CONTRIBUTION OF MICROORGANISMS TO THE OXIDATION OF PYRITE

Promotor: dr.ir. E.G. Mulder, hoogleraar in de algemene microbiologie
en de microbiologie van bodem en water

G.J.M.W. ARKESTEYN

CONTRIBUTION OF MICROORGANISMS TO THE OXIDATION OF PYRITE

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 15 februari 1980
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISN: 100693

This study was carried out at the Laboratory of Microbiology, Agricultural University, Wageningen, The Netherlands.

Cover designed by Rijk Verhoeven.

STELLINGEN

1. Bij de vorming van katteklei is de initiële verzuring een niet-biologisch proces.

   Dit proefschrift.

2. *Thiobacillus ferrooxidans* is in staat om de zwavelcomponent van pyriet via het direct-contact-mechanisme te oxyderen en de daarbij vrijkomende energie te benutten voor groei.

   Dit proefschrift.

3. De bepaling van het stikstofgehalte van ijzerhoudende sedimenten van *Thiobacillus ferrooxidans* cultures als parameter voor de groei van dit organisme, zoals uitgevoerd door McGoran e.a., leidt tot verkeerde conclusies.

   McGoran, C.J.M., Duncan, D.W. and Walden, C.C. (1969) Can. J. Microbiol. 15, 135-138.
   Ivarson, K.C. (1973) Can. J. Soil Sci. 53, 315-323.

4. De kwantitatieve bijdrage van de meeste kleurloze zwavelbacteriën tot de in de natuur voorkomende zwavelkringloop is onbekend.

5. Door de pH van potentiële katteklei via toediening van kleine hoeveelheden calciumcarbonaat hoger dan 5.5 te houden, kan de oxydatie van pyriet in belangrijke mate worden vertraagd en na enige jaren misschien zelfs geheel worden voorkomen.

6. Bij het onderzoek naar de factoren die het uiteenvallen van met zwaveldioxyde geconserveerde aardbeien bepalen, hebben Dennis e.a. de invloed van de pH sterk veronachtzaamd.

   Dennis, C., Davis, R.P., Harris, J.E., Calcut, L.W. and Cross, D. (1979) J. Sci. Food Agric. 30, 959-973.

7. Het verschil in eigenschappen tussen de pectine-esterasen van respectievelijk planten en schimmels wijst op een functioneel verschil van dit enzym bij beide typen van organismen.

Versteeg, C. Proefschrift LH Wageningen.

8. In deze tijd van normalisatie dient men in de conserventechnologie het gebruik van het begrip Z-waarde te vervangen door de temperatuurscoefficiënt Q_{10} en het begrip lethaal effect (uitgedrukt in procenten) door proceswaarde (uitgedrukt in minuten).

Leniger, H.A. en Beverloo, W.A. 1971 collegedictaat "Inleiding in de technologie" Afdeling Levensmiddelentechnologie LH Wageningen.

9. In de Beleidsnota Universitair Onderzoek had men bij de voorgestelde financieringsstructuur van het universitaire onderzoek meer rekening moeten houden met de consequenties voor het personeel.

Beleidsnota Universitair Onderzoek 1979. Staatsuitgeverij Den Haag.

10. Bij hun onderzoek naar de afbraakroute van propyleen hebben Cerniglia e.a. de betekenis van propyleen-oxyde sterk veronachtzaamd.

Cerniglia, C.E., Blevins, W.T. and Perry, J.J. (1976)
Appl. Environ. Microbiol. 32, 764-768.

11. "Regeren is vooruitzien" zal het devies geweest zijn van de gemeentelijke instantie die jongerenhuisvesting heeft gerealiseerd in een bejaardenhuis.

Hendrik Nanneshof Bolsward

G.J.M.W. Arkesteyn
Contribution of microorganisms to the oxidation of pyrite.
Wageningen, 15 februari 1980.

Aan mijn ouders
Aan Lieneke

wim van veen
prof.mulder
gijs kuenen
lieneke
jan de bont
chris schouten
j.c.v.velzen
aaldert
c.wessels
m.van de kolk
willy den
hartog roos
a.d.adamse
mevr.c.möller-mol
mevr.
v.d.zwaag
mevr. j.v.haperen
frits lap
l.t.beghein
arja
theo hansen
antoon akkermans
gerard kortstee
wout middel
hoven
alfons
jaap visser
nico v.breemen

CONTENTS

1. INTRODUCTION

1.1 GENERAL

Acid sulphate soils are situated in coastal regions and cover several million hectares of land distributed all over the world. However, the area of potentially acid sulphate soils is even larger. More than five-million hectares of acid sulphate soils are located in South-East and East Asia (11). A world map of such soils has been compiled in 1973 by Kawalec (36). It shows that the potentially acid sulphate soils and the acid sulphate soils are situated mainly in the tropics, but they occur in more temperate zones as well (Fig. 1).

The acid sulphate soils are not suitable for agricultural purposes and therefore the reclamation of potentially acid sulphate soils may give rise to many problems. Technically such soils could be improved by liming but the amounts of lime required (5-100 tons/ha) are not economically feasible for many countries (8).

Acid sulphate soils originate from anaerobic marine sediments containing substantial amounts of pyrite (FeS_2). When such soils become aerobic, pyrite is oxidized which results in the formation of sulphuric acid. The pH of the soils may thus decrease to 4.0 or even to as low as 3.0 when acid-neutralizing components e.g. calcium carbonate are not present. The acid attacks the clay minerals thereby causing the liberation of aluminium ions which are toxic to plants (10). Acid sulphate soils often show yellow mottles of the mineral jarosite [$KFe_3(SO_4)_2(OH)_6$] (see 1.3.4). The typical yellow colour led to the use by Dutch farmers of the term "cat clay" for these infertile soils. This word was (first) introduced into the literature when the regional soil survey started in the Netherlands (24); in more recent reports the term acid sulphate soils is used. Such soils have been known for centuries in the Netherlands and a historical review on the subject was published by Poelman (49). According to this study Linnaeus was the first to describe acid sulphate soils in his "Systema Naturae" in 1735 using the name "argilla vitriolaea" (vitriolic clay). At the same time Le Franc van Berkhey mentioned the term "katteklei" (cat clay) in the first part of his "Natural history of Holland" (49).

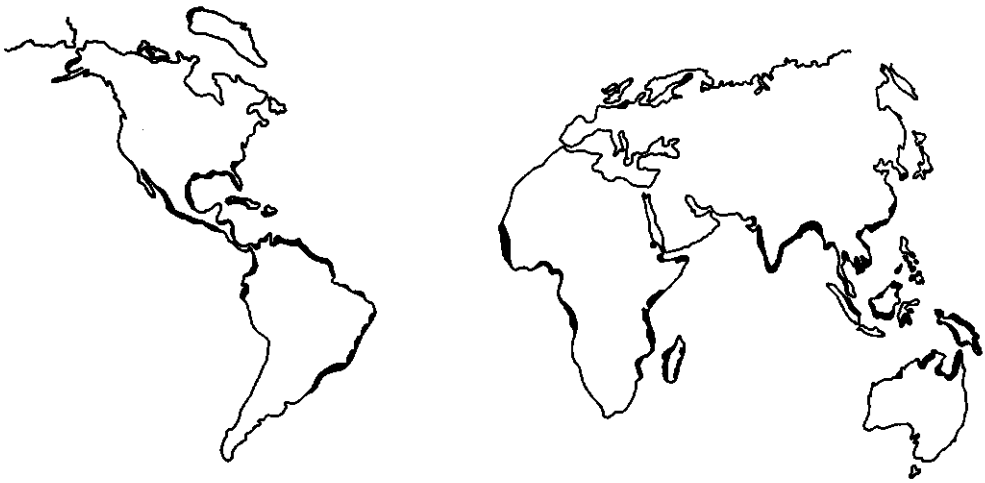

Fig. 1. The distribution of acid and potentially acid sulphate soils in the world (36)

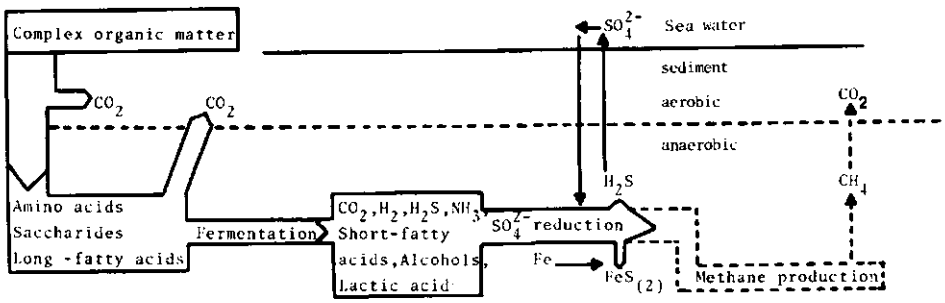

Fig. 2. The carbon cycle in estuarine areas

The first fundamental investigation of acid sulphate soils dates from 1886 (5). In that study an explanation was given for the oxidation-reduction cycle by which marine sulphate accumulated under reduced conditions as sulphides and the sulphidic sediments turned into acid sulphates on aeration. Many studies and literature reviews (8; 18) have appeared on this subject since then Most of the studies were carried out by soil scientists whose interests were mainly confined to geochemical processes. Surprisingly, little attention has been paid so far to the function of microorganisms in these processes, in spite of the important part bacteria play in the events leading to acidification of the soil. Therefore it was decided to set up a study on the role of microorganisms in the acidification of pyritic soils. Before presenting the results of this study a short review is given of the literature pertaining to the function of microorganisms in the formation and oxidation of pyrite.

