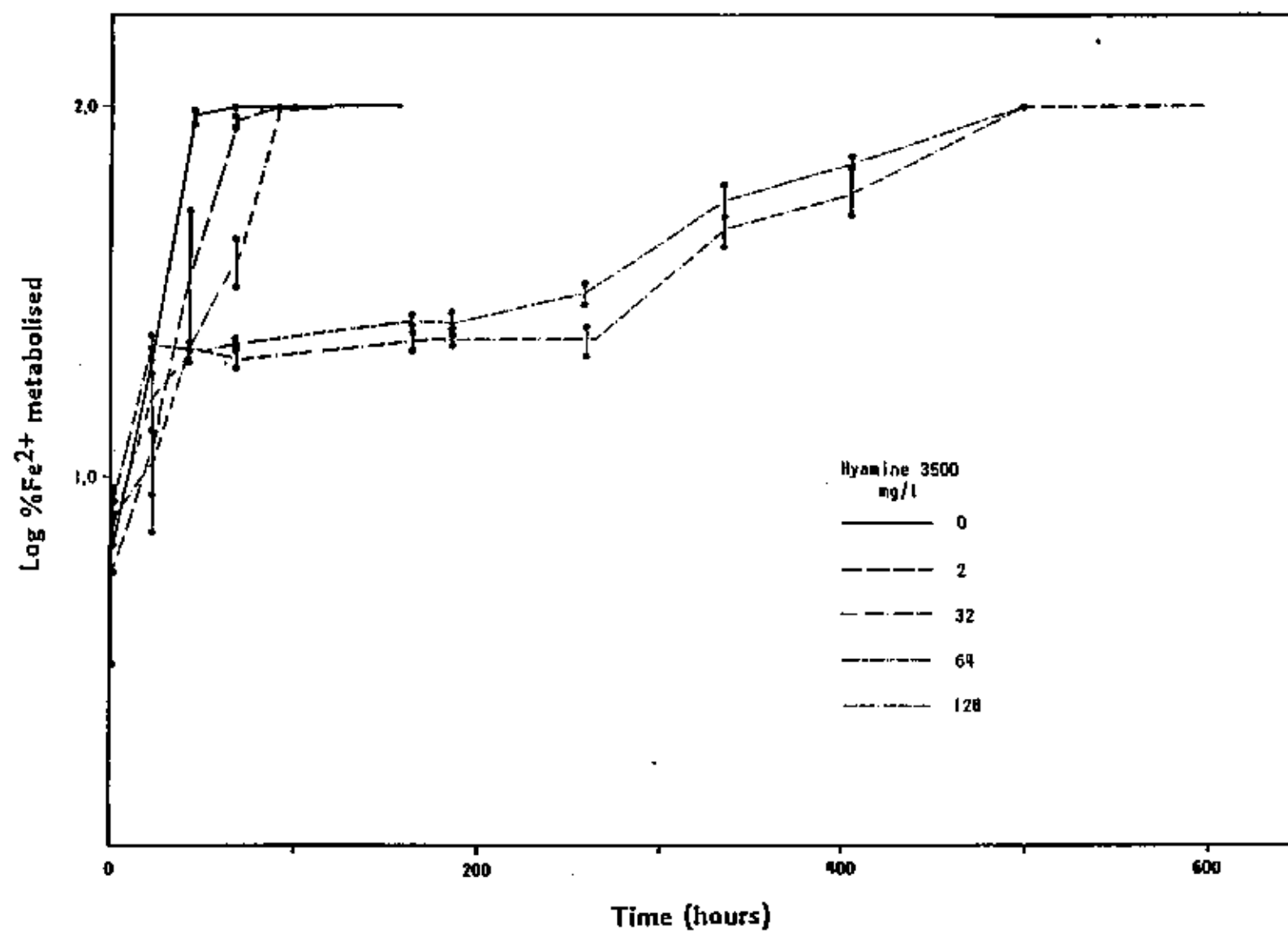
APPENDIX FIG. 20. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* WLR in HJJ medium. The Hyamine 3500 was added at the beginning of the experiment.



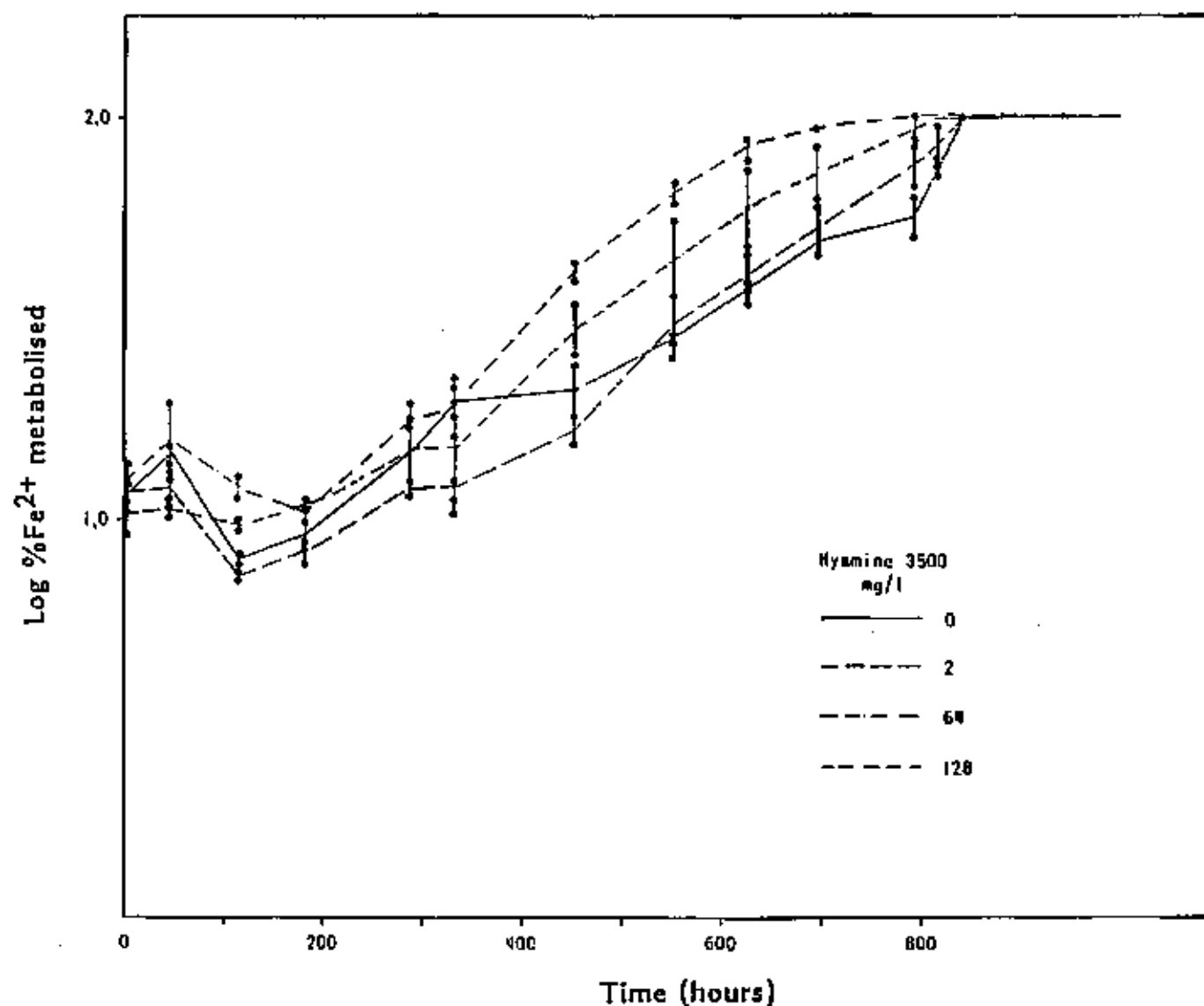
APPENDIX FIG. 21. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* WLR in HJJ medium. The Hyamine 3500 was added during the exponential phase of growth (18 h).



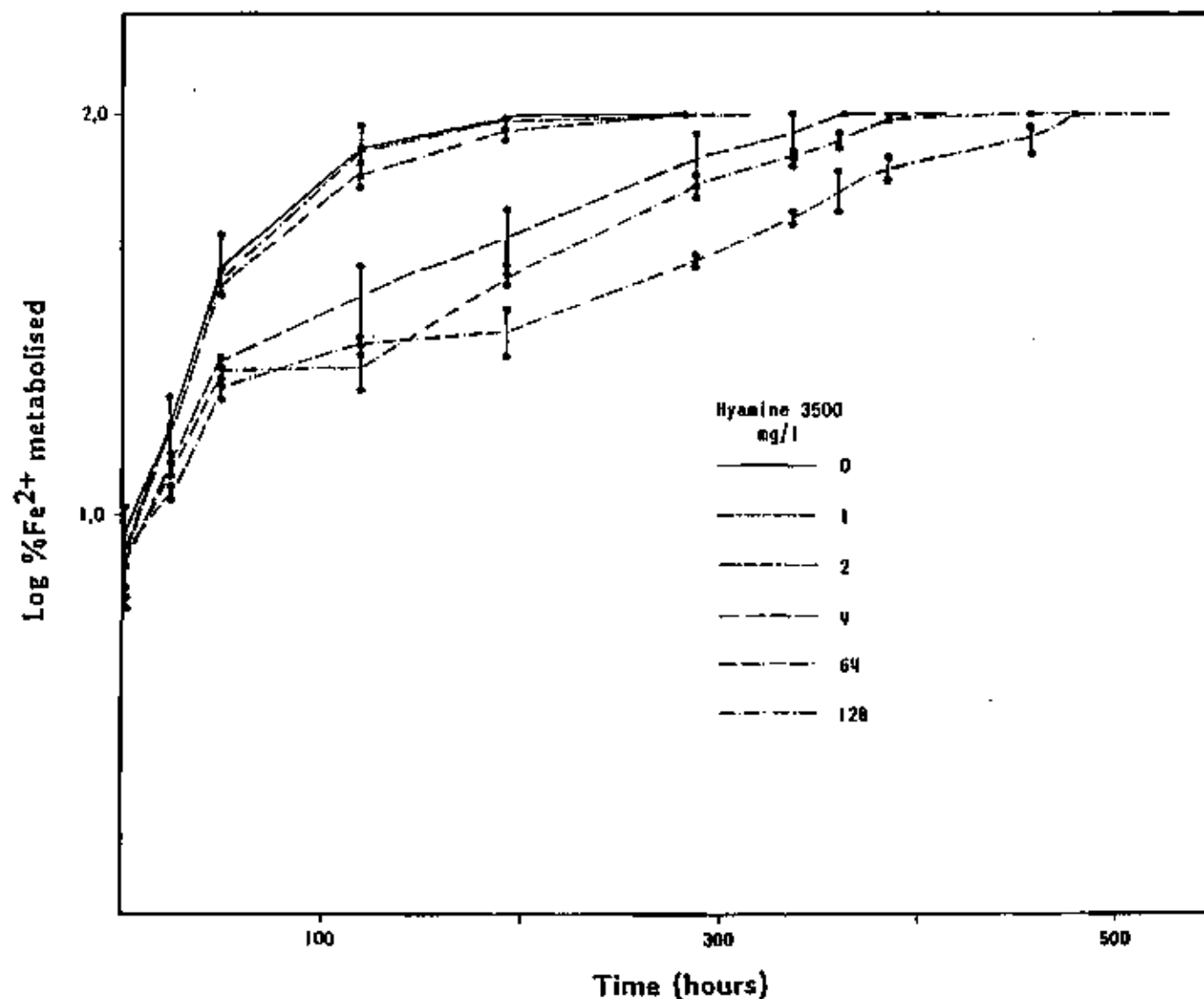
APPENDIX FIG. 22. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The Hyamine 3500 was added at the beginning of the experiment.



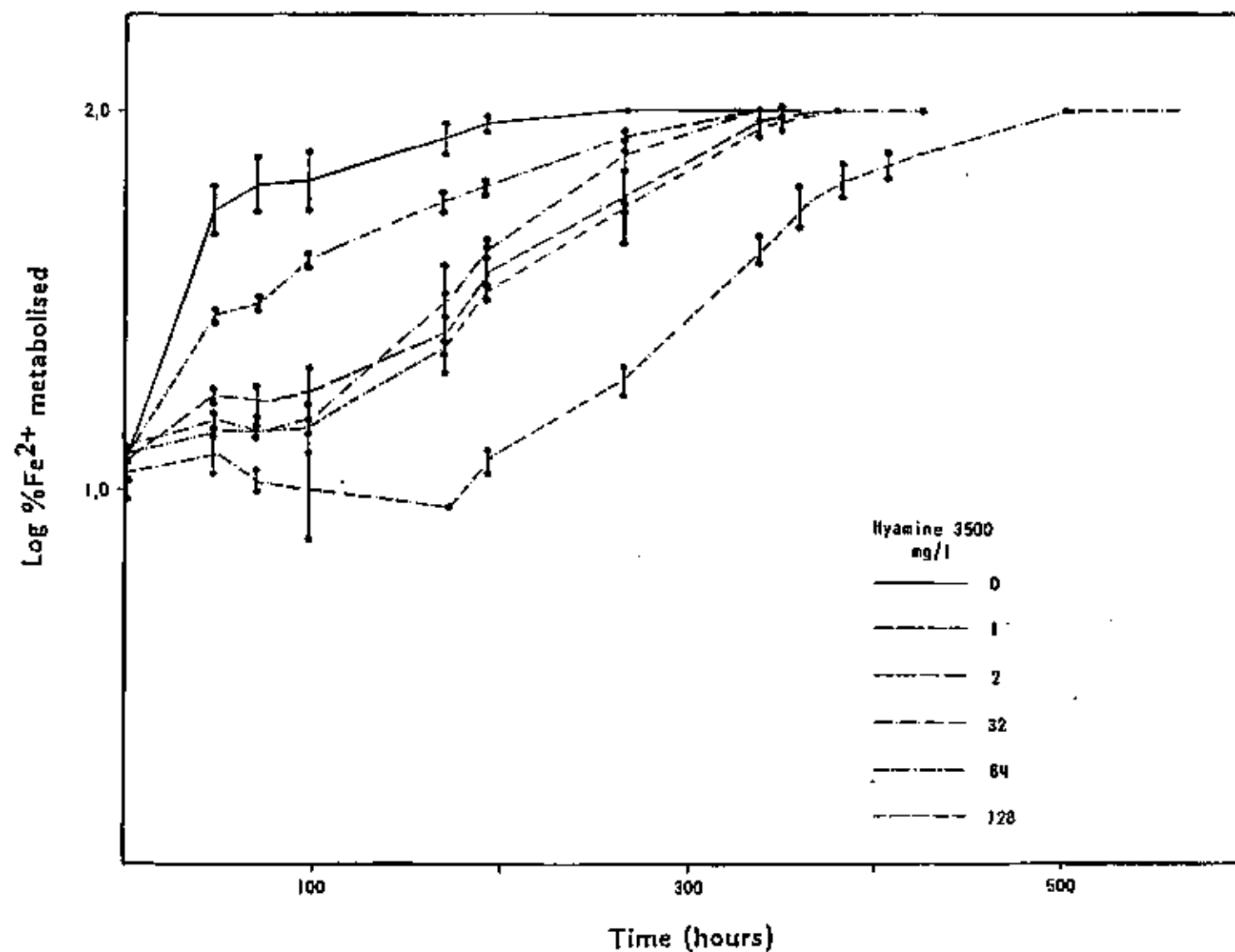
APPENDIX FIG. 23. Effect of increasing concentrations of Hyamine 3500 on the growth of *T ferrooxidans* ATCC 19859 in HJJ medium. The Hyamine 3500 was added during the exponential phase of growth (23 h).



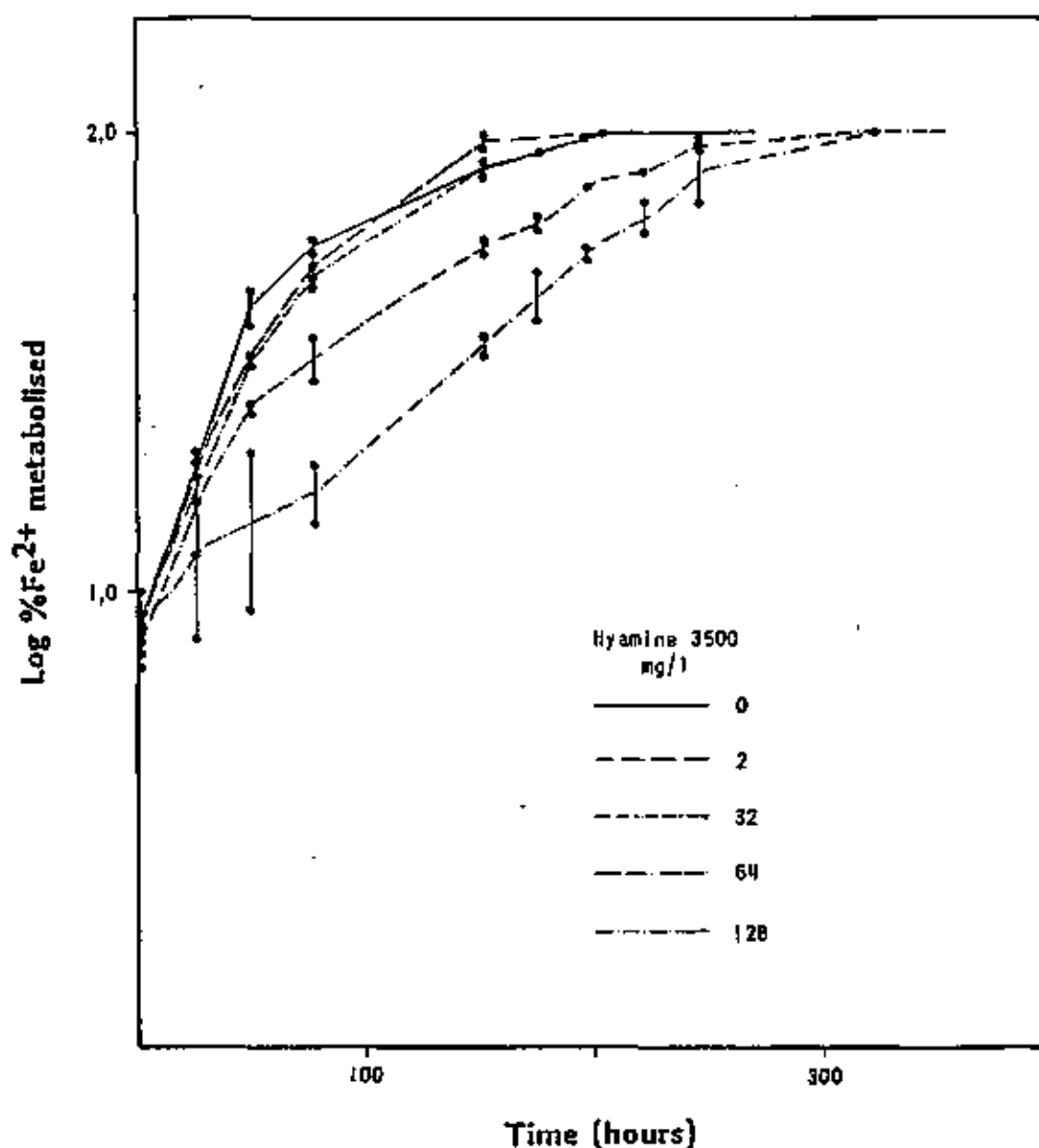
APPENDIX FIG. 24. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Hyamine 3500 was added at the beginning of the experiment.