1.2 THE FORMATION OF PYRITE

A number of processes mediated by microorganisms are of eminent importance for the formation of pyrite in anaerobic sediments. The first involve the anaerobic conversion of complex organic matter such as carbohydrates, proteins and lipids by organisms that hydrolyse these compounds to mono- and disaccharides, amino acids, glycerol and long-chain fatty acids. The hydrolysis products are fermented to organic acids, alcohols, carbon dioxide, hydrogen and ammonia. The sulphate-reducing bacteria in turn utilize these compounds as energy source using sulphate as electron acceptor which is reduced to sulphide. These processes are schematically summarized in Fig. 2. The sulphide can be precipitated as iron sulphide from which ultimately pyrite is formed. The various parts of this process will be discussed in the following paragraphs.

1.2.1 *The anaerobic digestion of complex organic matter*

Carbon dioxide is incorporated into organic compounds by autotrophic organisms such as algae and sea weeds in the oceans. When these organisms die they settle down. Especially in quiet coastal waters near shores deposition of large amounts of organic matter occurs (6). In estuarine marshes, the supply of organic material originates from both sedimentation of detritus and the natural salt-water vegetation. Only little information is available on the anaerobic decomposition of complex organic matter. Most of the present knowledge is derived from studies of the anaerobic digestion processes of activated sludge and the rumen

fermentation. The complex organic matter mainly consists of carbohydrates, lignin, proteins and lipids. Cellulolytic, proteolytic and lipolytic bacteria were observed and sometimes isolated from both anaerobic digesters and the rumen (32). Great differences exist between the anaerobic decay in sediments or waste-processing digesters and the anaerobic digestion in the rumen (41). Consequently, bacteria isolated from the rumen are not likely to be found in anaerobic marine sediments whereas the organisms isolated from anaerobic digesters, such as proteolytic clostridia or cellulolytic bacteria (32; 55), probably can be observed in marine sediments. Information on anaerobic starch or pectin fermentation in such environments is scarce (41). Lignin is probably not degraded anaerobically in nature (29). Extracellular enzymes from microorganisms including proteases and cellulases have been demonstrated in aquatic habitats rich in organic matter (42; 52). The products of hydrolysis of the organic matter, *viz.* amino acids, mono- and disaccharides and long-chain fatty acids, partly diffuse to the aerobic upper layer and are partly fermented to short-chain fatty acids e.g. formic, acetic, propionic, butyric and lactic acid and alcohols (25). During fermentation, hydrogen, carbon dioxide and ammonia are also produced. It has been shown that most of these acids and gasses can be utilized either by sulphate-reducing bacteria or by methanogenic bacteria. In natural sediments the zone of active methane production generally is found below the zone of sulphate reduction (15; 25). The methane-producing bacteria seem to be inhibited by the presence of sulphate owing to the production of hydrogen sulphide by sulphate-reducing bacteria. Competition for substrate, e.g. hydrogen, between both groups of bacteria may also occur (15). In the sediments of fresh-water lakes the sulphate concentration limits the presence of sulphate-reducing bacteria (15). In marine sediments sulphate is generally abundant and it is usually found down to 1 m depth or more (27). Therefore, in estuarine areas (see 1.2.4), methane formation if probably not very important during FeS_2 formation.

1.2.2 *The sulphate-reducing bacteria*

No chemical mechanism is known for the spontaneous reduction of sulphate by organic matter at normal temperature and pressure. The formation of hydrogen sulphide from sulphate in anaerobic sediments is brought about by the dissimilatory sulphate-reducing bacteria. Dissimilatory sulphate reduction is the process in which sulphate is used as the electron acceptor for the

oxidation of substrates from which energy is derived. Reviews on these bacteria were given by Postgate (50) and by Le Gall and Postgate (26). The sulphate-reducing bacteria may be classified according to the scheme presented in Table 1.

Table 1. The dissimilatory sulphate-reducing bacteria

Genus	Species	Spores	Habitat	References
Desulfotomaculum	*nigrificans*	+	soils, thermal springs	14
	orientis	+	tropical soil	14
	ruminis	+	rumen	14
	acetoxidans	+	muds	71
Desulfovibrio	*desulfuricans*	-	ubiquitous	14
	vulgaris	-	ubiquitous	14
	salexigens	-	sea water, marine and estuarine mud, pickling brines	14
	africanus	-	fresh and salt waters, Africa	14
	gigas	-	marine mucds	14
Desulfomonas	*pigra*	-	human faeces	47

Sulphate-reducing bacteria need a low redox potential (-200 mV) and are able to grow between pH 5.5 and 9.0. Ammonium salts and some amino acids are used as nitrogen source. Most of the sulphate-reducing bacteria are able to fix atmospheric nitrogen. The most commonly used energy and carbon sources are lactic and pyruvic acids, while some species utilize malic, fumaric and succinic acids. These compounds are oxidized according to the following equations (60).

Lactate $2(CH_3.CHOH.COOH) + SO_4^{2-} \rightarrow 2(CH_3.COOH) + 2CO_2 + S^{2-} + 2H_2O$ (Eq. 1)

Pyruvate $4(CH_3.CO.COOH) + SO_4^{2-} \rightarrow 4(CH_3.COOH) + 4CO_2 + S^{2-}$ (Eq. 2)

Malate $2(HOOC.CH_2.CHOH.COOH) + SO_4 \rightarrow 2(CH_3.COOH) + 4CO_2 + S^{2-} + 2H_2O$ (Eq. 3)

Fumarate $2(HOOC.CH.CH.COOH) + SO_4^{2-} \rightarrow 2(CH_3.COOH) + 4CO_2 + S^{2-}$ (Eq. 4)

Succinate $4(HOOC.CH_2.CH_2.COOH) + 3SO_4^{2-} \rightarrow 4(CH_3.COOH) + 8CO_2 + 3S^{2-} + 4H_2O$ (Eq. 5)

Some species are able to utilize choline and primary alcohols, including methanol, ethanol, propanol and butanol as energy and carbon source. Most *Desulfovibrio* spp. are also able to utilize molecular hydrogen as electron donor. Certain organic substrates *viz.* isobutanol formate and oxamate can be utilized as energy source but not as carbon source and, in fact, are organic substitutes for H_2 (50). Hydrogen-utilizing sulphate-reducing bacteria are unable to grow autotrophically (46); their metabolism represents one of the few examples of chemo-litho-heterotrophy.

The above-mentioned equations (1-5) show that the organic substrates are oxidized only to the acetate level. Consequently, the organic matter is only partially utilized by the sulphate-reducing microorganisms. Even if all of the organic material would be converted to lactate, the sulphate reducers could only oxidize it to about one third of completion (25). However, quantitative measurements indicated that up to 60% of the organic matter in marine sediments may be utilized by sulphate-reducing bacteria (35). These authors tried to explain the discrepancy by proposing a bacterial syntrophism in the sediment in which acetate is recycled into suitable substrates for the sulphate reducers. However, Widdel and Pfennig (71) isolated from mud *Desulfotomaculum acetoxidans*, an organism that has the capacity to oxidize acetate to carbon dioxide with the concomitant reduction of sulphate to hydrogen sulphide. This organism may account for the high amounts of organic material utilized by the sulphate-reducing bacteria in the marine sediment. Since virtually all of the strains described in the literature were isolated with lactate as the energy source, it is quite possible that the metabolic diversity of the sulphate reducers is greater than is presently thought (73).

1.2.3 *The formation of iron-sulphides*

The formation of iron sulphides has been extensively studied by van Breemen (10), Goldhaber and Kaplan (27) (and) Rickard (53). Ferrous ions are released from adsorbed coatings of colloidal ferric oxides, such as hematite (Fe_2O_3) and goethite (FeOOH), on detrital clays and silt grains which occur in large quantities in anaerobic sediments (6). They might also be released from the octahedral lattice of smectite clays and fine-grained ferric oxides (6; 20). Reduction of ferric to ferrous iron proceeds by hydrogen sulphide or by organic substances such as crysteine, phenols, polyphenols, gallic acid and tannic acid (61). As a consequence, relatively high steady-state concentrations of dissolved Fe^{2+} may occur in environments where the rate of oxidation of Fe^{2+} is slow compared with the rate of reduction of Fe^{3+}.

Ferrous ions reacts rapidly with sulphide to form either FeS (amorphous or mackinawite) or Fe_3S_4 (greigite) (53). Oxygen occasionally penetrates into the anaerobic sediment in some environments (see 1.2.4) and partially oxidizes sulphide to elemental sulphur (S^o), a process which can also be mediated by ferric iron as mentioned above. The elemental sulphur may further react with sulphide to form polysulphide, which is probably involved in the formation of

pyrite (53). Aqeous polysulphides may react with either FeS or Fe_3S_4 to form pyrite. The reaction mechanisms of these processes are not known in detail yet (53). The formation of pyrite schematically is presented in Fig. 3.

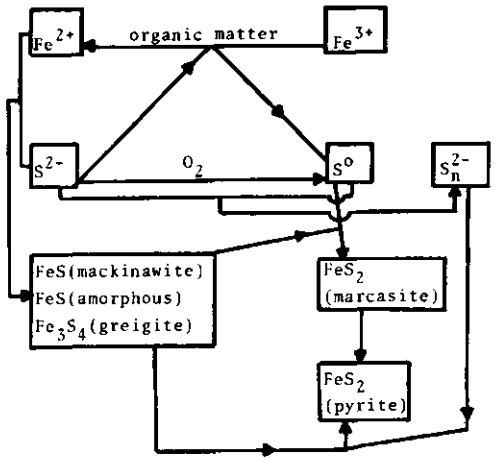

Fig. 3. Pathways in the formation of pyrite

1.2.4 *Accumulation of pytite in estuarine areas*

Pyrite may constitute up to 10% of the sediments in tidal muds above mean sea level (11), but its accumulation in both accretionary coasts and sea bottom sediments is much lower. This was reported by van Breemen (10) in a study on the formation of pyrite in tidal mangrove swamps in which he considered the influence of the presence of sulphate, organic matter and iron. He observed that despite the low redox potential at most 20% of the sulphate was removed in these swamps. The replacement of sulphate in the estuarine swamps was much faster than diffusion of SO_4^{2-} into the sea bottom, due to the tidal action in combination with the high permeability of the tidal muds. Large amounts of organic matter were supplied by the mangrove trees. Consequently, the limiting factor in the formation of pyrite was not the presence of either sulphate or organic matter but iron. This situation, however, was only met in estuarine areas that were intersected by numerous tidal channels. The accretionary coastal areas with relatively few of such creeks, anaerobic conditions prevailed and organic matter, iron and sulphate were abundant. However, the formation of pyrite was

slow, indicating that some other factors must be involved in the process. The striking differences between both types of coasts were attributed to the higher intensity of tidal flushing in the estuary. This resulted in (i) a partial oxidation of S^{2-} to S^{o} by O_2 during spring tides, whereas the accretionary coast always remained strictly anaerobic, so that S^{2-} could only be oxidized by ferric iron (ii) a better removal of dissolved alkalinity (HCO_3^-) which is formed during the sulphate reduction (see equation 1-5) (10). Partial oxidation of S^{2-} to S^{o} by an oxidant other than ferric iron is required for complete pyritization of a given amount of ferric oxide. Furthermore, a lower pH is kinetically more favourable for the formation of pyrite in the pH range 4-8 (27). In sea bottom sediments depletion of sulphate has never been observed. Here the supply of organic matter usually is the limiting factor in the sulphate reduction and consequently (in) the formation of pyrite (6; 53). The diffusion of sulphate into the sediment may only be limiting when excessive amounts of organic matter enter the system, e.g. due to pollution (53).

1.3 THE OXIDATION OF PYRITE

Pyritic sediments that become aerated by natural or artificial drainage will be oxidized either non-biologically or by microbial activity. As pyrite contains a sulphur and an iron moiety which both can be oxidized, sulphur-oxidizing bacteria as well as iron-oxidizing organisms might be involved in its oxidation. Both groups of bacteria will shortly be reviewed whereafter the microbial oxidation of pyrite and the formation of acid sulphate soils will be discussed.

1.3.1 *Sulphur-oxidizing microorganisms*

Sulphur-oxidizing bacteria can be divided into the phototrophic sulphur bacteria and the colourless sulphur bacteria. Organisms of the first group will not be important in the oxidation of pyrite because light cannot penetrate deep enough into the soil and in the absence of light, these bacteria cannot oxidize reduced sulphur compounds (30). Colourless sulphur bacteria might be involved in the oxidation of elemental sulphur which is presumably formed in the soil from pyrite according to the equation:

$FeS_2 + \frac{1}{2}O_2 + 2H^+ \rightarrow Fe^{2+} + 2S^o + H_2O$ (Equation 6) (10).

The group of the colourless sulphur-bacteria includes many genera of sulphur-oxidizing bacteria of which the representatives of the genus *Thiobacillus* have been studied in detail (cf the review of Kuenen, 39). Table 2 gives a

survey of the bacteria belonging to this genus.

The thiobacilli are able to utilize several reduced sulphur compounds, including S^0, as energy source. *Thiobacillus thioparus* and several other thiobacilli are able to grow at neutral pH, but *Thiobacillus thiooxidans*, *Thiobacillus ferrooxidans* and the recently isolated *Thiobacillus acidophilus* require a low pH for growth. The properties of the last two organisms will be discussed in a separate paragraph (see 1.3.2).

Table 2. The genus *Thiobacillus*

	Species	Habitat	References
Thiobacillus	*perometabolis*	soil	43
	novellus	soil	33
	denitrificans	sea water, peat, mud, compost	33
	thioparus	water, mud, soil	33
	neapolitanus	soil, (sea)water	33
	thiooxidans	soil	33
	ferrooxidans	acid soils, acid waters	33
	acidophilus	acid soils, acid waters	28

Hardly anything is known about the extent to which the colourless bacteria contribute to the oxidation of sulphur compounds in soils. Only under extreme conditions, like low pH it has become more clear which bacteria were responsible for the oxidation of the reduced sulphur compounds e.g. oxidation of pyrite by *Thiobacillus ferrooxidans* in acid sulphate soils or acid mine-water.

1.3.2 *Iron-oxidizing microorganisms*

Bacteria involved in the accumulations of large amounts of ferric iron in nature include: (a) sheath-forming bacteria of the *Sphaerotilus-Leptothrix* group, (b) bacteria of the genus *Gallionella*, (c) *Metallogenium* spp., (d) a *Sulfobolus*-type bacterium, (e) *Leptospirillium ferrooxidans* and (f) bacteria belonging to the genus *Thiobacillus*.

The organisms of the first group grow optimally at neutral pH at which ferrous iron is readily oxidized non-biologically. Therefore the contribution of these bacteria to the accumulation of ferric hydroxide may be confined to their capacity to bind large amounts of ferric iron (69).

Gallionella spp. are micro-aerophilic bacteria which oxidize ferrous ions at low pO_2 values. Their chemoautotrophic growth is highly probable but is not proved (72).

Bacteria of the *Sphaerotilus-Leptothrix* group as well as *Gallionella* spp. occur in aquatic habitats and consequently, have not to be considered in the oxidation of pyrite in the soil.

A member of the genus *Metallogenium* were isolated from mine water sediment (70). The organism, an acid-tolerant filamentous bacterium, was proposed to catalyse the oxidation of iron in the pH range of 3.5-4.5. Whether or not *Metallogenium* occurs in acid sulphate soils is not known.

A microorganism capable of oxidizing iron and sulphur and of growing at high temperatures (45-70^{o}C) and low pH was isolated from a thermal region in Yellowstone Park (13). This organism resembles *Sulfolobus* very much; in view of its obligately high growth temperature it is probably not involved in the formation of acid sulphate soils.

Another iron-oxidizing microorganism recently isolated is *Leptospirillum ferrooxidans*. (Markosyan. ref. 1). This organism grows below pH 2.0 with ferrous iron as the energy source. It was reported not to oxidize reduced sulphur compounds.

Iron-oxidizing bacteria belonging to the genus *Thiobacillus* were first isolated from acid mine effluent (17). These bacteria were able to use ferrous iron as energy source under acid conditions where non-biological oxidation did not occur. As it was subsequently shown that the organisms were also able to grow on reduced sulphur compounds, the name *Thiobacillus ferrooxidans* was proposed for these iron bacteria (63). After the first description of *Thiobacillus ferrooxidans*, many other reports have appeared. The organism is a Gram-negative, non-sporulating, motile rod. It is an obligately autothroph deriving its energy from the oxidation of ferrous iron and several reduced sulphur compounds. The optimum temperature for growth is between 25 and 30^{o}C. Growth can be observed between pH 1.5 and 5.0. The physiology, particularly the iron and sulphur metabolism, of this organism has been reviewd by Tuovinen and Kelly (66) and more recently by Silver (56).

In 1967 it was suggested that the organism currently known as *Thiobacillus ferrooxidans* probably is a heterogeneous culture consisting of different metabolically similar, bacteria (45). This hypothesis was supported by the fact that bacteria grown on different substrates were found to contain different DNA-base compositions (56). Furhtermore that a different organism *viz.* *Thiobacillus acidophilus* was isolated from a presumably pure iron-grown culture of *Thiobacillus ferrooxidans* (28). Adaptation of *Thiobacillus ferrooxidans* to growth on organic substrates had been reported earlier (54; 62). After a few

transfers onto glucose medium the organism had lost its ability to grow on ferrous iron. *Thiobacillus acidophilus* probably is similar to both the glucose-grown organism of Shafia and Wilkinson (54) and the glucose-adapted strain of Tabita and Lundgren (62) with regard to metabolic properties and enzyme content (28). Emphasis in studying *Thiobacillus acidophilus* was layed on the taxonomic and metabolic properties of the organism (28). The organism is a Gram-negative, non-sporulating motile rod. Except from elemental sulphur, it obtains energy from various organic compounds. According to Guay and Silver (28) ferrous iron, thiosulphate, sulphite and metalsulphides did not support growth of this organism. Therefore it is most intriguing what could explain the presence of the bacterium in iron grown cultures of *Thiobacillus ferrooxidans*.

1.3.3 *Microbial oxidation of pyrite*

Early studies on the microbial oxidation of pyrite were aimed at the elucidation of the formation of sulphuric acid from the mineral in coal-mine waste (2; 4; 9; 16; 18; 21; 44; 58; 67; 68). Later on attention was directed towards bacterial oxidation of other sulphide minerals and low grade ores followed by leaching of their sulphates. These processes have recently been reviewed by Brierly (12), Kelly (37) and Kelly et al. (38). At present microbial desulphurization of coal is of interest in respect of the increasing need of coal in the future (22).

In 1953 it was demonstrated that *Thiobacillus ferrooxidans* causes sulphate formation from pyrite concretions obtained from coal and from pure pyrite (64). Till then it was generally believed that *Thiobacillus thiooxidans* was involved in the oxidation of pyrite but it was shown that this organism was without any effect on pyrite (64).

Different opinions exist about the mechanism of FeS_2 oxidation by *Thiobacillus ferrooxidans* (57). Temple and Delchamps (64) postulated an overall sequence of acid-forming reactions in coal-mine effluent which was adopted later by many other researchers. The first step is the non-biological oxidation of fine-grained pyrite to ferrous sulphate.

$$FeS_2 + H_2O + 3\tfrac{1}{2}O_2 \rightarrow FeSO_4 + H_2SO_4 \quad \text{(Equation 7)}$$

The ferrous sulphate is oxidized by *Thiobacillus ferrooxidans* according to the equation:

$$2FeSO_4 + \tfrac{1}{4}O_2 + H_2SO_4 \rightarrow Fe_2(SO_4)_3 + H_2O \quad \text{(Equation 8)}$$

The ferric iron formed reacts with pyrite yielding ferrous sulphate and elemental sulphur which is further oxidized to H_2SO_4.