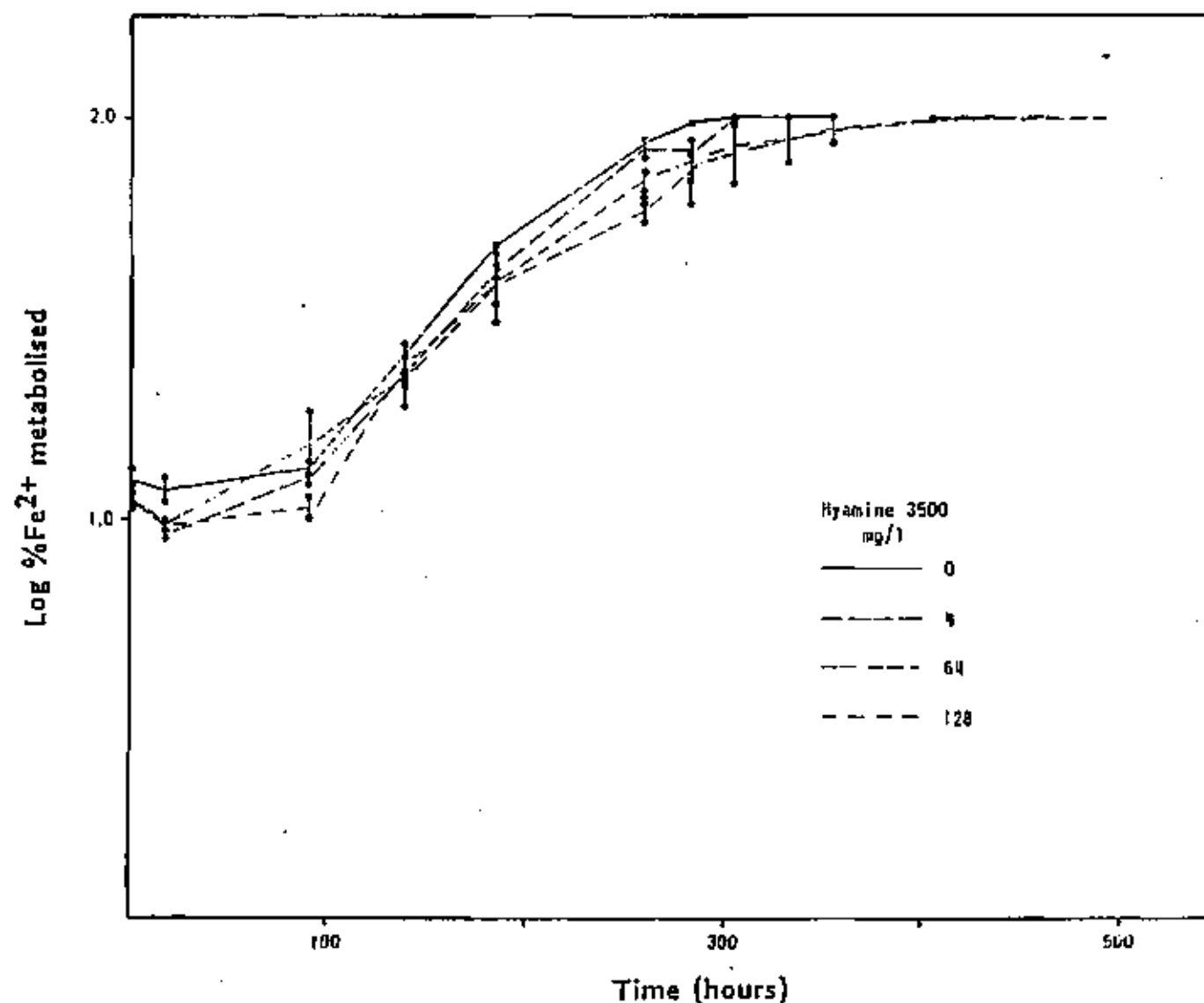
APPENDIX FIG. 25. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Hyamine 3500 was added during the exponential phase of growth (24 h).



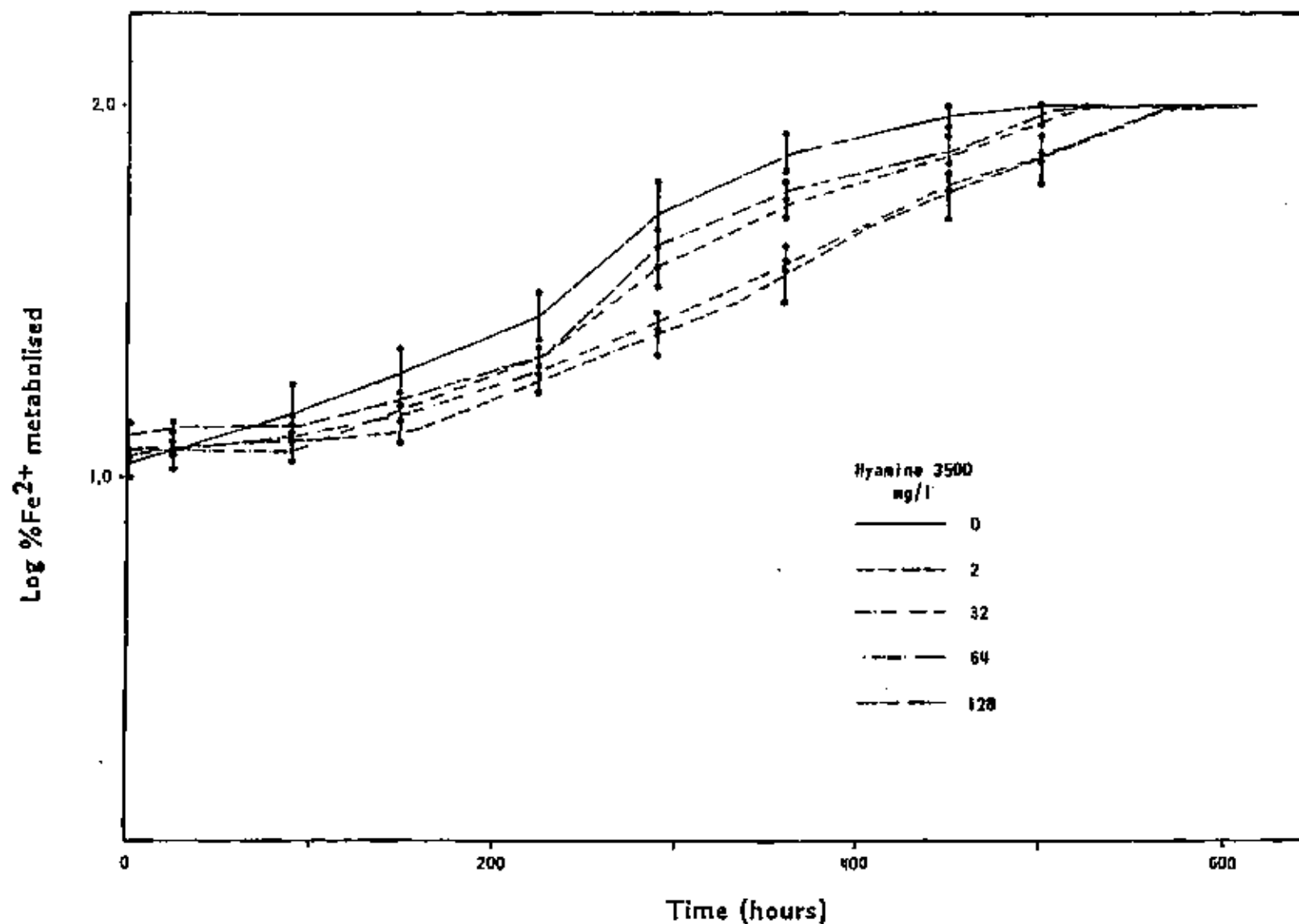
APPENDIX FIG. 26. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air dried mine dump sand G5. The Hyamine 3500 was added at the beginning of the experiment.



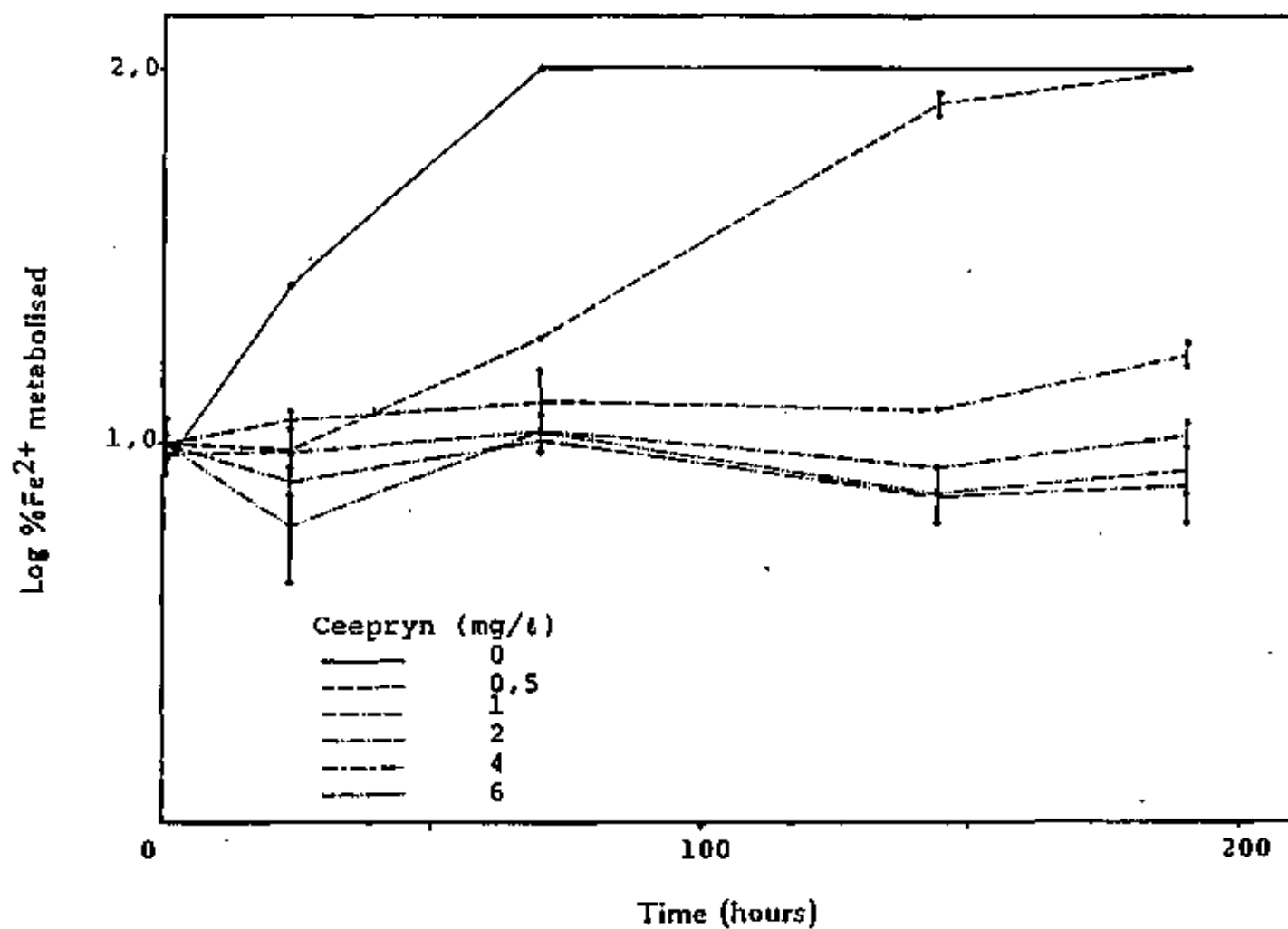
APPENDIX FIG. 27. Effect of increasing concentrations of Hyamine 3500 on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Hyamine 3500 was added during the exponential phase of growth (26 h).



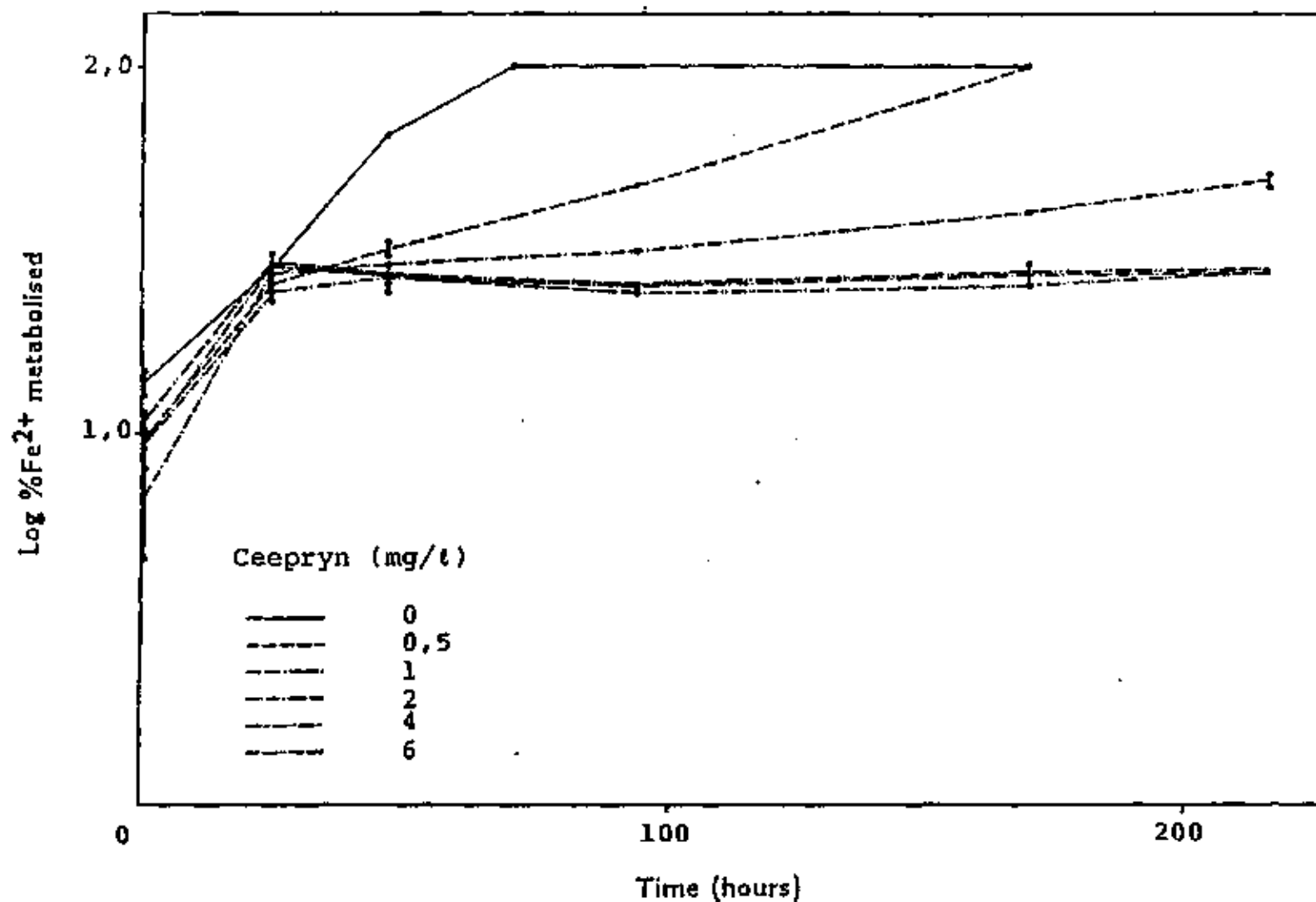
APPENDIX FIG. 28. Effect of increasing concentrations of Hyamine 3500 on the growth of enrichment cultures of *T. ferrooxidans*, derived from soil G4 10^{-1} MPN cultures, in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Hyamine 3500 was added at the beginning of the experiment.