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3FeSO_4 + 2S^o \quad \text{(Equation 9)}$$

$$2S^o + 6Fe_2(SO_4)_3 + 8H_2O \rightarrow 12FeSO_4 + 8H_2SO_4 \quad \text{(Equation 10)}$$

Equations 9 and 10 results in the overall reaction:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-} \quad \text{(Equation 11)}$$

The validity of the overall equation (Eq. 11) has been demonstrated even under anaerobic conditions whereas accumulation of intermediate elemental sulphur has not been shown hitherto. The role of *Thiobacillus ferrooxidans* is to provide a continuous supply of ferric ions which in turn oxidize the pyrite yielding ferrous sulphate to complete a cyclic process. The influence of the organism in promoting the oxidation of pyrite is indirect according to the above-described mechanism which is therefore called the indirect contact mechanism (57). Some scientists contended that direct microbial oxidation of pyrite, the direct contact mechanism, must be discounted because the rate-determining step in the oxidation of pyrite is the oxidation of ferrous to ferric iron at low pH values (61).However, observations that support direct oxidation of FeS_2 by *Thiobacillus ferrooxidans* have been made as well. Inhibitors of Fe^{2+} and S^o oxidation, NaN_3 and N-ethylmaleimide (NEM), respectively, partially abolished FeS_2 oxidation (23). From the ratio oxygen consumption/CO_2 fixation Beck and Brown (3) concluded that in addition to iron the sulphur moiety of pyrite was most likely utilized as energy source. *Thiobacillus ferrooxidans* is able to utilize the energy released during the oxidation of metal sulphides that are free of iron, indicating that it grows on the sulphur moiety of the minerals (65).

The only other known microorganisms associated with the oxidation of pyrite are *Metallogenium* and *Leptospirillum ferrooxidans*. The latter organism catalyses the oxidation of pyrite when grown in mixed cultures with sulphur-oxidizing thiobacilli, indicating that microbial oxidation of the sulphur as well as the iron moiety must be involved (1; 48). The oxidation of pyrite by a mixed culture of *Leptospirillum* and members of the genus *Thiobacillus* was more extensive than that brought about by a pure culture of *Thiobacillus ferrooxidans* (48). The significance of *Leptospirillum ferrooxidans* in the oxidation of pyrite needs further research.

1.3.4 *The oxidation of pyrite in acid sulphate soils*

When pyritic sediments are exposed to air due to drainage, a complex sequence of reactions is started. Initially, when the pH of the soil is near neutrality, *Thiobacillus ferrooxidans* is not involved in the oxidation of pyrite. As mentioned earlier, (see 1.2.4), during pyrite formation in estuarine areas most of the alkalinity is removed by tidal action. A low buffering capacity of the soil has therefore resulted and a rapid decrease of pH takes place when some pyrite is oxidized. Virtually nothing is known at present about the processes in these initial stages. The thiobacilli that can grow at neutral pH (see 1.3.1) might be involved in oxidizing the elemental sulphur which is formed according to equation 6. However, once the pH has dropped below a value of about 4.0 *Thiobacillus ferrooxidans* starts its contribution to the further oxidation of FeS_2 according to one or both of the mechanisms outlined in section 1.3.3. In slowly acidifying acid sulphate soils, there exists a distinct separation between pyrite within the soil and the ferric precipitates near channels and cracks, through which oxygen penetrates into the soil. Oxygen is probably inactivated by reaction with dissolved ferrous iron before it can reach the pyrite. The resulting ferric ions diffuse towards pyrite especially at pH below 4.0. This implies that ferric iron must be the immediate oxidant of pyrite in these soils (10) and that the direct contact mechanism probably is not very important.

The application of bactericides might be a successful means in lowering the rate of oxidation of pyrite in the soil by *Thiobacillus ferrooxidans* (59). Treatment of acid soils with the detergent dodecyl benzene sulfonate (DBS) was effective in lowering the numbers of iron-oxidizing bacteria. This decrease in numbers lasted only from two to six weeks and treatment of the soil in the field with DBS which is a strong inhibitor of iron oxidation in the laboratory had no significant effect on either pH or total pyrite oxidation (40).

One of the most characteristic products of pyrite oxidation in the soil is jarosite ($KFe_3(SO_4)_2(OH)_6$). The mechanism according to which this mineral is formed is not well known. Jarosite can be synthesized by aeration of a solution of ferrous sulphate and potassium sulphate at pH 0.8-1.7 (Brown ref. 10). Jarosite can also be formed in cultures of *Thiobacillus ferrooxidans* growing on ferrous sulphate (34). The organism may be involved in the formation of jarosite in the acid sulphate soils (10).

The reactions presumably involved in the oxidation of pyrite are depicted in Fig. 4.

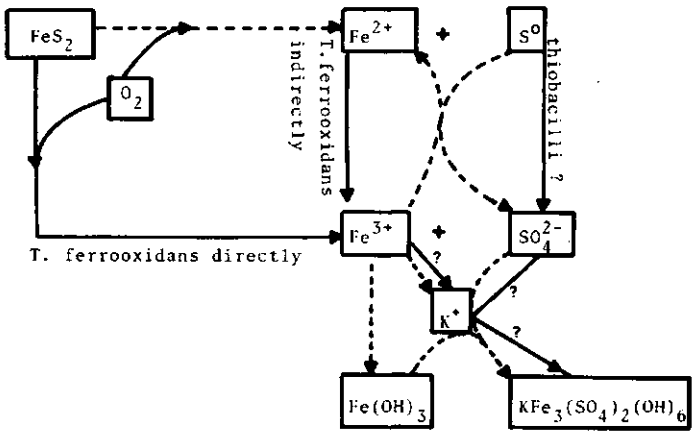

Fig. 4. The pathways of the oxidation of pyrite. ----- non-biological processes ——— microbial processes

The rate-limiting step in the oxidation of pyrite in the soil is the supply of oxygen (10). In young acid sulphate soils the oxidation of the mineral is more rapid because oxygen has to diffuse less deep into the soil than in old acid sulphate soils. Typical profile morphologies of young and old acid sulphate soils are given in Fig. 5. When pyrite is oxidized by ferric ions, ferrous sulphate is formed according to equation 11. Part of the ferrous sulphate diffuses upwards where it is oxidized and jarosite can be formed. This explains why jarosite is found at shallower depths than pyrite. In the upper part of the soil jarosite is not stable and it is slowly hydrolysed to ferric ions which partly precipitate as ferric oxide. Part of the ferric ions migrate downwards upon flooding. The different horizons, ferric oxide, jarosite and pyrite, are found at progressively greater depths when the soil becomes older (Fig. 5B) (11). The less acid surface layer in older acid sulphate soils may be due to (i) the weathering of clay and other silicates in which process cations are released from the mineral lattice, (ii) neutralization by alkalinity in flood

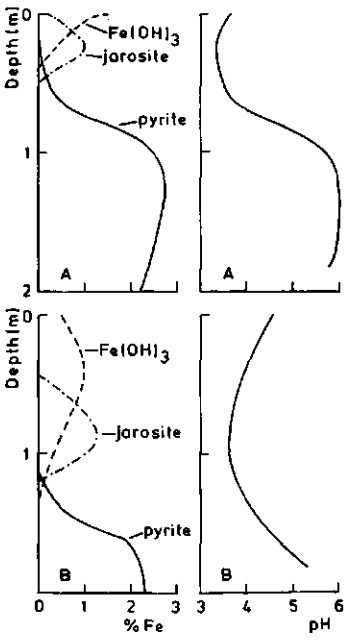

Fig. 5. Prophile morphology of (A) a young acid sulphate soils and (B) an old acid sulphate soil.(11).

water, (iii) deacidification during soil reductions. In the latter process ferric iron is reduced to ferrous ions which is attended with description of SO_4^{2-} and hydrolysis of basic sulphate. The formed ferrous sulphate is partly oxidized at the soil water interface to ferric hydroxide and sulphuric acid. The acid is removed laterally with the flood water (10).

It is surprising that only few reports deal with the microbial processes in acid soils. The oxidation of pyrite in the IJsselmeelpolders was studied by Quispel et al (51). They concluded that oxidation of the mineral is not strictly dependent on the activity of bacteria but that microorganisms have a stimulating effect on the process. *Thiobacillus thiooxidans* was isolated from these soils and it was concluded that the organism accelerates the oxidation of pyrite. Hart (31) showed that the oxidation of ferrous to ferric iron in pyritic soils from Sierra Leone was azide-sensitive and that iron-oxidizing bacteria could be cultured from the soil (31). He concluded that bacterial iron oxidation might be important in intensifying the acid conditions but that

the bacterial sulphur oxidation by *Thiobacillus thiooxidans* was the main process in the oxidation of pyrite. In 1972 it was shown that *Thiobacillus ferrooxidans* catalyses the oxidation of pyrite in the soil at a pH below 4.0 (7).

1.4 OUTLINE OF THE PRESENT INVESTIGATION

Although a great deal is known about the formation of acid sulphate soils, relatively little attention has been paid so far to the role of microorganisms in this process. The microbial activity has been treated as a black box in most reports. In particular, hardly anything is known about the initial pH drop from near neutral to pH 4.0 when pyritic soils become exposed to oxygen. Therefore a study was carried out to elucidate microbial processes which occur in the initial stages of acidification of potentially acid sulphate soils. The results of this part of the research are presented in Chapter 2.

Below pH 4.0, mainly *Thiobacillus ferrooxidans* is reponsible for the oxidation of pyrite. Up till now there is no consensus whether the organism oxidizes the mineral according to the direct or indirect contact mechanism; therefore, it was decided to investigate this matter in detail. Chapter 3 was devoted to this subject.

Thiobacillus acidophilus was isolated from each of the three cultures of *Thiobacillus ferrooxidans* employed during these investigations. The former organism has often been met within cultures of iron-oxidizing autotrophs by other investigators (see 1.3.2) but nothing is known what could explain its presence in *Thiobacillus ferrooxidans* cultures. Therefore, Chapter 4 of this thesis deals with the question why *Thiobacillus acidophilus* so often accompanies *Thiobacillus ferrooxidans*.