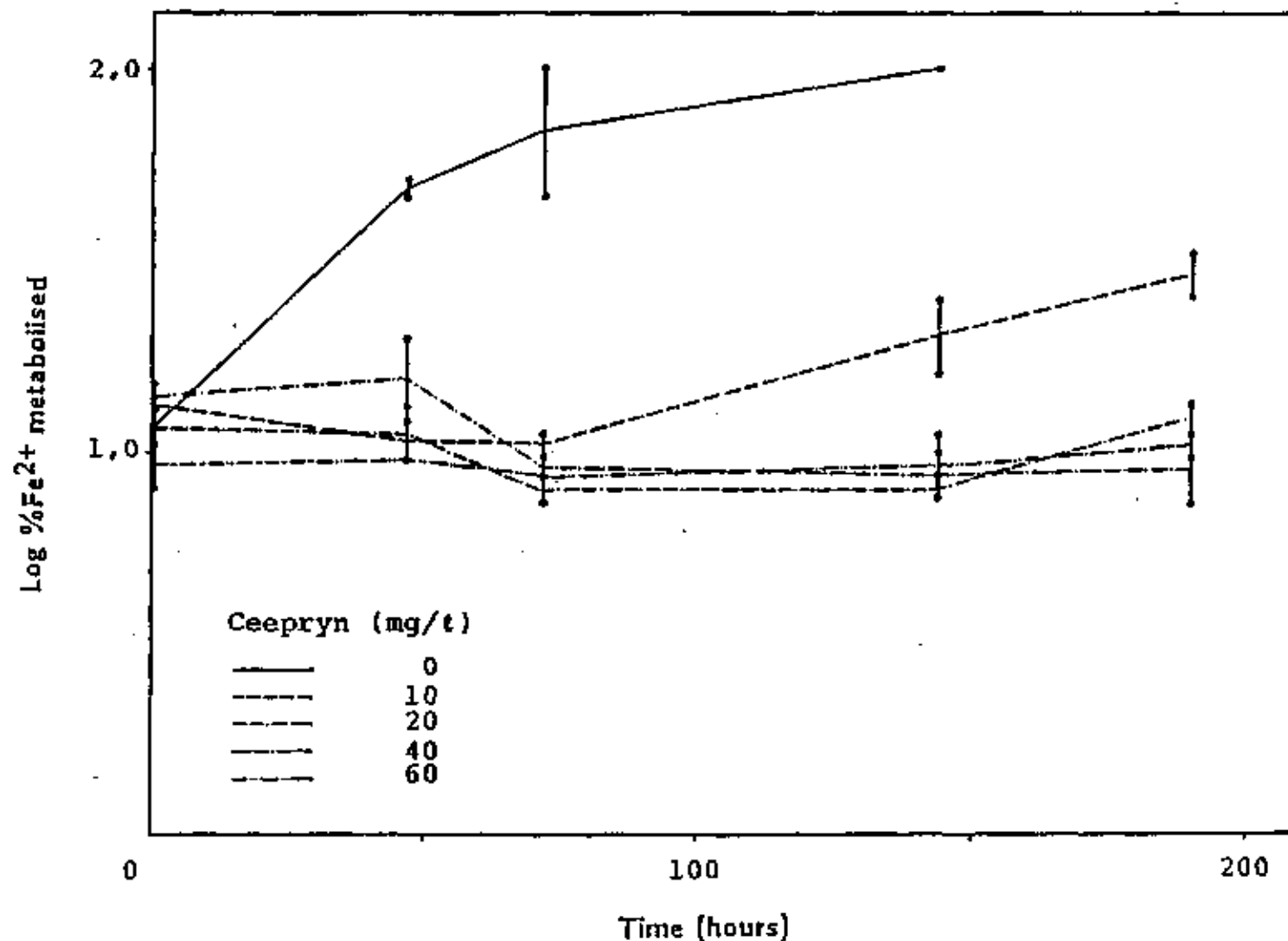
APPENDIX FIG. 29. Effect of increasing concentrations of Hyamine 3500 on the growth of enrichment cultures of *T. ferrooxidans*, derived from soil G4 10^{-1} MPN cultures, in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Hyamine 3500 was added during the exponential phase of growth (25 h).



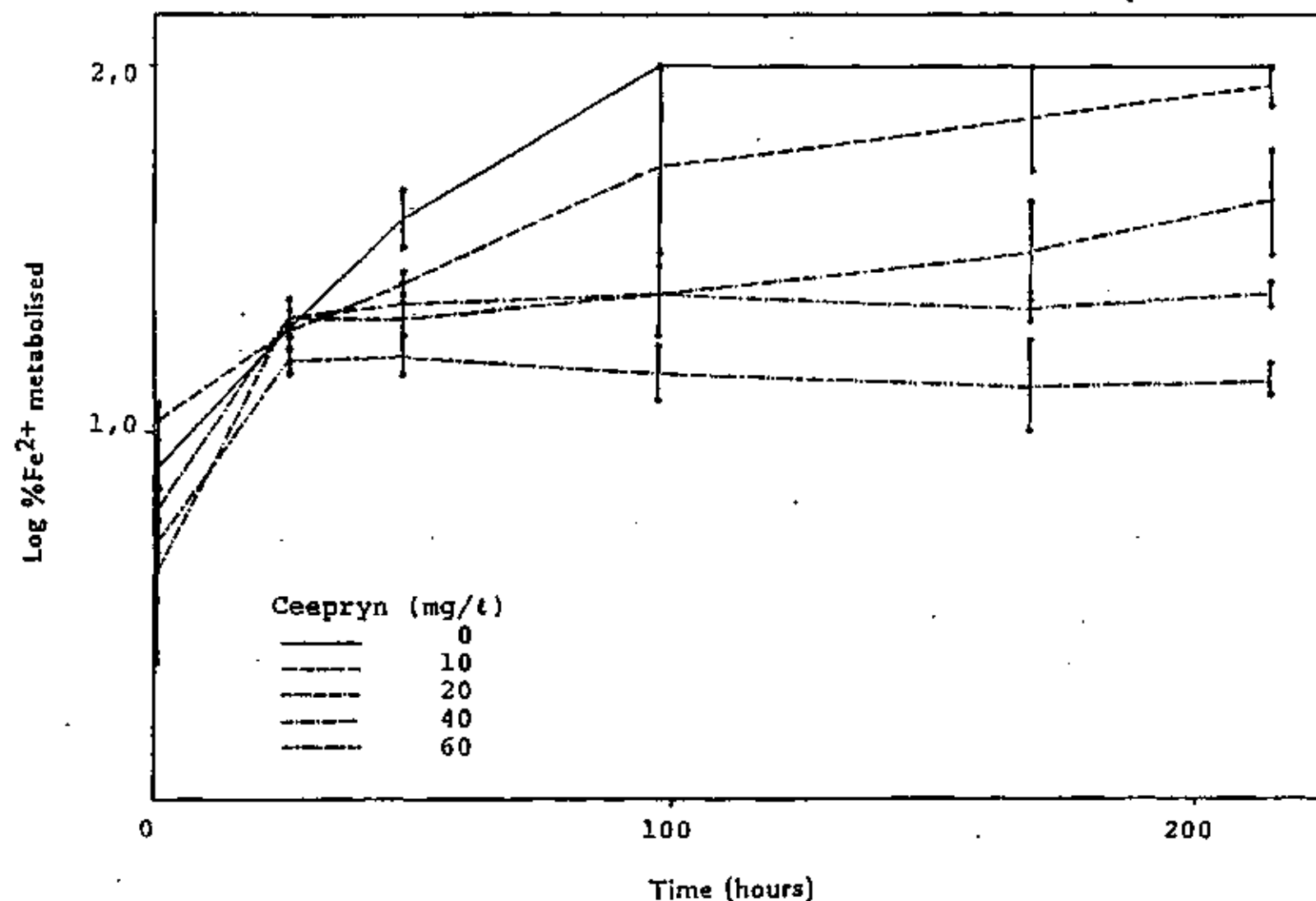
APPENDIX FIG. 30. Effect of increasing concentrations of Ceepryn on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium when the Ceepryn was added at the beginning of the experiment.



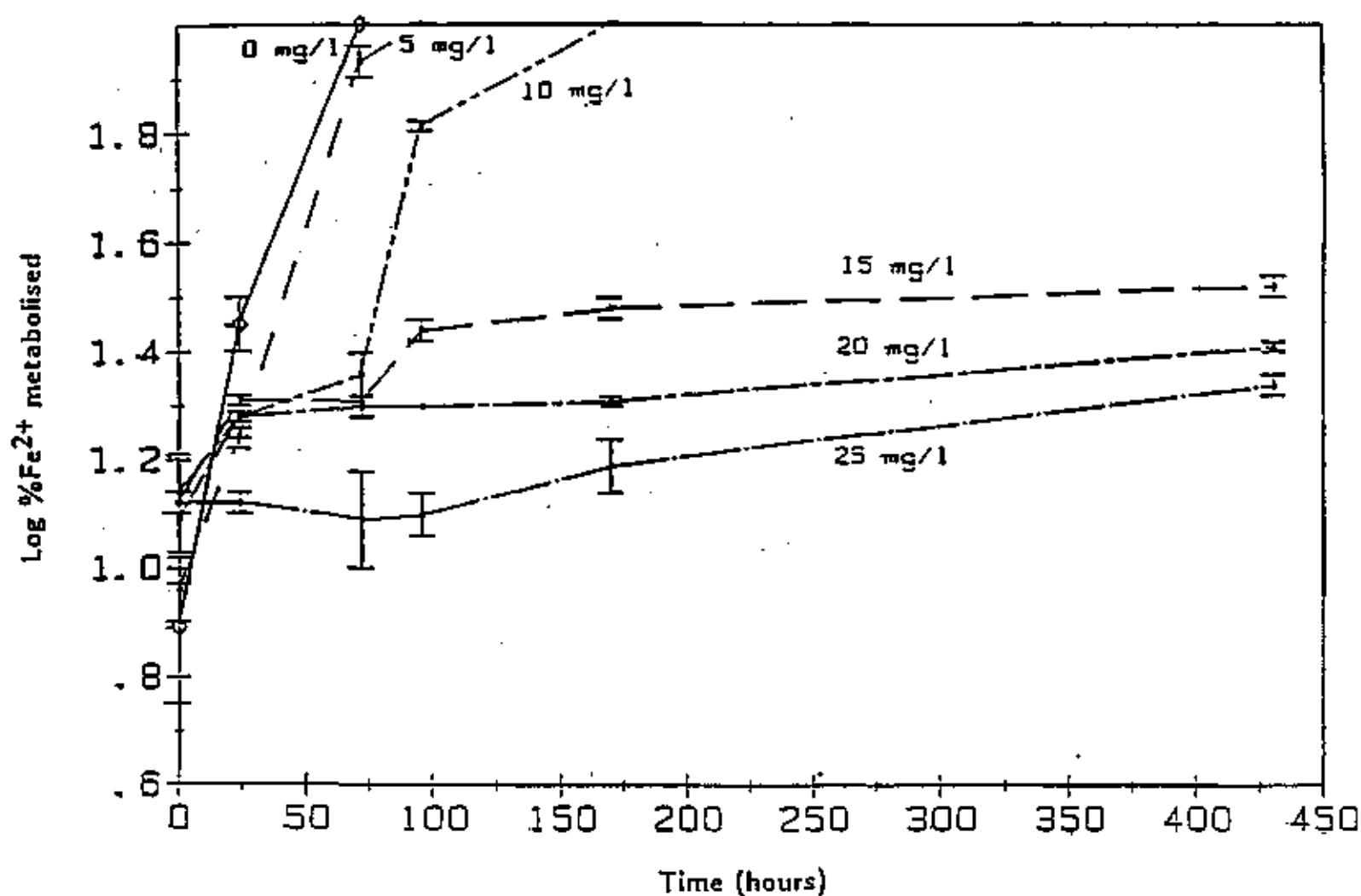
APPENDIX FIG. 31. Effect of increasing concentrations of Ceepryn on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium when the Ceepryn was added during the exponential phase of growth (25 h).



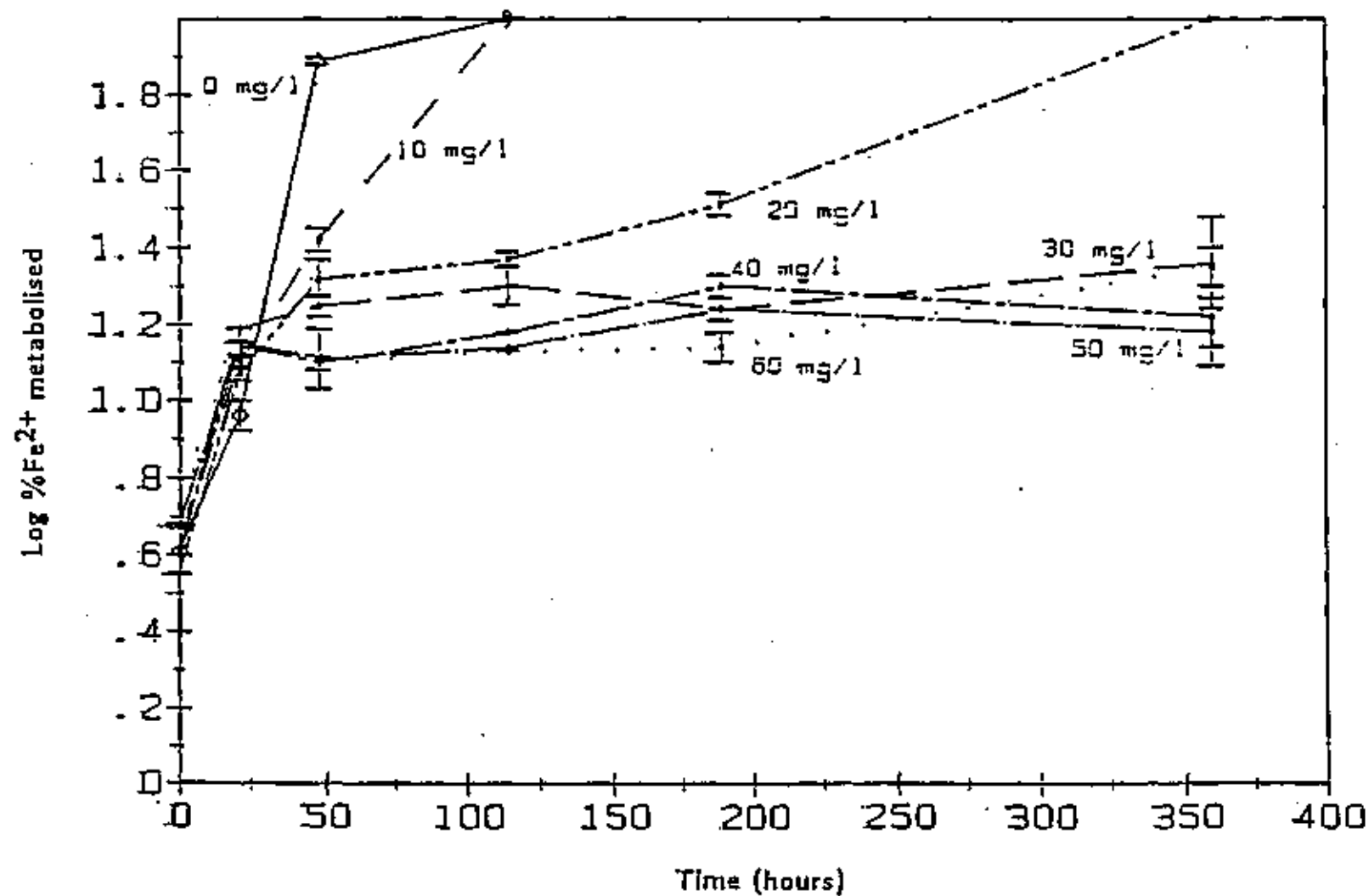
APPENDIX FIG. 32. Effect of increasing concentrations of Ceepryn on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Ceepryn was added at the beginning of the experiment.