REFERENCES

1. Balashova, V.V., Vedenina, I.Ya., Markosyan, G.E. and Zavarzin, G.A. 1974 The auxotrophic growth of *Leptospirillum ferrooxidans*. Mikrobiologiya *43*, 581-588 (Eng. trans. pp. 491-494).
2. Beck, J.V. 1960 A ferrous-ion-oxidizing bacterium; I. Isolation and some general physiological characteristics. J. Bacteriol. *79*, 502-509.
3. Beck, J.V. and Brown, D.G. 1968 Direct sulfide oxidation in the solubilization solubilization of sulfide ores by *Thiobacillus ferrooxidans*. J. Bacteriol. *96*, 1433-1434.
4. Belly, R.T. and Brock, T.D. 1974 Ecology of iron-oxidizing bacteria in pyritic materials associated with coal. J. Bacteriol. *117*, 726-732.

5. Bemmelen, J.M. van, 1886 Bijdragen tot de kennis van den alluvialen bodem in Nederland. Derde Verhandeling: De samenstelling en de vorming van de zure gronden in het Nederlandse alluvium. Verh. Acad. Wet. Amsterdam XXV B: 33-105.
6. Berner, R.A. 1972 Sulfate reduction, pyrite formation, and the oceanic sulfur budget. *In* The changing of the oceans. Eds. D. Dyrsen and D. Jagner. John Wiley. New York.
7. Bloomfield, C. 1972 The oxidation of iron sulphides in soils in relation to the formation of acid sulphate soils and of ochre deposits in field draines. J. Soil Science *23*, 1-16.
8. Bloomfield, C. and Coulter, J.K. 1973 Genesis and management of acid sulfate soils. Adv. Agron. *25*, 265-326.
9. Braley, S.A. 1960 The oxidation of pyritic conglomerates. Industrial Wastes *5*, 89-91.
10. Breemen, N. van 1976 Genesis and solution chemistry of acid sulphate soils in Thailand. Thesis. Agricultural University. Wageningen.
11. Breemen, N. van, and Pons, L.J. 1977 Acid sulphate soils and rice. Paper presented at the soils and rice symposium. International Rice Research Institute Los Banos. Philippines.
12. Brierley, C.L. 1978 Bacterial leaching. C.R.C. Critical reviews in microbiology *6* (3), 207-262.
13. Brierley, C.L. and Brierley, J.A. 1973 A chemoautotrophic and thermotrophic microorganism isolated from an acid hot spring. Can. J. Microbiol. *19*, 183-188.
14. Buchanan, R.E. and Gibbons, N.E. 1974 Bergey's manual of determinative bacteriology. 8th ed. The Williams and Wilkins Company. Baltimore.
15. Cappenberg, Th.E. 1975 Interrelations between sulphate-reducing and methane producing bacteria in bottom deposits of a fresh-water lake. Thesis. Free University. Amsterdam.
16. Clark, C.S. 1965 The oxidation of coal mine pyrite. Thesis. The Johns Hopkins University. Baltimore. Maryland.
17. Colmer, A.R. and Hinkle, M.E. 1947 The role of microorganisms in acid mine drainage; a preliminary report. Science *106*, 253-256.
18. Colmer, A.R., Temple, K.T. and Hinkle, M.E. 1950 An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. J. Bacteriol. *59*, 317-328.
19. Dost, H. (ed) 1973 Acid sulphate soils. Proceeding of an international symposium. Wageningen. I.L.R.I. publ. 18. 2 vols. (295 pp; 406 pp.).
20. Drever, J.I. 1971 Magnesium-iron replacement in clay minerals in anoxic marine sediments. Science *172*, 1334-1336.
21. Dugan, P.R. 1975 Bacterial ecology of strip mine areas and its relationship to the production of acidic mine drainage. Ohio J. Science *75*, 266-279.
22. Dugan, P.R. and Apel, W.A. 1978. Microbial desulfurization of coal. *In* Metallurgical applications of bacterial leaching and related microbial phenomena. Eds. L.E. Murr, A.E. Torma and J.A. Brierley: Academic Press N.Y. (In press).
23. Ducan, D.W., Landesman, J. and Walden, C.C. 1967 Role of *Thiobacillus ferrooxidans* in the oxidation of sulfide minerals. Can. J. Microbiol. *13*, 397-403.
24. Edelman, C.H. 1948 Katteklei. Boor en Spade *1*, 172-176.
25. Fenchel, T.M. and Jørgensen, B.B. 1977. Detritus food chains of aquatic ecosystems: The role of bacteria. *In* Advances in microbial Ecology Vol. 1. Ed. M. Alexander. Plenum Press, New York 1-58.

26. Gall, J. le and Postgate, J.R. 1973. The physiology of sulphate-reducing bacteria. Adv. Microb. Physiol. *10*, 81-133.
27. Goldhaber, M.B. and Kaplan, I.R. 1974. The sulfur cycle. *In* The sea, vol. 5. Marine Chemistry. Ed. E.D. Goldberg. John Wiley. New York p. 569-655.
28. Guay, R. and Silver, M. 1975 *Thiobacillus acidophilus* sp. nov.; isolation and some physiological characteristics. Can. J. Microbiol. *21*, 281-288.
29. Hackett, W.E., Connors, W.J., Kirk, T.K. and Zeikus, J.G. 1977 Microbial decomposition of synthetic ^{14}C-labeled lignins in nature: lignin biodegradation in a variety of natural materials. Appl. Environm. Microbiol. *33*, 43-51.
30. Hansen, T.A. 1974 Sulfide als electronen donor voor *Rhodospirillaceae*. Thesis. University Groningen.
31. Hart, M.G.R. 1959 Sulphur oxidation in tidal mangrove soils of Sierra Leone. Plant and Soil *11*, 215-236.
32. Hobson, P.N., Bousfield, S. and Summers, R. 1974. Anaerobic digestion of organic matter. C.R.C. Critical Reviews in Environmental Control 131-191.
33. Hutchinson, M., Johnstone, K.I. and White, D. 1969. Taxonomy of the genus *Thiobacillus*: the outcome of numerical taxonomy applied to the group as a whole. J. Gen. Microbiol. *57*, 397-410.
34. Ivarson, K.C. 1973 Microbiological formation of basic ferric sulfates. Can. J. Soil Sci. *53*, 315-323.
35. Jørgensen, B.B. and Fenchel, T. 1974 The sulfur cycle of a marine sediment model system. Mar. Biol. *24*, 189-201.
36. Kawalec, A. 1973. World distribution of acid sulfate soils, references and map. *In* Acid Sulphate soils proceedings of an International symposium Wageningen. Ed. H. Dost. I.L.R.I. publ. 18 vol. 1 275-295.
37. Kelly, D.P. 1977 Extraction of metal from ores by bacterial leaching: Present status and future prospects. *In* Microbial energy conversion. Eds. H.G. Schlegel and J. Barnea. Pergamom press Oxford 329-338.
38. Kelly, D.P., Norris, P.R. and Brierley, C.L. 1979 Microbiological methods for the extraction and recovery of metals. *In* S.G.M. symposium Book *29* Eds. A.T. Bull, D.C. Ellwood and C. Ratledge. Cambridge University Press 263-308.
39. Kuenen, J.G. 1975 Colourless sulfur bacteria and their role in the sulfur cycle. Plant and Soil *43*, 49-76.
40. Kugatow, M.A. 1977 Mediation of acid strip mine pollution by the attempted inhibition of the iron-oxidizing autotroph, *Thiobacillus ferrooxidans*. Thesis. The Pennsylvenia State University U.S.A.
41. Laanbroek, H.J. 1978 Ecology and physiology of L-aspartate- and L-glutamate fermenting bacteria. Thesis. University of Groningen.
42. Lichtfield, C.D. 1973 Interactions of amino acids and marine bacteria. *In* Estuarine Microbial Ecology. Eds. L.H. Stevenson and R.R. Colwell. University of South Caroline press. Colombia S.C. p. 145-164.
43. London, J. and Rittenberg, S.C. 1967 *Thiobacillus perometabolis* nov. sp., a non-autotrophic *Thiobacillus*. Arch. Mikrobiol. *59*, 218-225.
44. Lorenz, W.C. and Tarpley, E.C. 1963 Oxidation of coal mine pyrites. *In* Bureau of mines. Report of investigations 6247. U.S. Dept. of the interior Bureau of mines.
45. Margalith, P., Silver, M. and Lundgren, D.G. 1966 Sulfur oxidation by the iron bacterium *Ferrobacillus ferrooxidans*. J. Bacteriol. *92*, 1706-1709.
46. Mechalas, B.J. and Rittenberg, S.C. 1960 Energy coupling in *Desulfovibrio desulfuricans*. J. Bacteriol. *80*, 501-507.

47. Moore, W.E.C., Johnson, J.L. and Holdeman, L.V. 1976 Emendation of *Bacteroidaceae* and *Butyrivibrio* and descriptions of *Desulfomonas* gen. nov. and ten new species in the genera *Desulfomonas*, *Butyrivibrio*, *Eubacterium*, *Clostridium* and *Ruminicoccus*. Int. J. Syst. Bacteriol. *26*, 238-252.
48. Norris, P.R. and Kelly, D.P. 1978 Dissolution of pyrite (FeS_2) by pure and mixed cultures of some acidophilic bacteria. FEMS Microbiol. Lett. *4*, 143-146.
49. Poelman, J.N.B. 1972 Het klattekleiverschijnsel in oude natuurwetenschappelijke literatuur. Boor en Spade *18*, 41-49.
50. Postgate, J.R. 1965 Recent advances in the study of the sulphate-reducing bacteria. Bacteriol. Rev. *29*, 425-441.
51. Quispel, A., Harmsen, G.W. and Otzen, D. 1952 Contribution to the chemical and bacteriological oxidation of pyrite in soils. Plant and Soil *4*, 43-55.
52. Reichardt, W. and Simnon, M. 1972 Die Mettma ein Gebrigsbach als Brauerei vorflutter. Mikrobiologische Untersuchungen entlang eines Abwasser-substratgradienten. Arch. Hydrobiol. Suppl. *42*, 125-138.
53. Rickard, D.T. 1973 Sedimentary iron sulphide formation. *In* Acid sulphate soils. Proceedings of an International symposium Wageningen. Ed. H. Dost. I.L.R.I. publ. 18 vol. 1, 28-65.
54. Shafia, F. and Wilkinson, R.F. 1969 Growth of *Ferrobacillus ferrooxidans* on organic matter. J. Bacteriol. *97*, 256-260.
55. Siebert, M.L. and Toerien, D.F. 1969 The proteolytic bacteria present in the anaerobic digestion of raw seawage sludge. Wat. Res. *3*, 241-250.
56. Silver, M. 1978 Metabolic mechanism of iron oxidizing thiobacilli. *In* Metallurgical applications of bacterial leaching and related microbial phenomena. Eds. L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press New York 3-17.
57. Silverman, M.P. 1967 Mechanism of bacterial pyrite oxidation. J. Bacteriol. *94*, 1046-1051.
58. Silverman, M.P., Rogoff, M.H. and Winder, I. 1961 Bacterial oxidation of pyrite materials in coal. Appl. Microbiol. *9*, 491-496.
59. Singer, P.C. and Stumm, W. 1970 Acid mine drainage: The rate limiting step. Science *167*, 1121-1123.
60. Starkey, R.L. 1961 Sulfate-reducing bacteria, their production of sulphide and their economic importance. Tappi. *44*, 493-496.
61. Stumm, W. and Morgan, J.J. 1970 Aquatic chemistry. An introduction emphasizing chemical equilibria in natural waters. John Wiley Interscience, New York 583 pp.
62. Tabita, R. and Lundgren, D.G. 1971 Utilization of glucose and the effect of organic compounds on the chemolithotroph *Thiobacillus ferrooxidans*. J. Bacteriol. *108*, 328-333.
63. Temple, K.L. and Colmer, A.R. 1951 The autotrophic oxidation of iron by a new bacterium: *Thiobacillus ferrooxidans*. J. Bacteriol. *62*, 605-611.
64. Temple, K.L. and Delchamps, E.W. 1953 Autotrophic bacteria and the formation of acid in bituminous coal mines. Appl. Microbiolcgy *1*, 255-258.
65. Torma, A.E. and Legault, G. 1973 Role de la surface des minerais sulfurés lors de leur biodegradation par *Thiobacillus ferrooxidans*. Ann. Microbiol. Inst. Pasteur *124A*, 111-121.
66. Tuovinen, O.H. and Kelly, D.P. 1972 Biology of *Thiobacillus ferrooxidans* in relation to the microbiological leaching of sulphide ores. Z. f. Allgem. Mikrobiologie *12*, 311-346.

67. Tuttle, J.H., Dugan, P.R., Macmillan, C.B. and Randles, C.I. 1969 Microbial dissimilatory sulfur cycle in acid mine water. J. Bacteriol. *97*, 594-602.
68. Tuttle, J.H., Randles, C.I. and Dugan, P.R. 1968 Activity of microorganisms in acid mine water. 1. Influence of acid water on aerobic heterotroph of a normal stream. J. Bacteriol. *95*, 1495-1503.
69. Veen, W.L. van, Mulder, E.G. and Deinema, M.H. 1978 The *Sphaerotilus-Leptothrix* group of bacteria. Microbiol. Rev. *42*, 329-356.
70. Walsh, F. and Mitchell, R. 1972 A pH-dependent succession of iron bacteria. Environm. Sci. Technol. *6*, 809-812.
71. Widdel, F. and Pfennig, N. 1977 A new anaerobic, sporing, acetate-oxidizing, sulphate-reducing bacterium, *Desulfotomaculum* (emend.) *acetoxidans*. Arch. Microbiol. *112*, 119-122.
72. Wolfe, R.S. 1958 Cultivation morphology, and classification of the iron bacteria. J. Am. Wat. Wks. Ass. *50*, 1241-1249.
73. Zinder, S.M. and Brock, T.D. 1978 Microbial transformations of sulfur in the environment. *In* Sulfur in the environment part II Ecological impacts. Ed. J.O. Nriagu. John Wiley Sons inc. New York, 445-466.

2. PYRITE OXIDATION IN ACID SULPHATE SOILS: THE ROLE OF MICROORGANISMS

ABSTRACT

A study has been made of microbial processes in the oxidation of pyrite in acid sulphate soil material. Such soils are formed during aeration of marine muds rich in pyrite (FeS_2). Bacteria of the type of *Thiobacillus ferrooxidans* are mainly responsible for the oxidation of pyrite, causing a pronounced acidification of the soil. However, because *Thiobacillus ferrooxidans* functions optimally at pH values below 4.0, its activity cannot explain the initial pH drop from approximately neutral to about 4. This was shown to be a non-biological process, in which bacteria play an insignificant part. Although *Thiobacillus thioparus* and *Thiobacillus thiooxidans* were isolated from the acidifying soil, they did not stimulate oxidation of FeS_2, but utilized reduced sulphur compounds, which are formed during the non-biological oxidation of FeS_2.

Ethylene-oxide-sterilized and dry-sterilized soil inoculated with pure cultures or mixtures of various thiobacilli or with freshly sampled acid sulphate soil did not acidify faster than sterile blanks. *Thiobacillus thiooxidans*, *Thiobacillus thioparus*, *Thiobacillus intermedius* and *Thiobacillus perometabolis* increased from about 10^4 to 10^5 cells/ml in media with FeS_2 as energy source. However, FeS_2 oxidation in the inoculated media was not faster than in sterile blanks.

Attempts to isolate microorganisms other than *Thiobacillus ferrooxidans*, like *Metallogenium* or *Leptospirillum ferrooxidans*, which might also be involved in the oxidation of FeS_2 were not successful.

Addition of $CaCO_3$ to the soil prevented acidification but did not stop non-biological oxidation of FeS_2.

2.1 INTRODUCTION

Acid sulphate soils are formed during aeration of marine muds rich in pyrite (FeS_2), when insufficient amounts of neutralizing components are present. The acidification is caused by the oxidation of pyrite, a process that has been studied extensively in connection with the formation of acidic mine effluents. Our present knowledge of the microbial oxidation of FeS_2 under natural conditions has largely been derived from the efforts of workers on acid mine drainage (5, 9, 10, 11). Their consensus is that *Thiobacillus ferrooxidans*, an autotrophic microorganism that derives energy from the oxidation of Fe^{2+} or reduced sulphur compounds, is mainly responsible for the oxidation of FeS_2 at a pH below 4.0.

Relatively few publications deal with microbial FeS_2 oxidation in acid sulphate soils. Some researchers concluded that the oxidation of FeS_2 in soil was catalysed by *Thiobacillus thiooxidans* which would oxidize S^o (elemental sulphur) as an intermediate in the non-biological oxidation of FeS_2 (12, 19). In a later study iron-oxidizing microorganisms were found in soils from mangrove swamps in Sierra Leone but their significance in pyrite oxidation was underestimated because it was concluded that *Thiobacillus thiooxidans* was mainly responsible for the oxidation of pyrite (13). In later reports it was shown that *Thiobacillus ferrooxidans* catalyses the oxidation of FeS_2 at pH below 4.0 (6).

Little is known about the initial pH drop from near neutral to pH 4.0. It was postulated that the oxidation of FeS was necessary as a first step to make the soil acid enough for FeS_2 oxidation (14). S^o also might be important in this respect (19). However, the amounts of FeS and S^o normally present in potentially acid sulphate soils are far too small to explain the drop in pH (6). The presence of a certain fraction very fine grained, probably poorly cristallized pyrite that would be relatively susceptible to non-biological or microbial oxidation might be important (8). It is unknown whether bacteria are responsible for the initial pH drop. The aim of this study was to elucidate microbial processes, which occur in the acidification of potentially acid sulphate soils. Special attention was given to the initial pH drop, from near neutral to pH 4.0 and the role of thiobacilli and possibly other microorganisms.

2.2 MATERIALS AND METHODS

2.2.1 *Cultures and media*

Cultures of the following bacteria were used in this study: *Thiobacillus ferrooxidans*, strain ATCC 13598; *Thiobacillus thiooxidans*, strain DSM 504; *Thiobacillus thioparus*, strain DSM 505; *Thiobacillus intermedius*, strain ATCC 15466 and *Thiobacillus perometabolis*, strain ATCC 27793.

Medium 9K was used for most probable number (MPN) determinations of iron-oxidizing bacteria and for growing *Thiobacillus ferrooxidans* (21). The medium for the MPN determinations of acidophilic sulphur-oxidizing autotrophs was based on medium S5 (15) with elemental sulphur as energy source and a pH of 4.0. *Thiobacillus thiooxidans* was maintained in this medium. In the medium

for the MPN determinations of thiosulphate oxidizing autotrophs the S^0 was replaced by $Na_2S_2O_3.5H_2O$ and the pH was 7.0. *Thiobacillus thioparus* was maintained and grown in this medium. *Thiobacillus intermedius* and *Thiobacillus perometabolis* were maintained and grown on media described by London (16, 17). The medium for the plate counts of heterotrophic bacteria was the same as used for *Thiobacillus thioparus*, but supplemented with 0.01% yeast extract and 0.1% glucose and with an initial pH of 7.0. Colonies of the final dilutions were tested for thiosulphate oxidation by transfer to 10 ml liquid media. After one-week incubation at 30°C the pH was measured, tetrathionate was assayed qualitatively according to the procedure of Koh (ref. 20, p. 73) and thiosulphate was determined iodometrically.