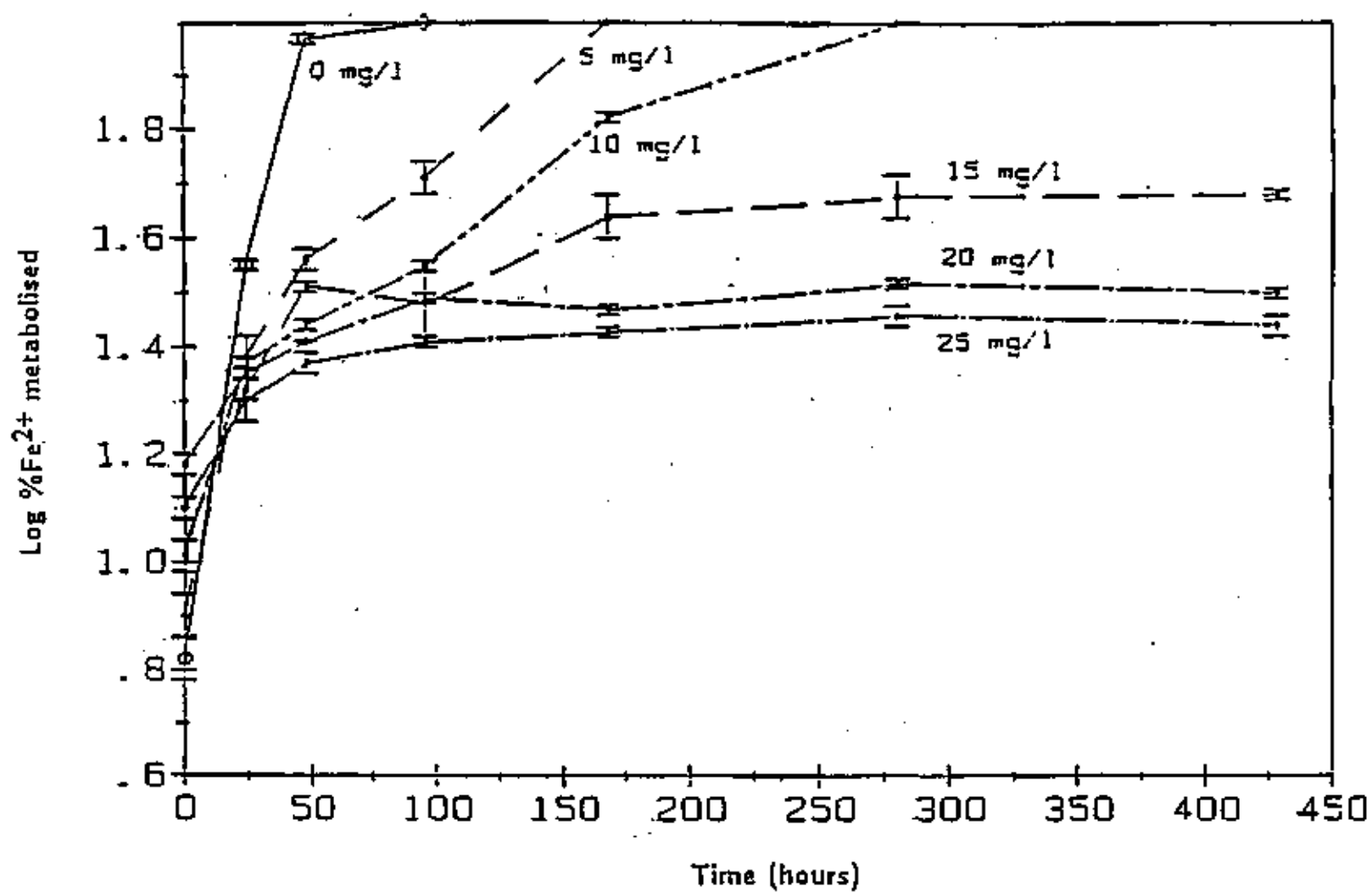
APPENDIX FIG. 33. Effect of increasing concentrations of Ceepryn on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The Ceepryn was added during the exponential phase of growth (25,5 h).



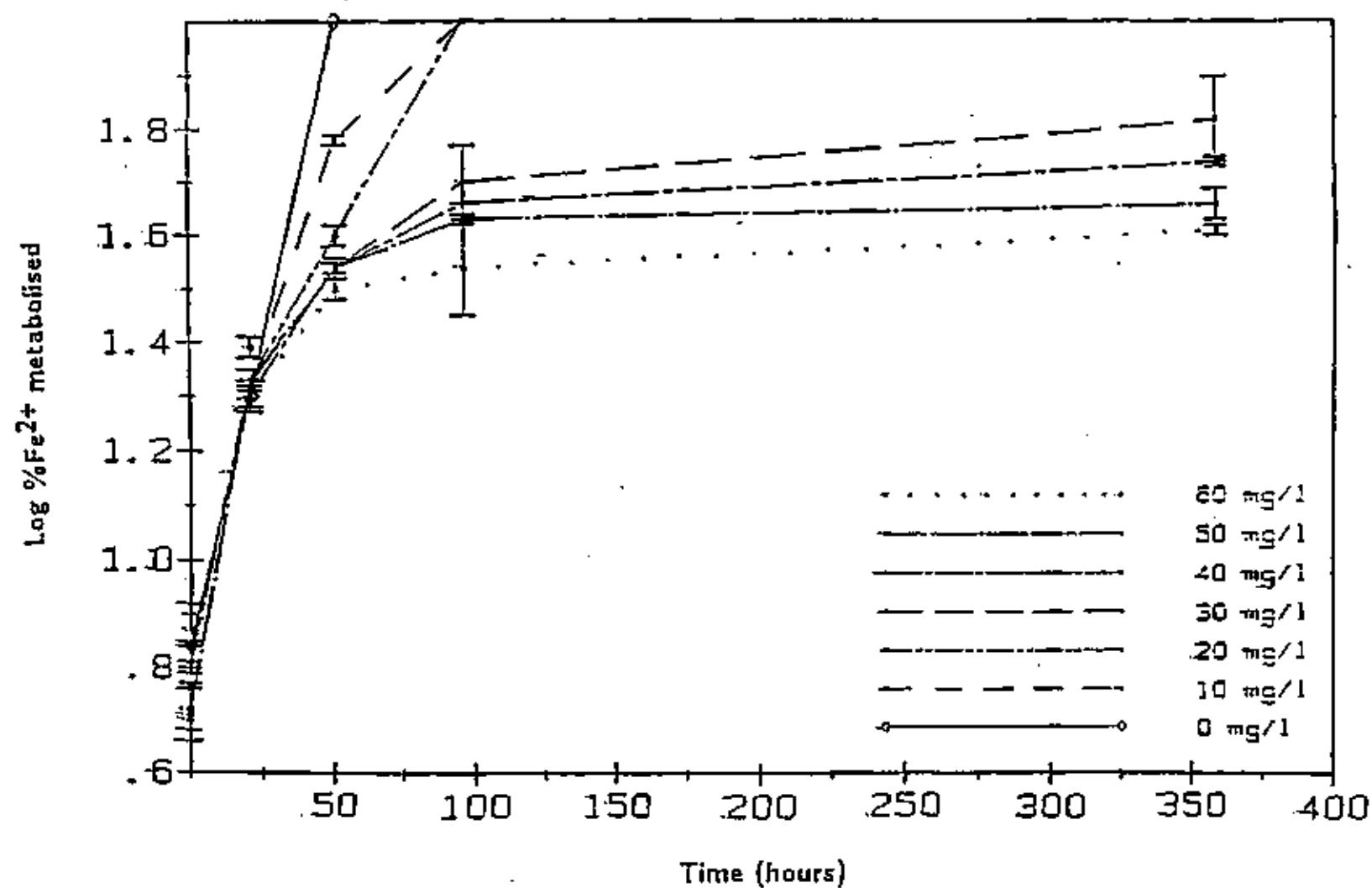
APPENDIX FIG. 34. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* WLR in HJJ medium when the sodium benzoate was added at the beginning of the experiment.



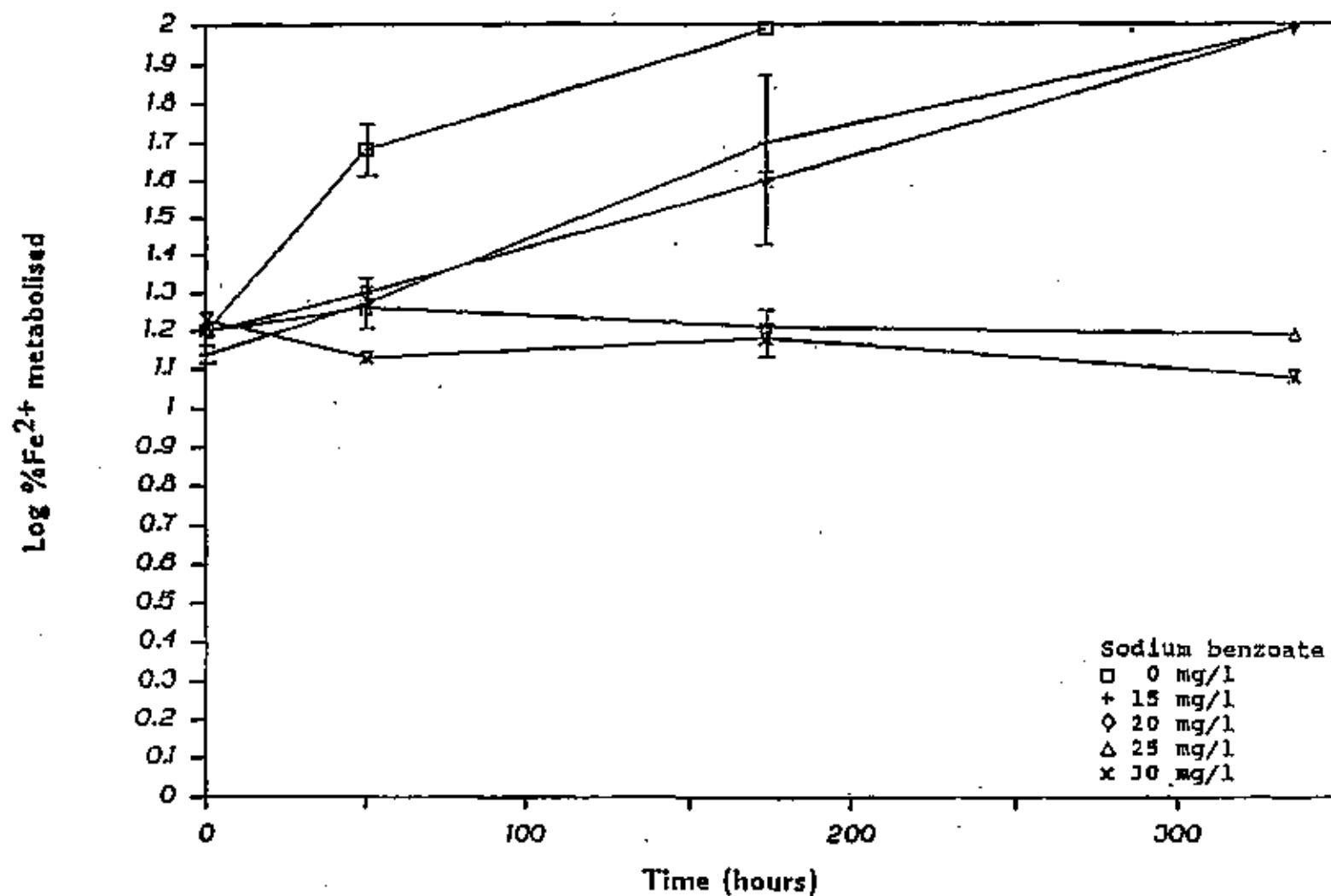
APPENDIX FIG. 35. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* WLR in HJJ medium when the inhibitor was added during the exponential phase of culture growth (21 h).



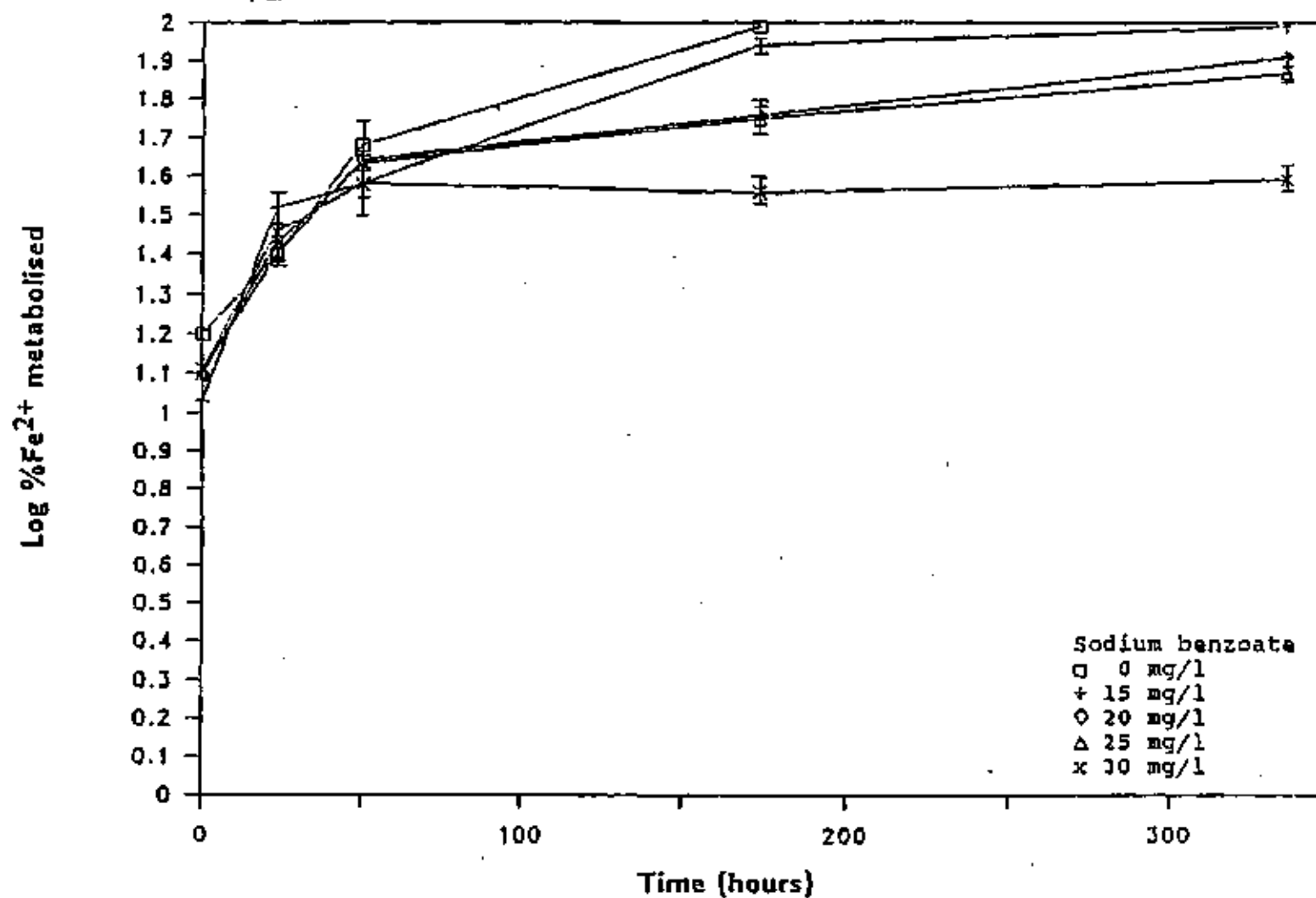
APPENDIX FIG. 36. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium when the inhibitor was added at the beginning of the experiment.



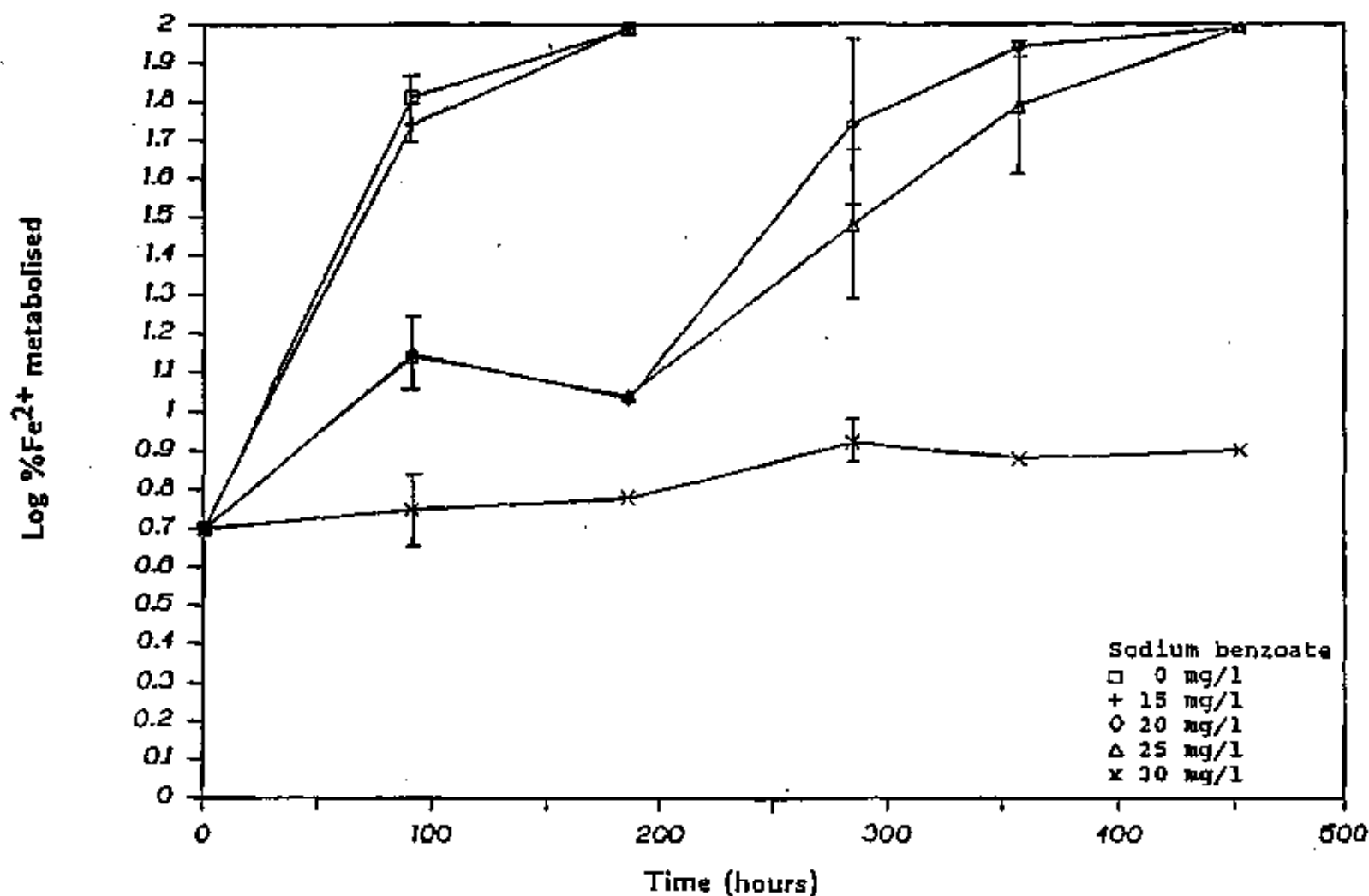
APPENDIX FIG. 37. Effect of increasing concentrations of sodium benzoate on the growth of *T ferrooxidans* ATCC 19859 in HJJ medium when the inhibitor was added during the exponential phase of culture growth (21 h).



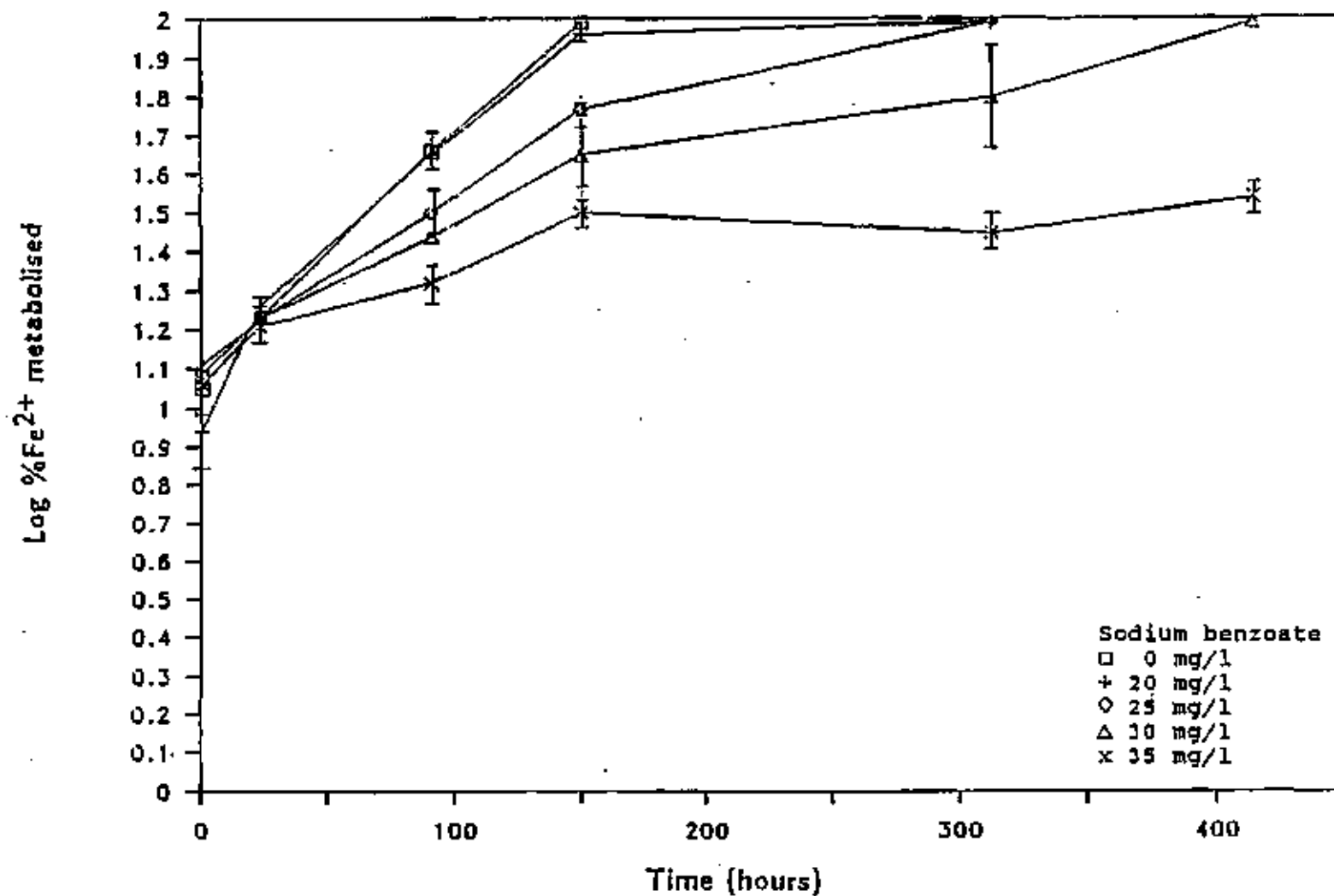
APPENDIX FIG. 38. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sodium benzoate was added at the beginning of the experiment.



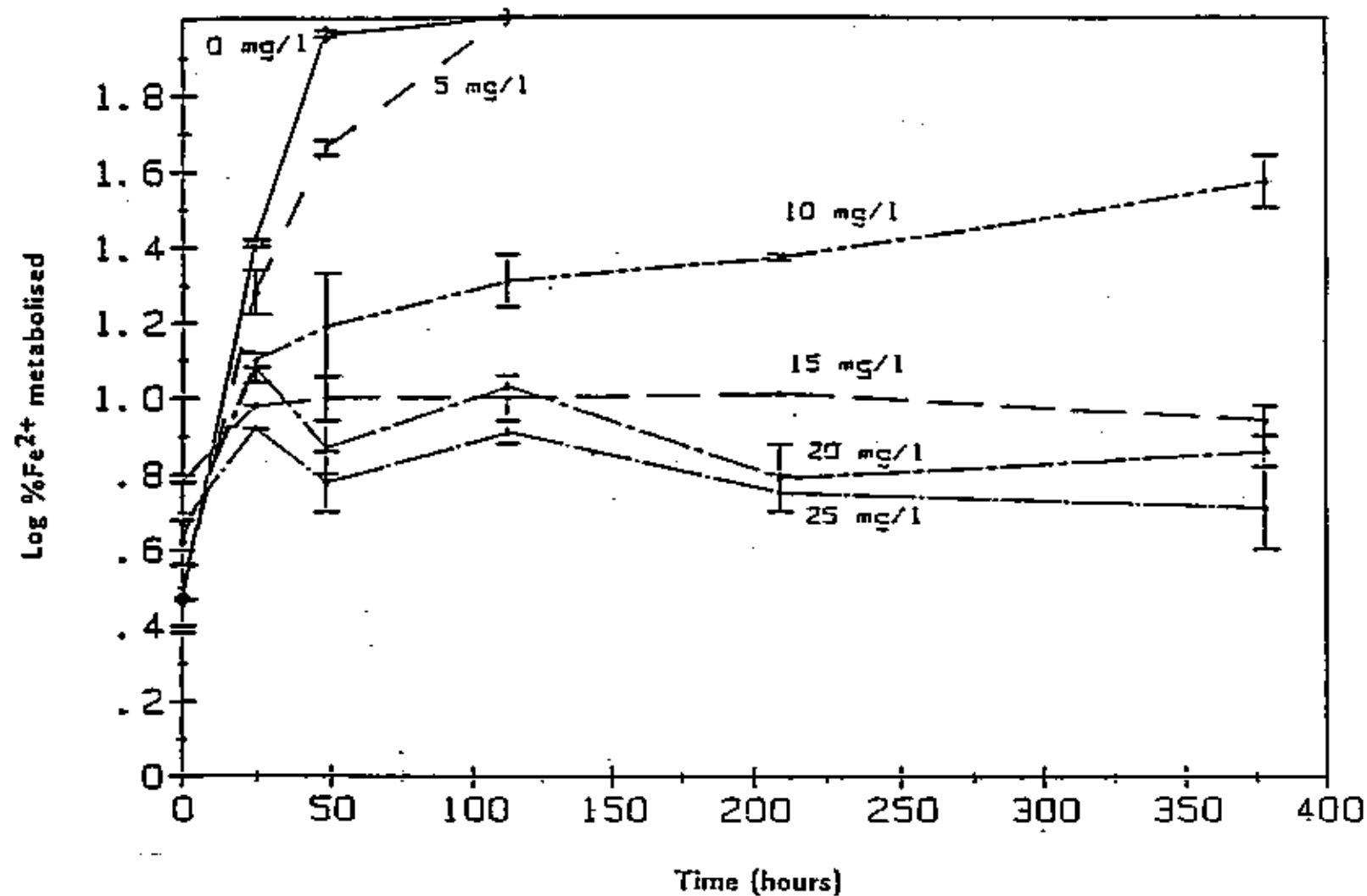
APPENDIX FIG. 39. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sodium benzoate was added during the exponential phase of culture growth (23 h).



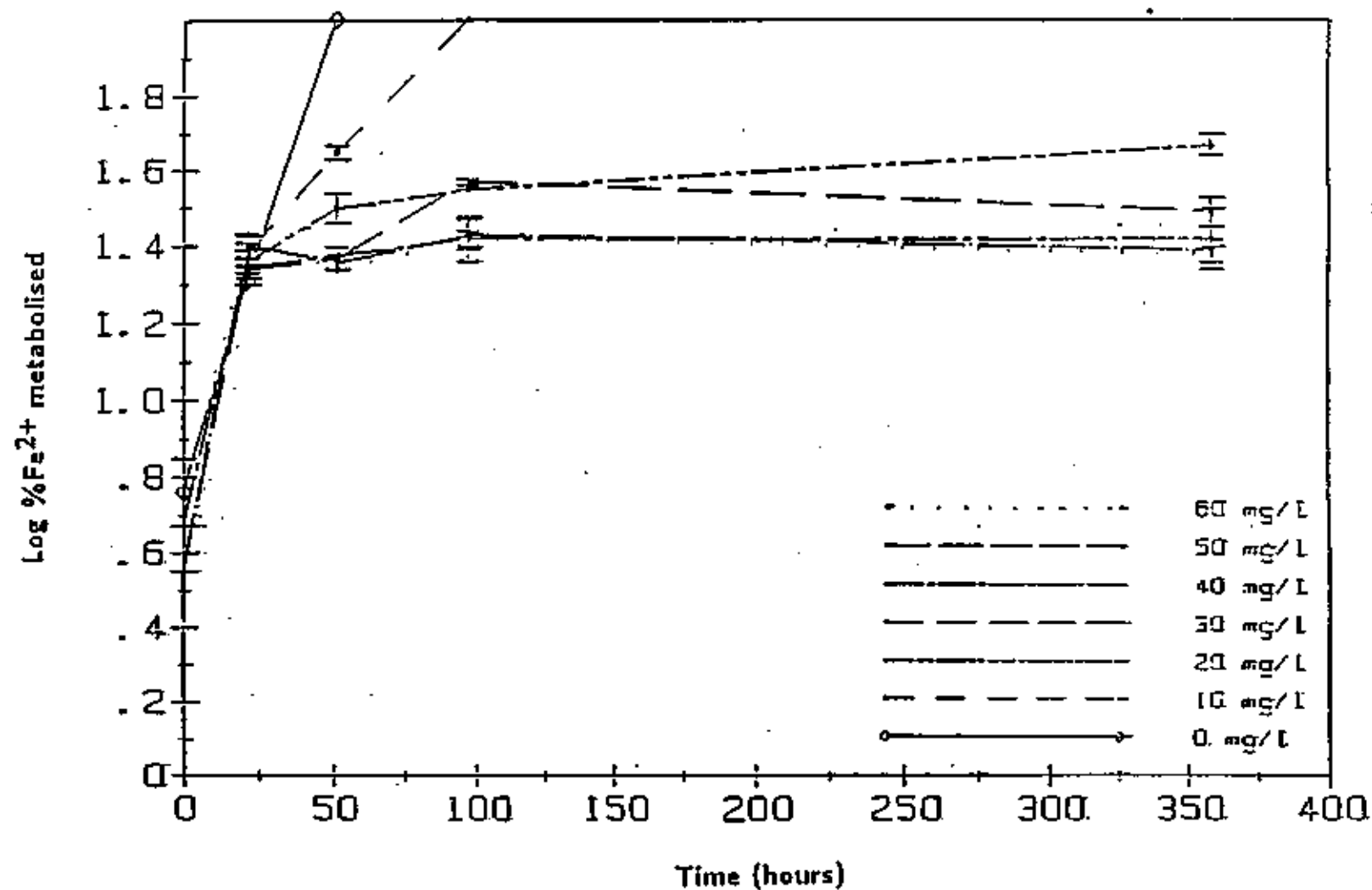
APPENDIX FIG. 40. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sodium benzoate was added at the beginning of the experiment.



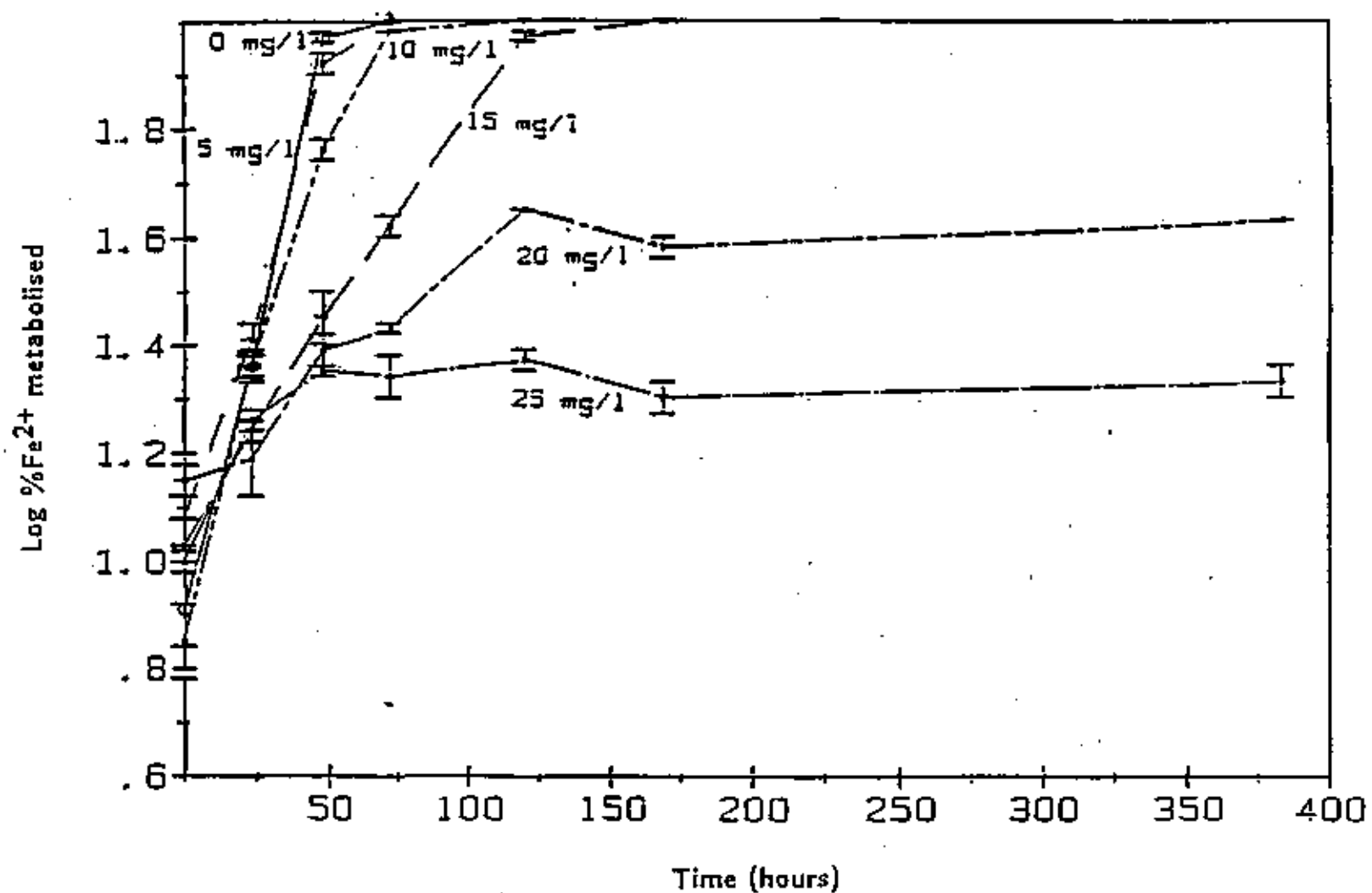
APPENDIX FIG. 41. Effect of increasing concentrations of sodium benzoate on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sodium benzoate was added during the exponential phase of culture growth (23 h).



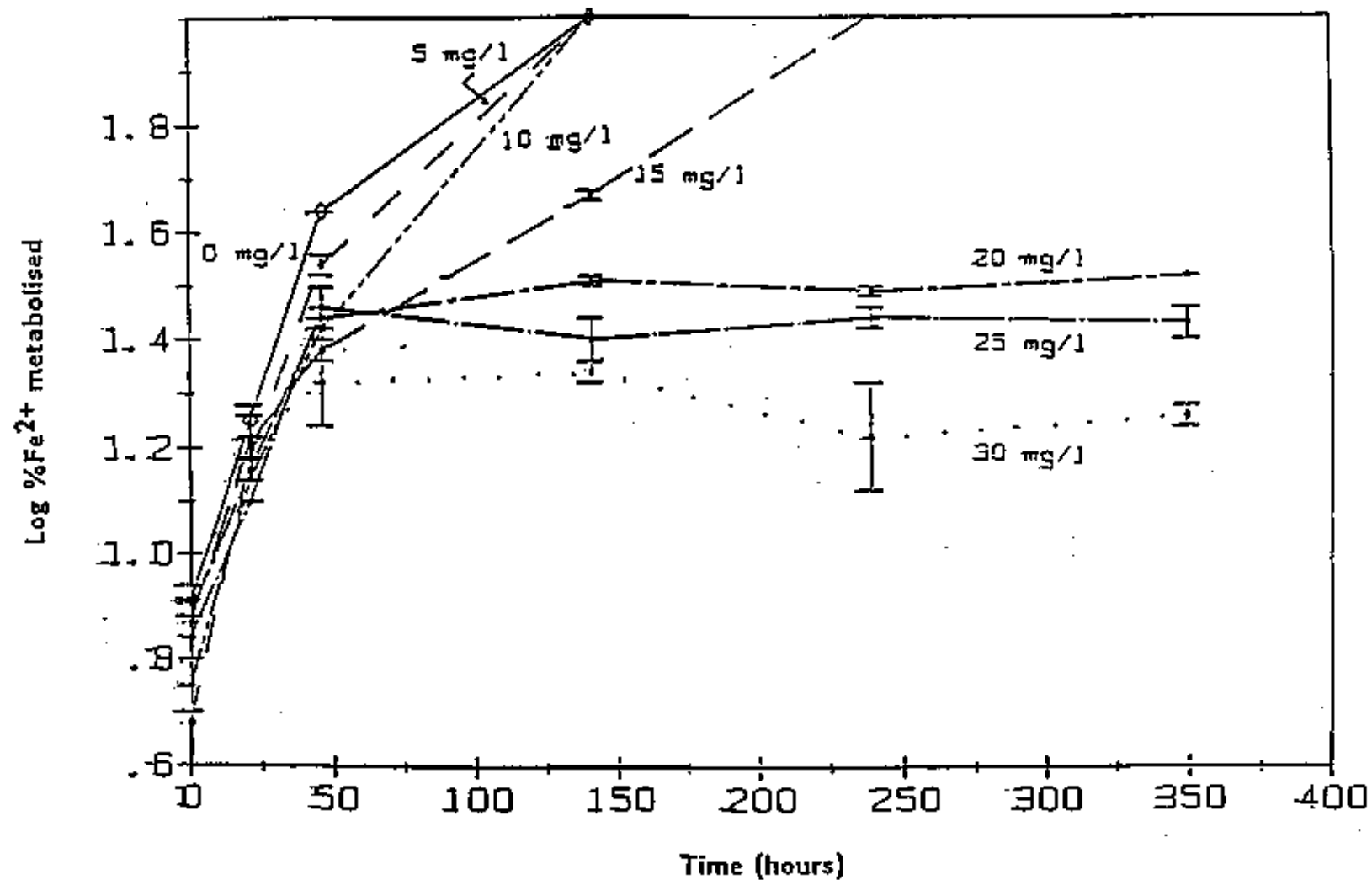
APPENDIX FIG. 42. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* WLR in HJJ medium when the sorbic acid was added at the beginning of the experiment.