2.2.2 *Soil samples*

Oxidation of FeS_2 was studied in two samples from the substrata of acid sulphate soils in the Haarlemmermeerpolder. One sample contained $CaCO_3$, the other no carbonate. Immediately after sampling, the soil was divided into 6 portions of 130 grams, which were placed into 500-ml jars. The initial water content was about 200% (mass fraction to ovendried soil). After evaporation of most of the water until field capacity the samples were moistened regularly with small amounts of distilled water. Periodically, one sample of each series was made up with distilled water to the original water content, and thoroughly mixed whereafter cell counts of different microorganisms were made. The pH was measured after three days whereupon the soil was freeze-dried and stored at -20°C under an argon atmosphere. After treatment of the last jar the soil was assayed for total Fe II, total Fe III, water-soluble sulphate, HCl-soluble sulphate (jarosite), FeS_2, S^0 and total S according to the procedure of Begheyn et al. (4).

2.2.3 *Sterilization of the soil*

Non-calcareous samples from the substrata of acid sulphate soils in the Haarlemmermeerpolder were freeze-dried. The dried soil was transferred to glass-tubes (2 g/tube) and sterilized under an argon atmosphere at 160°C for two hours or under an ethylene-oxide atmosphere at room temperature for 24 hours. The sterile soil was inoculated with different thiobacilli and the moisture content adjusted to 50% with sterile distilled water. Periodically the contents of a tube were analysed for pH and 6 N HCl-extractable Fe as a measure of the amount of pyrite oxidized.

2.2.4 *Enrichment cultures in a chemostat*

Two 2-l fermentors were used (Eschweiler, Kiel, West Germany) for the enrichment of FeS_2-oxidizing bacteria. The working volume of the fermentor was 1.0 l and the dilution rate was 0.005 h^{-1}. One litre of the medium contained: $(NH_4)_2SO_4$, 0.2 g; KH_2PO_4, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; KCl, 0.2 g; yeast extract, 0.05 g; FeS_2 (particles below 42 µm), 5 g and 1 ml of a trace element solution consisting of (per litre): $ZnSO_4$, 0.3 g; $MnSO_4.4H_2O$, 2.2 g; $CuSO_4.5H_2O$, 0.3 g; $CoCl_2.6H_2O$, 0.2 g; H_3BO_3, 2.0 g and $Na_2MoO_4.2H_2O$, 0.75 g. The medium without pyrite was sterilized by autoclaving. FeS_2 was sterilized separately at 160°C under an argon atmosphere for 2 h and added to the basal medium. The fermentors were aerated with a gas mixture consisting of 1% O_2, 2% CO_2 and 97% N_2. The gas flow was 20 ml.min^{-1}. The pH was fixed at different values by a pH meter with a titration unit.

2.2.5 *Pyrite*

Pyrite was obtained from Dr. Krantz, Mineraliën, Versteinerungen, und Geogeräte, D53, Bonn, Germany. Its composition and treatment before use were described earlier (1) (see also section 3.2.7).

2.3 RESULTS

2.3.1 *FeS_2 oxidation in aerated soil samples*

Samples from the substrata of an acid sulphate soil from the Haarlemmermeer-polder were exposed to air as described in section 2.2.2. Periodically the contents of one jar of each series were screened for different thiobacilli whilst iron and sulphur balances were made. Relevant analytical data of the soil at the beginning of the experiment are given in Table 1. Before bacterial counts were performed the media used for the MPN-determinations of the thiobacilli were tested for selectivity. *Thiobacillus ferrooxidans* grew only in the 9K medium, *Thiobacillus thiooxidans* only in the medium used for acidophilic sulphur oxidizers and *Thiobacillus thioparus* only in the medium for autotrophic thiosulphate oxidizers.

Fig. 1 shows that pyrite oxidation in the calcareous and non-calcareous soils proceeded in a different way. After an initial lag phase during which the moisture content of the soil dropped to about 50%, the pyrite content of both

Table 1. Analytical data of soil samples from the substrata of acid sulphate soils. Percentages refer to ovendried soil

pH	FeS_2 (%S)	Water soluble SO_4^{2-} (%S)	Total S%	$CaCO_3$	Particles < 2 µ	FeO	Fe_2O_3	organic matter
6.7	1.11	0.15	1.28	5.1	57.1	0.77	3.14	3.2
6.3	0.96	0.15	1.22	0.0	60.3	0.70	3.37	3.8

samples decreased. In the calcareous soil this drop was slight but non-calcareous soil lost more than half of its original pyrite content during the 140-day incubation period.

The results of the cell counts are shown in Fig. 2. Heterotrophic bacteria as well as *Thiobacillus thioparus* and *Thiobacillus thiooxidans* increased significantly in numbers in both the calcareous and non-calcareous soils during the first 40 days of incubation. About 5% of the heterotrophic bacteria

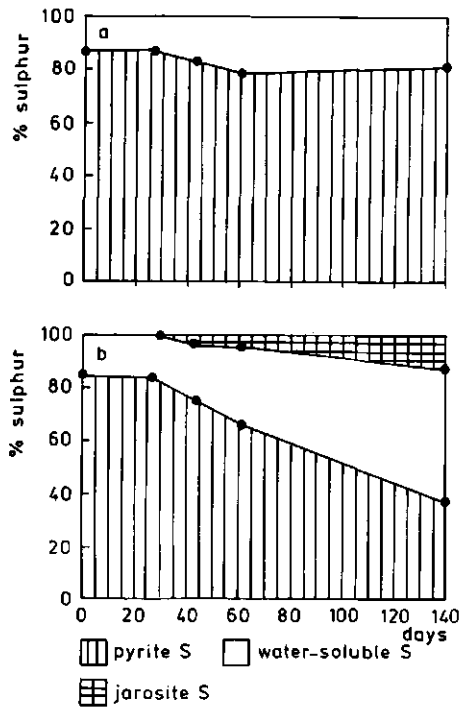

Fig. 1. Changes of different sulphur fractions in: a) calcareous and b) non-calcareous soil; percentages refer to total sulphur determined.

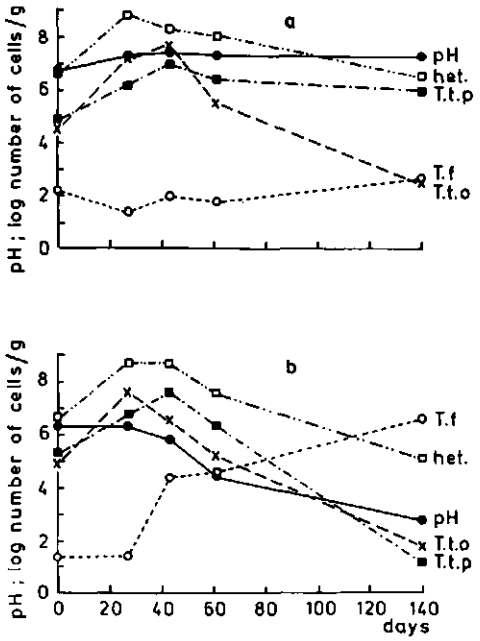

Fig. 2. Numbers/g of wet soil of different sulphur- and iron-oxidizing autotrophic and heterotrophic bacteria and pH at different time intervals in a) calcareous and b) non-calcareous pyritic soils.
T.f. = *Thiobacillus ferrooxidans*, T.t.o = *Thiobacillus thiooxidans*, T.t.p = *Thiobacillus thioparus* and het. = heterotrophic microorganisms.

was able to oxidize thiosulphate to tetrathionate and to decrease the pH of their medium. *Thiobacillus ferrooxidans* increased in numbers only when the pH had decreased; it was the predominant microorganisms in the non-calcareous soil after 140 days but did not develop in the calcareous soil. The presence and growth of *Thiobacillus thioparus* and *Thiobacillus thiooxidans* in the first 40 days of incubation is puzzling. Their presence may have been due to a small amount of a sulphur compound with an oxidation state intermediate between sulphide and sulphate such as elemental sulphur, sulphite, thiosulphate or tetrathionate. The sum of the analysed sulphur fractions, *viz*: FeS_2, elemental sulphur (S^o) water-soluble SO_4^{-2} and HCl-soluble SO_4^{2-} (jarosite) was almost equal to total sulphur determined separately (Fig. 1). The only detectable reduced sulphur compound was FeS_2, S^o was never detected. From the sulphur balance it was concluded that other reduced compounds never exceeded 200 ppm S relative to oven-dried soil. It is unlikely that such small amounts can explain the growth of *Thiobacillus thioparus* and *Thiobacillus thiooxidans*.

Therefore an alternative explanation was investigated *viz.* that the organisms benefitted from reduced S-compounds released during the non-biological oxdiation of FeS_2 by dissolved oxygen.

2.3.2 *Growth of thiobacilli in liquid media with FeS_2 as energy source*

FeS_2 can be oxidized by dissolved oxygen according to the Equation:

$$FeS_2 + \tfrac{1}{2}O_2 + 2H^+ \rightarrow Fe^{2+} + 2S^o + H_2O \quad \text{(equation 1) (8)}$$

At pH values above 4.0, Fe^{2+} is instantaneously oxidized non-biologically to Fe^{3+}. The S^o formed is further oxidized by Fe^{3+} ions according to the Equation:

$$S^o + 6Fe^{3+} + 4H_2O \rightarrow 6Fe^{2+} + SO_4^{2-} + 8H^+ \quad \text{(equation 2)}$$

or when starting from FeS_2:

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

However, the mobility of ferric iron is low at near neutral pH and consequently it might be possible for the sulphur-oxidizing bacteria to use the elemental sulphur formed according to Eq. 1 for growth. The validity of this assumption was investigated by growing several thiobacilli on FeS_2 *viz.* *Thiobacillus thioparus*, *Thiobacillus thiooxidans*, *Thiobacillus intermedius* and *Thiobacillus perometabolis*. These organisms are able to oxidize S^o or S^{2-}. They were inoculated in their respective basal salts media with FeS_2 as energy source and incubated at 30°C on a gyrotory shaker. The medium for *Thiobacillus perometabolis* contained 0.005% yeast extract as sole organic material. The medium for *Thiobacillus intermedius* did not contain organic matter. The experiment lasted about 40 days. A slight pyrite oxidation took place in the culture flasks, but there was no difference between the inoculated flask (Fig. 3). In most of the cultures cell numbers increased by a factor of about 10, only in the case of *Thiobacillus thioparus* this increase was more substantial. The bacteria apparently utilized the small amount of S^o released during the non-biological FeS_2 oxidation. To investigate this phenomenon some further experiments were carried out.