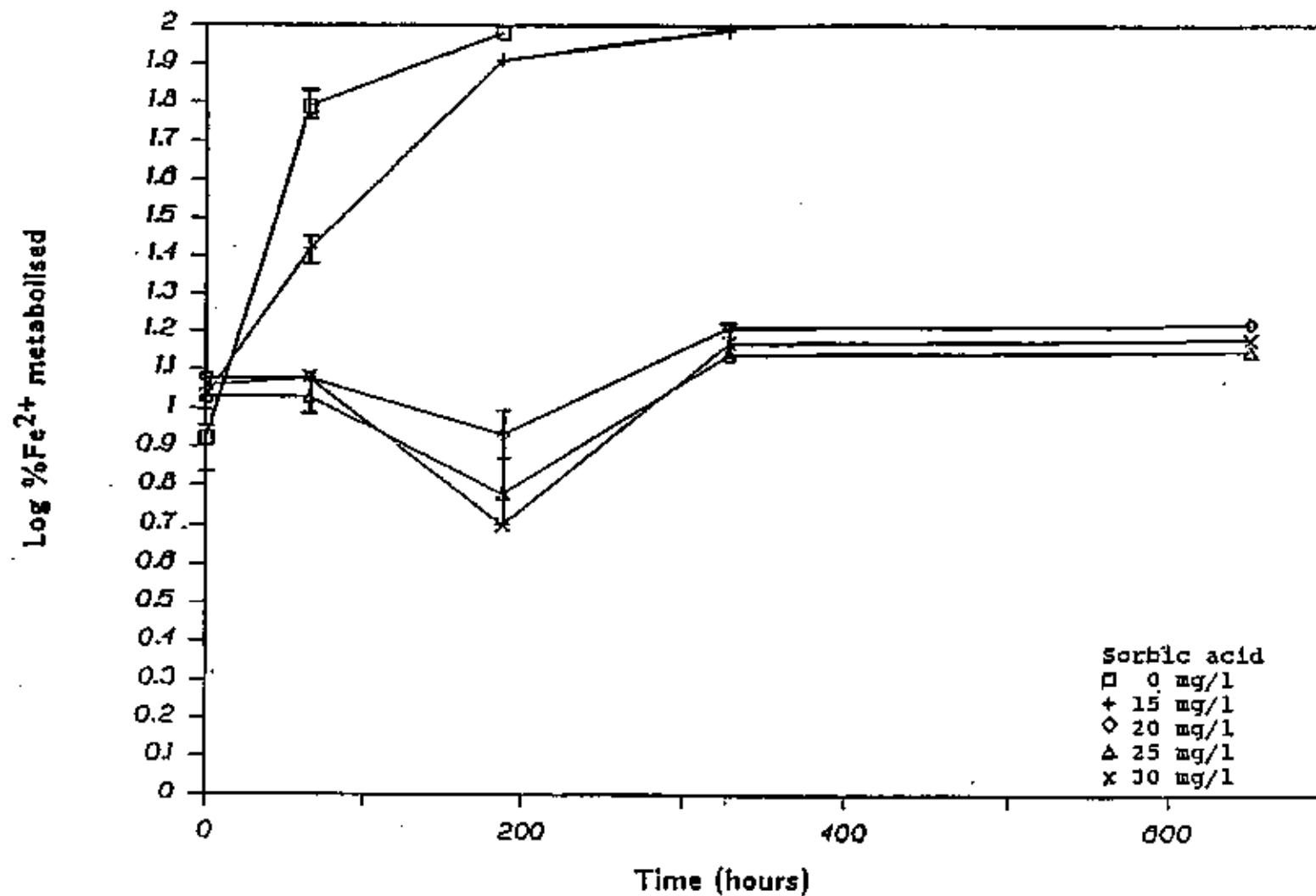
APPENDIX FIG. 43. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* WLR in HJJ medium when the inhibitor was added during the exponential phase of culture growth (21 h).



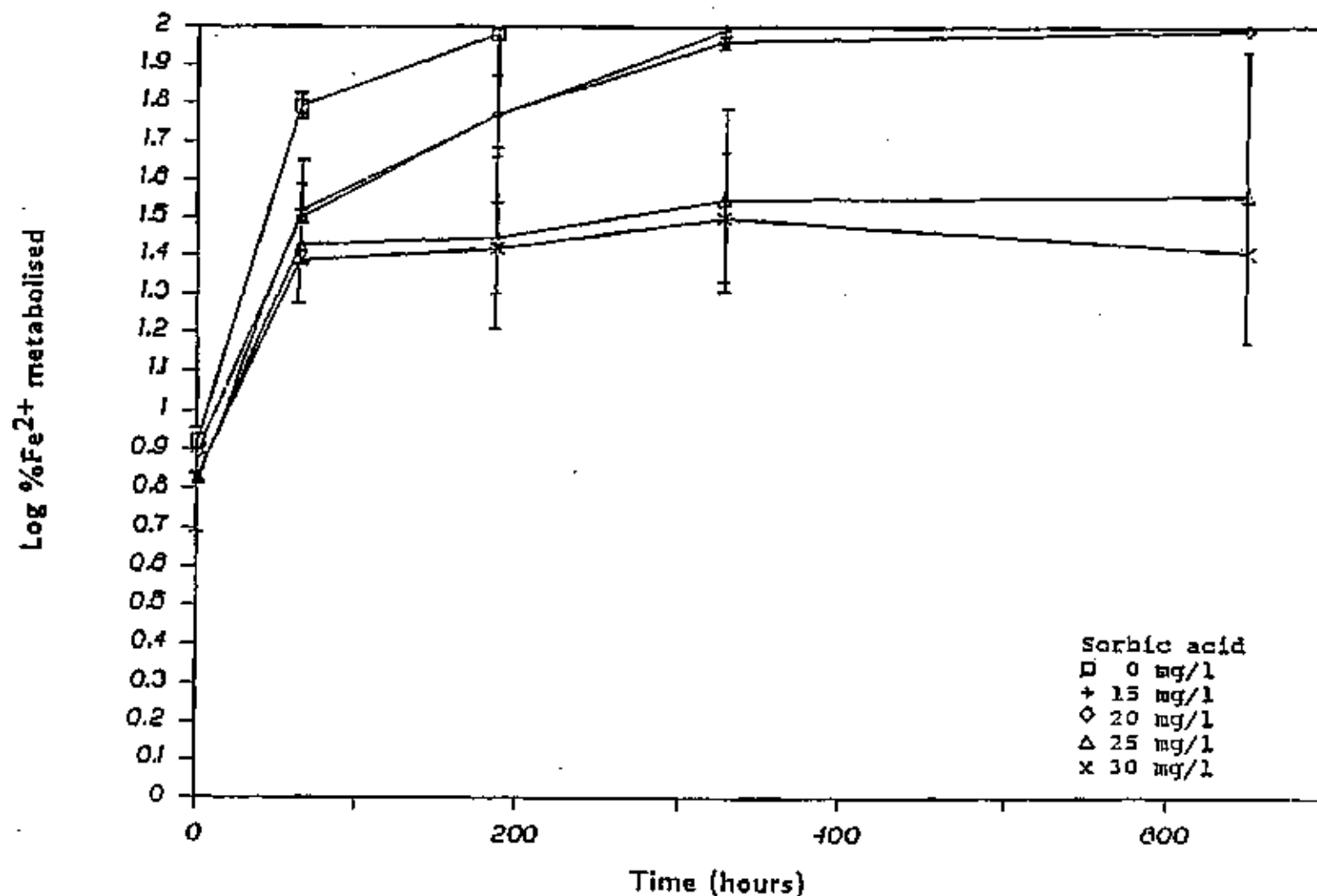
APPENDIX FIG. 44. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium when the sorbic acid was added at the beginning of the experiment.



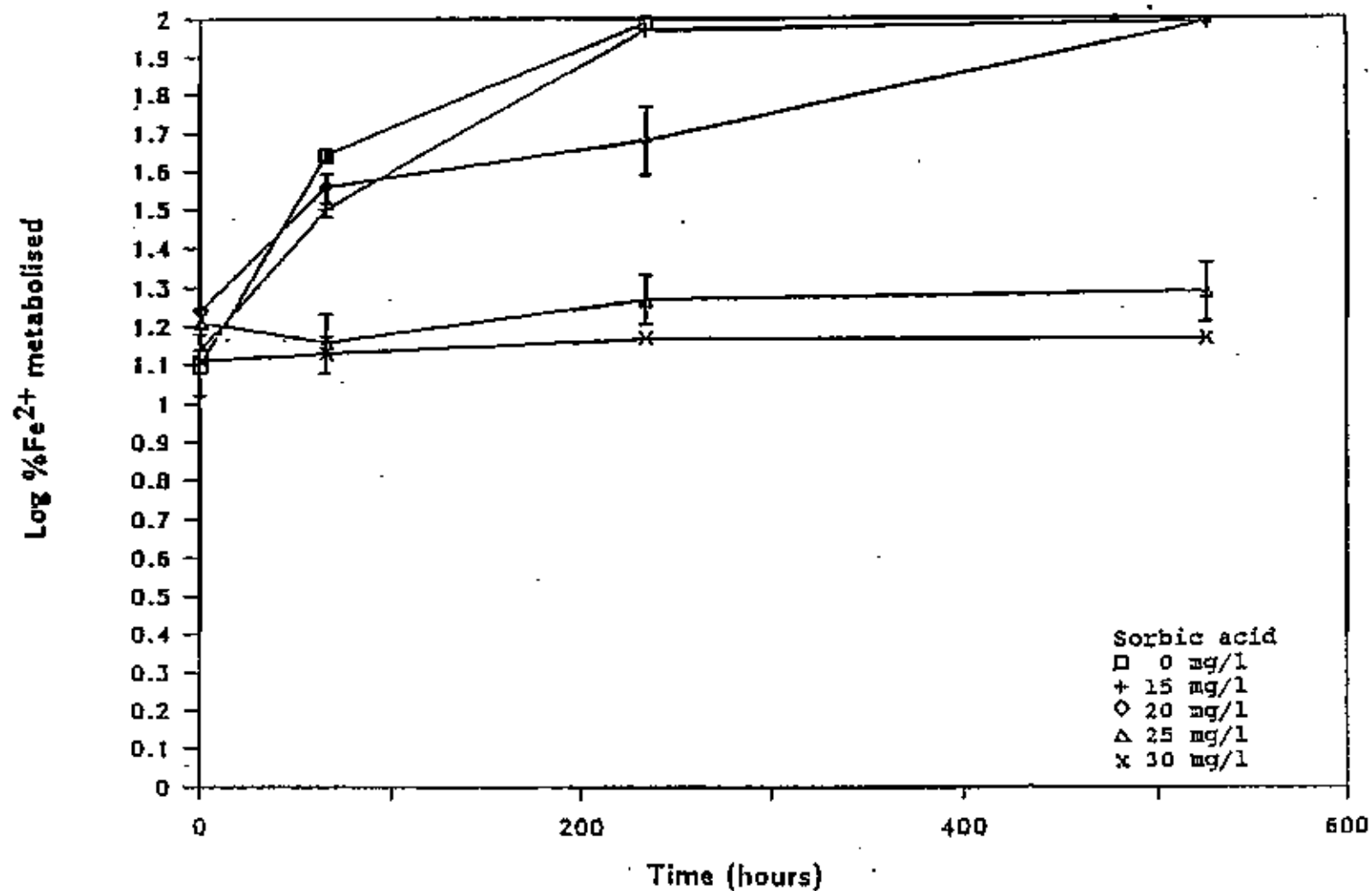
APPENDIX FIG. 45. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium when the inhibitor was added during the exponential phase of culture growth (21 h).



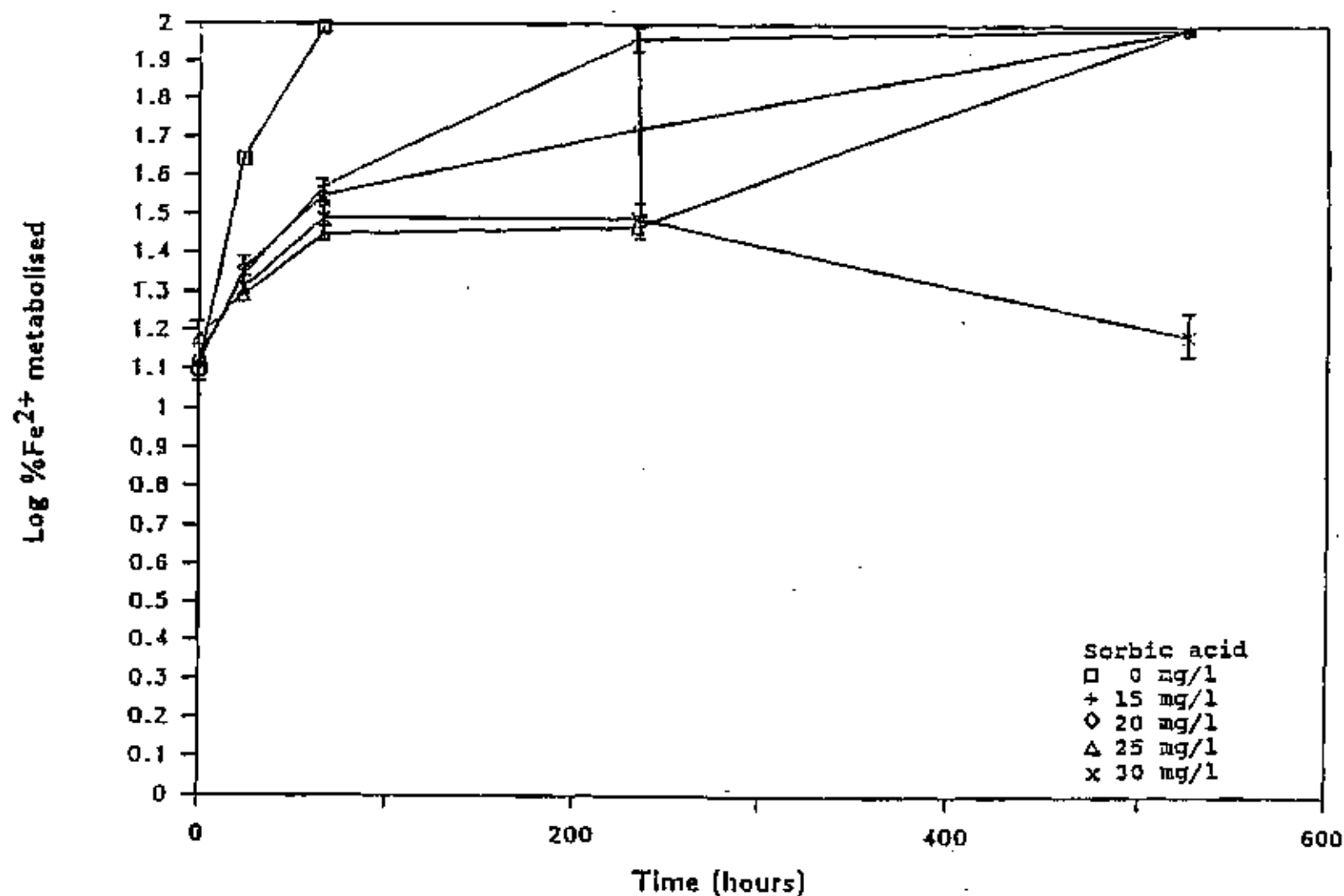
APPENDIX FIG. 46. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sorbic acid was added at the beginning of the experiment.



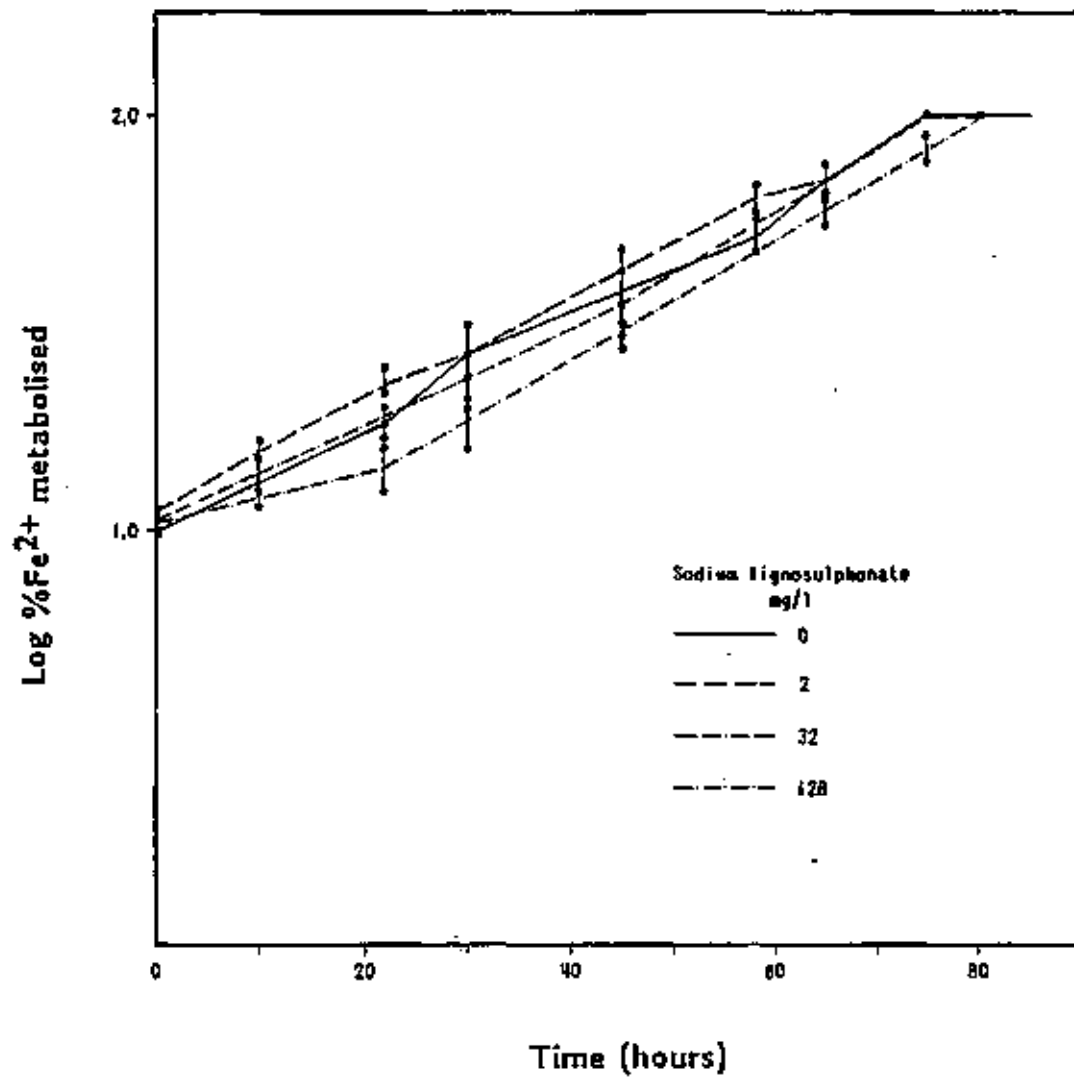
APPENDIX FIG. 47. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* WLR in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sorbic acid was added during the exponential phase of culture growth (23 h).



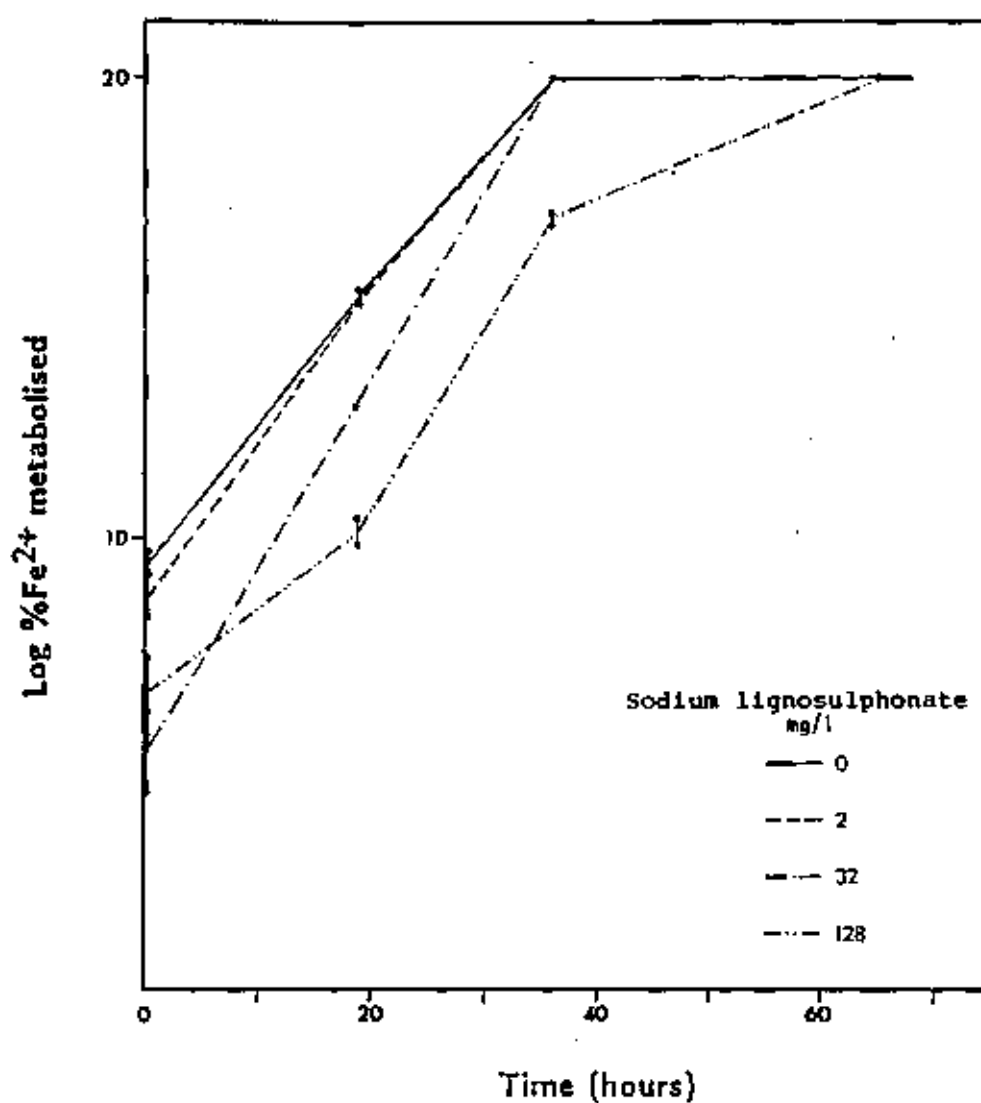
APPENDIX FIG. 48. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sorbic acid was added at the beginning of the experiment.



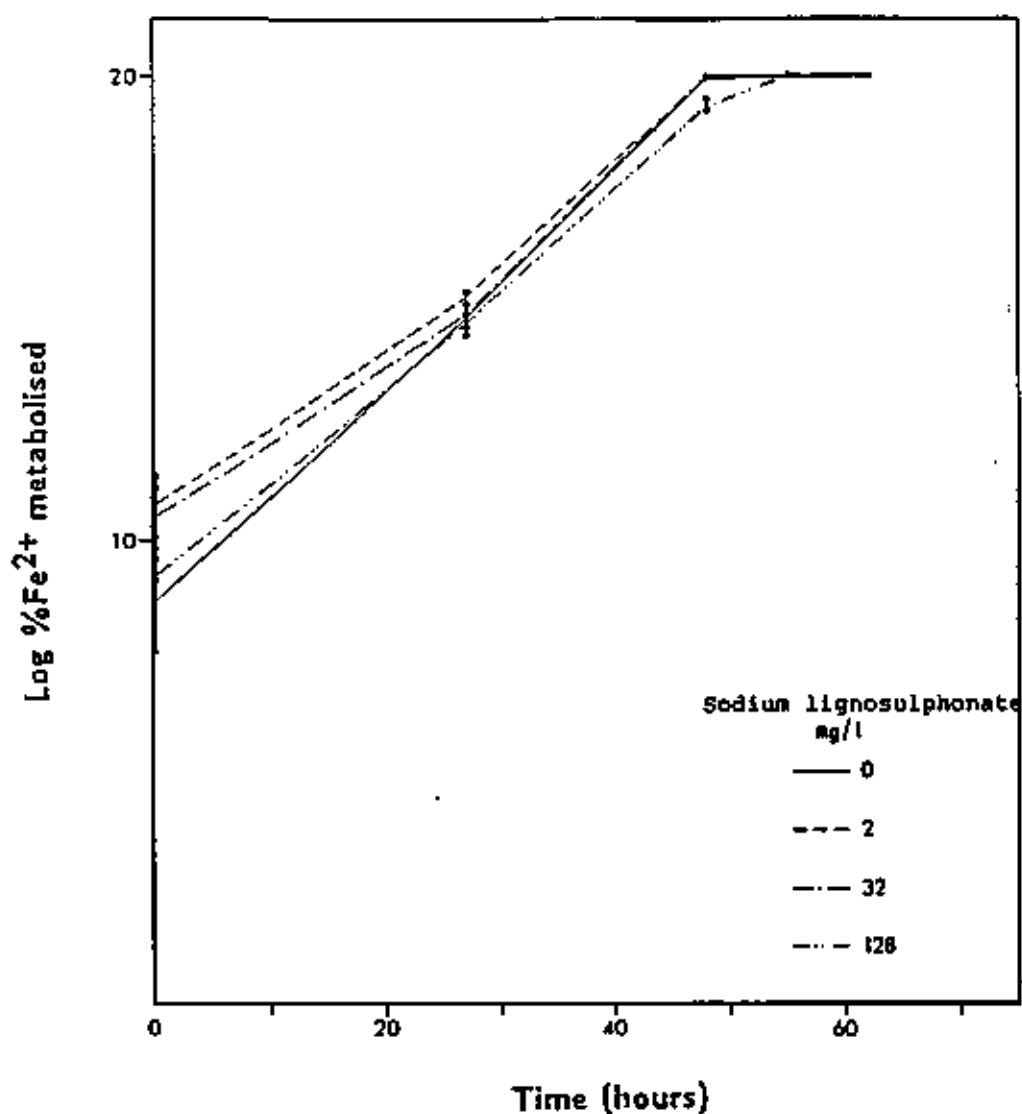
APPENDIX FIG. 49. Effect of increasing concentrations of sorbic acid on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The sorbic acid was added during the exponential phase of culture growth (23 h).



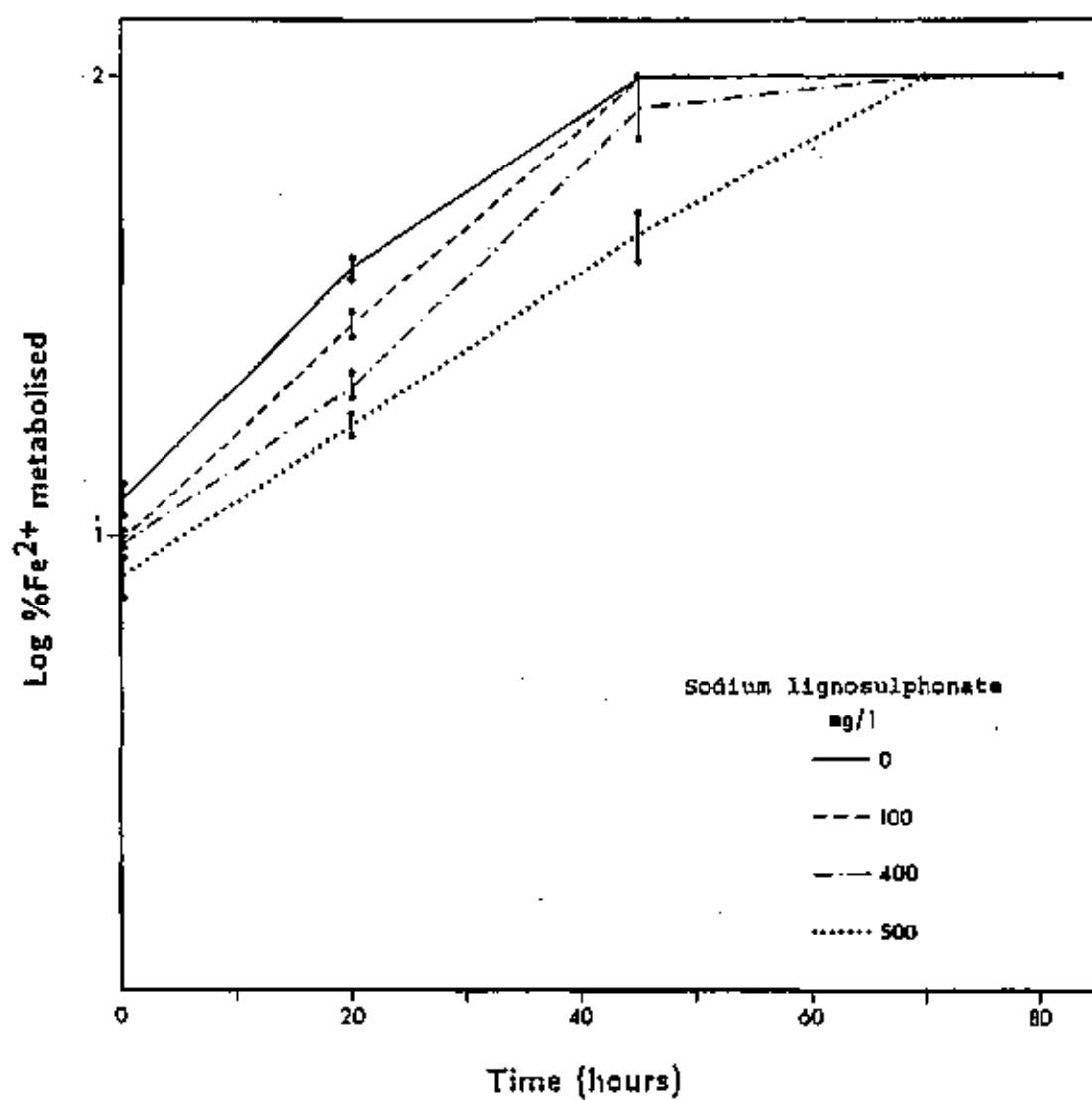
APPENDIX FIG. 50. Effect of increasing concentrations of sodium lignosulphonate on the growth of *T. ferrooxidans* WLR in HJJ medium. The sodium lignosulphonate was added during the exponential phase of growth (30 h).



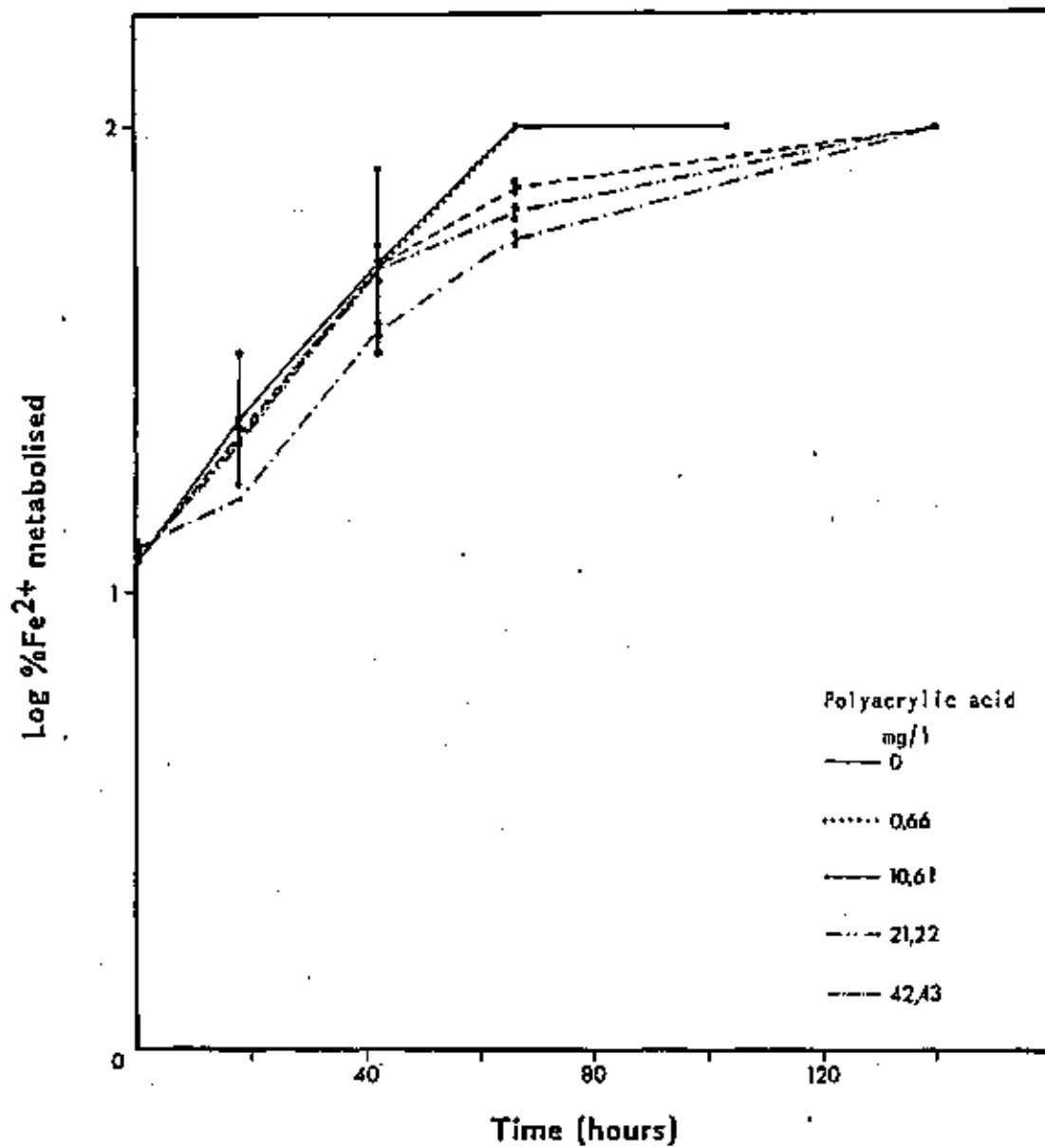
APPENDIX FIG. 51. Effect of increasing concentrations of sodium lignosulphonate on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The sodium lignosulphonate was added at the beginning of the experiment.



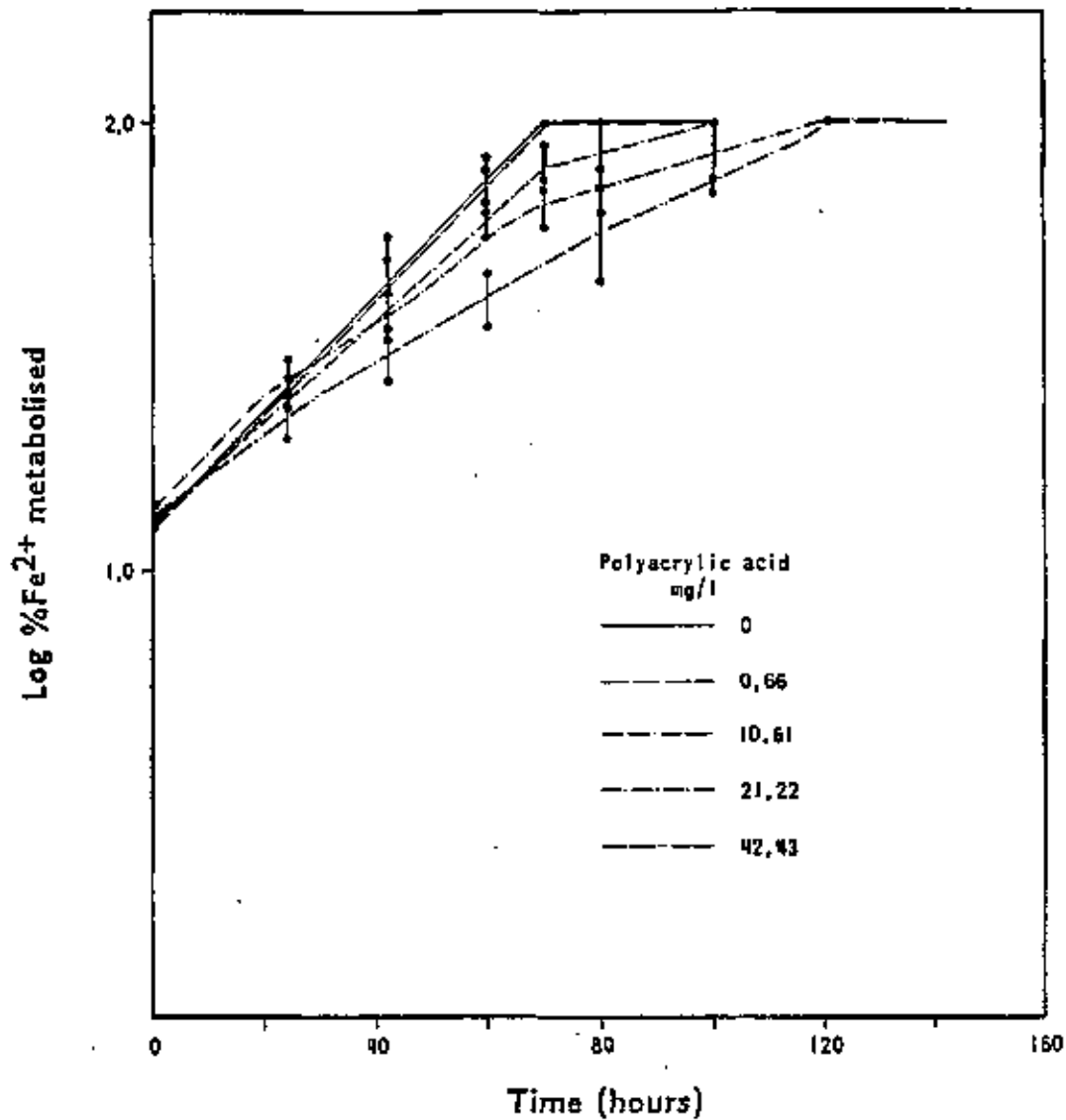
APPENDIX FIG. 52. Effect of increasing concentrations of sodium lignosulphonate on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The sodium lignosulphonate was added during the exponential phase of growth (27 h).



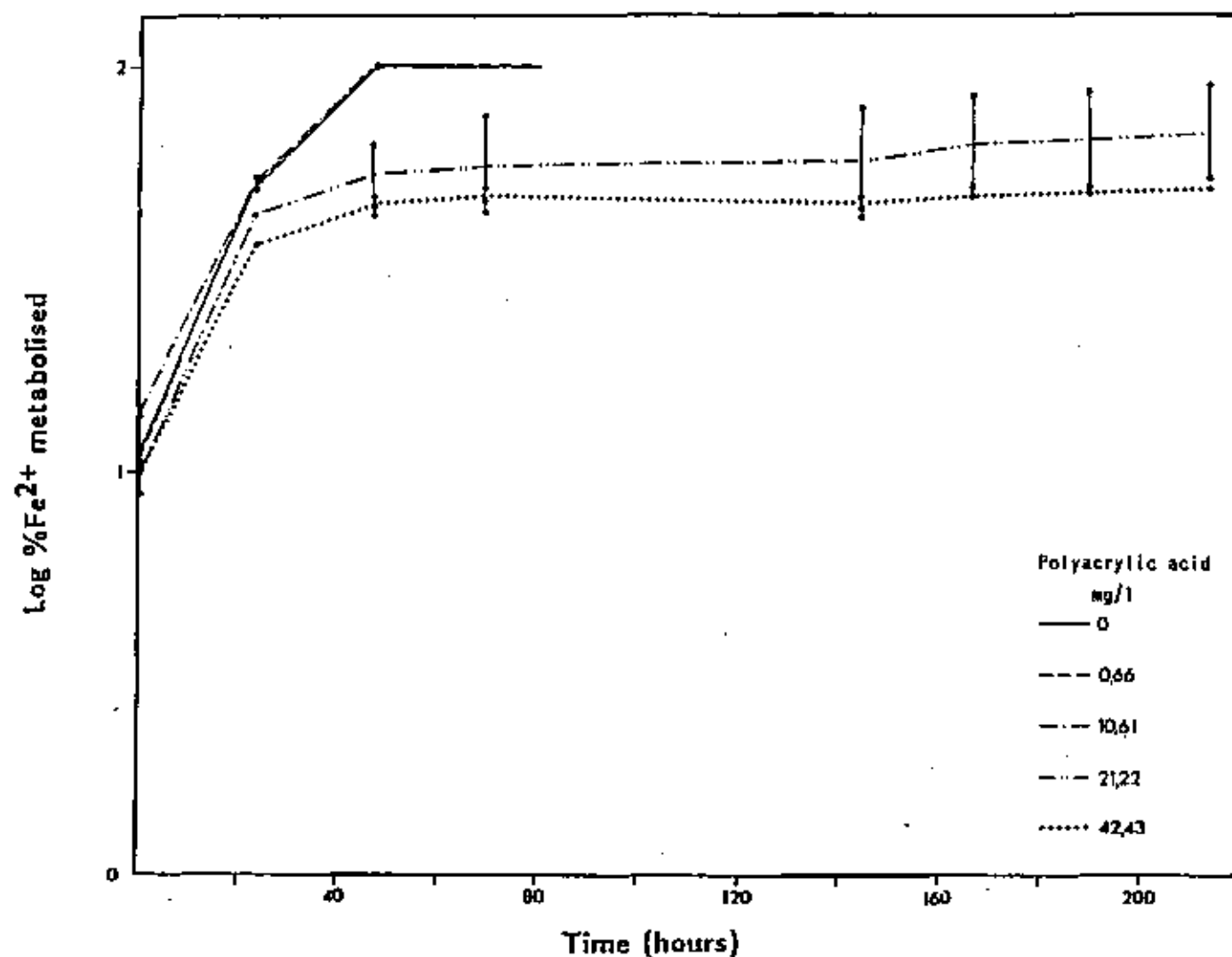
APPENDIX FIG. 53. Effect of increasing concentrations of sodium lignosulphonate on the growth of *T. ferrooxidans* WLR in HJJ medium. The sodium lignosulphonate was added at the beginning of the experiment.



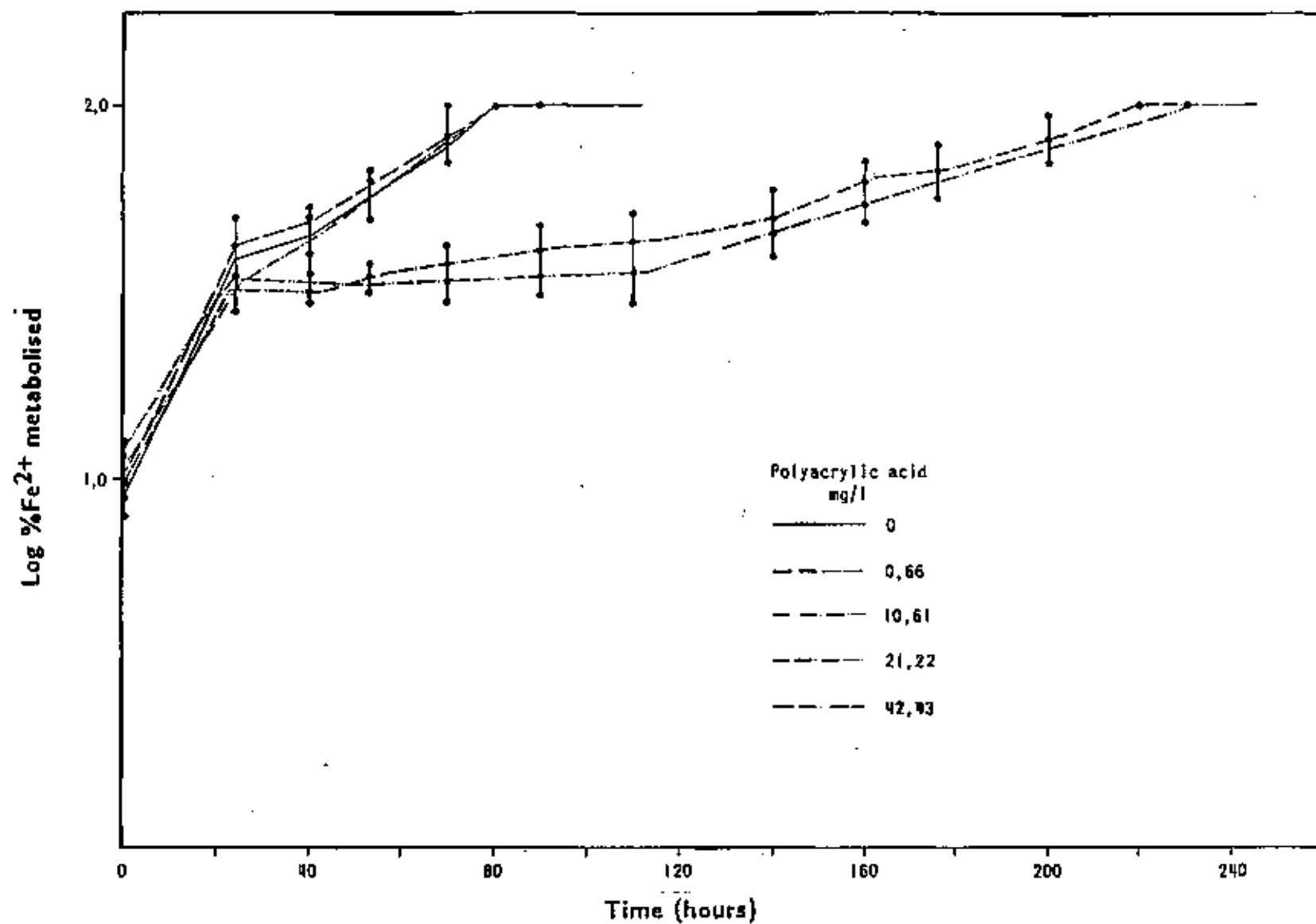
APPENDIX FIG. 54. Effect of increasing concentrations of polyacrylic acid on the growth of *T. ferrooxidans* WLR in HJJ medium. The polyacrylic acid was added at the beginning of the experiment.



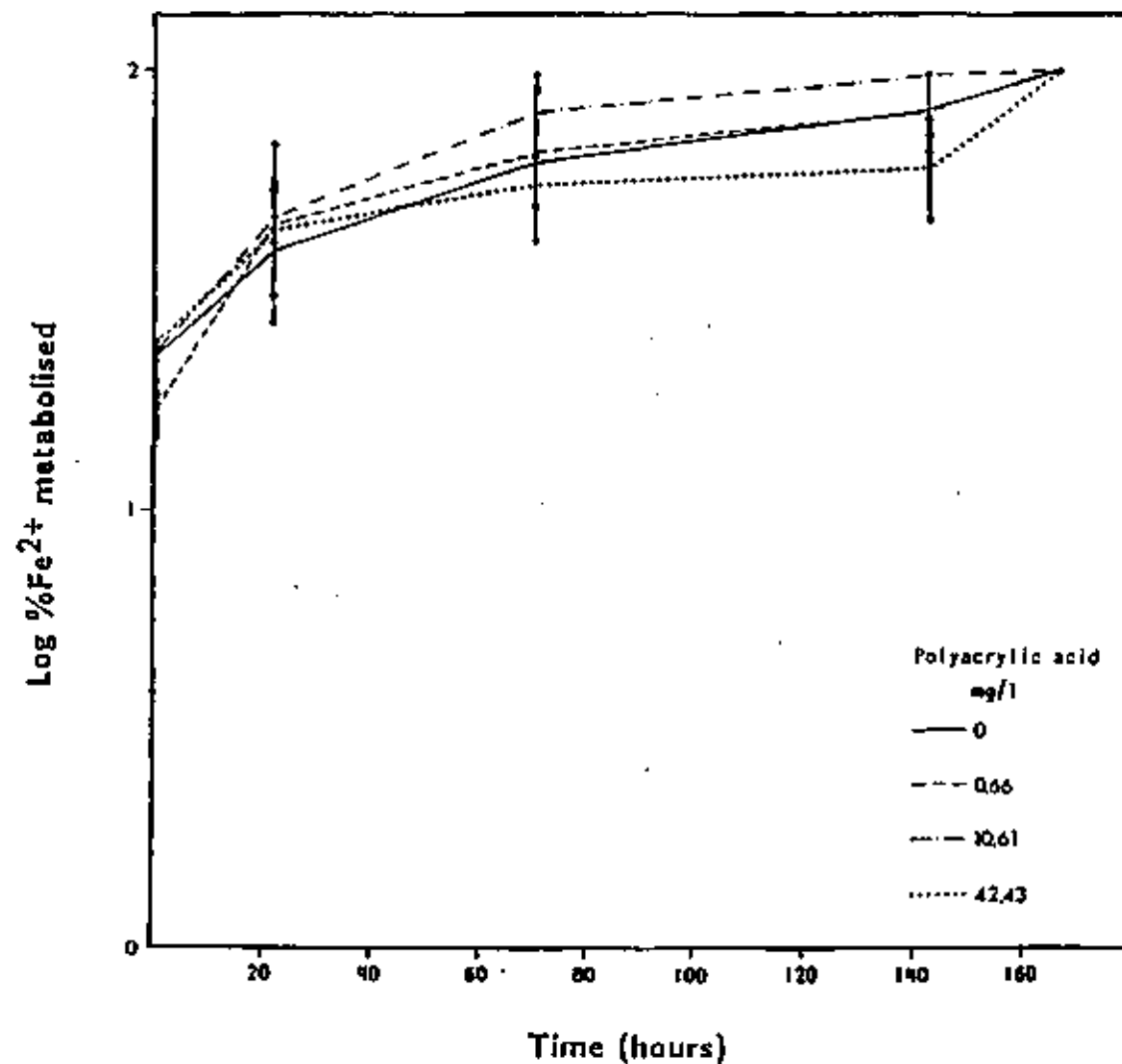
APPENDIX FIG. 55. Effect of increasing concentrations of polyacrylic acid on the growth of *T. ferrooxidans* WLR in HJJ medium. The polyacrylic acid was added during the exponential phase of growth (24 h).



APPENDIX FIG. 56. Effect of increasing concentrations of polyacrylic acid on the growth of *T. ferrooxidans* ATCC 19859 in HJJ medium. The polyacrylic acid was added at the beginning of the experiment.



APPENDIX FIG. 57. Effect of increasing concentrations of polyacrylic acid on the growth of *T ferrooxidans* ATCC 19859 in HJJ medium. The polyacrylic acid was added during the exponential phase of growth (24 h).



APPENDIX FIG. 58. Effect of increasing concentrations of polyacrylic acid on the growth of *T. ferrooxidans* ATCC 19859 in 100 ml HJJ medium supplemented with 50 g air-dried mine dump sand G5. The polyacrylic acid was added at the beginning of the experiment.