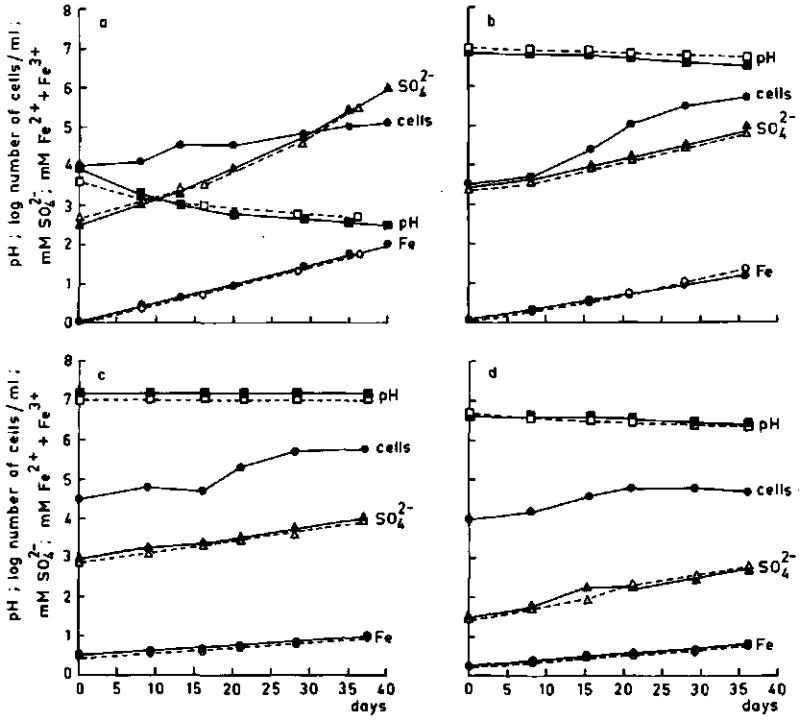

Fig. 3. Growth of a) *Thiobacillus thiooxidans*; b) *Thiobacillus thioparus*; c) *Thiobacillus perometabolis* and d) *Thiobacillus intermedius* in liquid media with FeS_2 as energy source.
—— inoculated flasks; ------ uninoculated blanks.

2.3.3 *Simultaneous growth of Thiobacillus ferrooxidans and Thiobacillus thiooxidans in liquid medium with FeS_2 as energy source*

In acid environments Fe^{3+} ions react rapidly with FeS_2 according to the Equation:

$$FeS_2 + 2Fe^{3+} \rightarrow 3Fe^{2+} + 2S^o \qquad \text{(Equation 4) (8)}$$

This elemental sulphur might be utilized by *Thiobacillus thiooxidans* and explain the growth of this bacterium in acid sulphate soils provided that S^o is not instantaneously oxidized non-biologically by Fe^{3+}. However, attempts to grow *Thiobacillus thiooxidans* on FeS_2 at 18 mM Fe^{3+} were not successful. This concentration of iron did not inhibit growth of the organism on S^o, but within half a day all the Fe^{3+} was reduced to Fe^{2+}, so that the circumstances became similar to those of the preceding experiment (Fig. 3a).

To improve the growth of *Thiobacillus thiooxidans* on pyrite a constant addition of Fe^{3+} would be necessary, resulting in a constant supply of S^{o} which might be utilized by the organism.

To realize this situation, *Thiobacillus thiooxidans* together with *Thiobacillus ferrooxidans* was inoculated into 9K FeS_2 medium. The iron oxidizer provided Fe^{3+} ions that released S^{o} from FeS_2. In this way there might be a constant supply of useful substrate for the sulphur oxidizer. Three Erlenmeyer flasks containing 9K medium were sterilized by autoclaving. One flask was inoculated with *Thiobacillus ferrooxidans* and contained FeS_2 as the energy source, the second was inoculated with a mixture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* and contained also FeS_2. The third flask was inoculated with a mixed culture of both bacteria but contained only $FeSO_4.7H_2O$ as the energy source. Periodically, cells were counted and estimations were made of the amount of FeS_2 or Fe^{2+} oxidized. The mixed population did not oxidize FeS_2 faster than the pure culture of *Thiobacillus ferrooxidans* (Fig. 4). Consequently *Thiobacillus thiooxidans* which grow slightly under these circumstances did not have any positive effect upon the oxidation of pyrite. The low increase in cell numbers of *Thiobacillus thiooxidans* may have been due to the low pH, which favours the oxidation of FeS_2 by Fe^{3+} ions according to Equation 3.

To prevent the pH drop the experiment was repeated and carried out in pH-controlled fermentors (Eschweiler, Kiel). One fermentor was inoculated with *Thiobacillus ferrooxidans*, a second with a mixture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. The pH was fixed at 4.0. The results of this experiments (Fig. 5) show that the increase in number of cells of *Thiobacillus thiooxidans* was more pronounced than it was in the preceding experiment (Fig. 4b). However, also at pH 4.0 *Thiobacillus thiooxidans* did not accelerate oxidation of pyrite by *Thiobacillus ferrooxidans*.

2.3.4 *The role of thiobacilli in the initial acidification of pyritic soils from pH 7.0 to pH 4.0.*

The assumption that thiobacilli would not affect FeS_2-oxidation between pH 7.0 and 4.0 was further examined. Soil samples of 2 g, sterilized by heat or with ethylene-oxide were inoculated with pure cultures or mixtures of different thiobacilli or with small amounts of freshly taken samples of acid sulphate soil. Periodically the pH of the samples was measured by adding

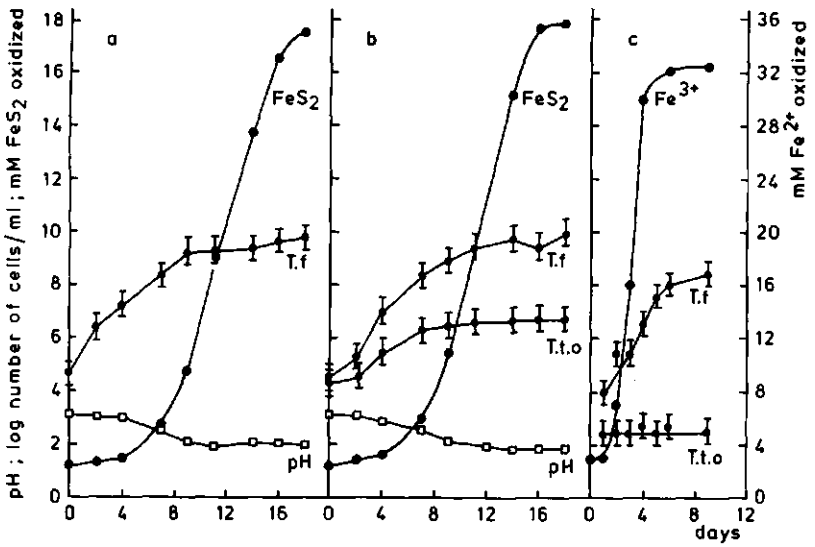

Fig. 4. a) *Thiobacillus ferrooxidans* growing on FeS_2; b) mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* growing in liquid media with FeS_2 as energy source; c) mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* growing in liquid medium with $FeSO_4.7H_2O$ as energy source.
T.f. = *Thiobacillus ferrooxidans* and T.t.o. = *Thiobacillus thiooxidans*

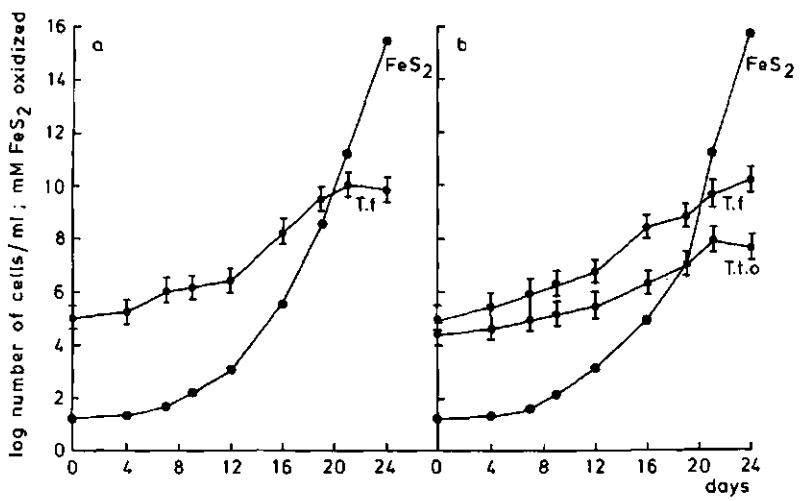

Fig. 5. Growth of a) *Thiobacillus ferrooxidans* and b) a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* in liquid medium with FeS_2 as energy source at constant pH of 4.0.
T.f. = *Thiobacillus ferrooxidans* and T.t.o. = *Thiobacillus thiooxidans*.

2.5 ml of demineralized water and extractable iron was estimated by adding 5 ml of 6 N HCl. Marked differences between the two ways of sterilization were not observed. The figures given in Table 2 represent data of the ethylene-oxide-sterilized soil. Neither thiobacilli nor other bacteria were involved in the initial pH drop (Table 2a). There were no significant differences between the sterile soil and the soils inoculated with various thiobacilli or with unsterilized acid sulphate soil. An acceleration of the oxidation of pyrite was observed when *Thiobacillus ferrooxidans* was present and when the pH decreased below 4.0 (Table 2a). $CaCO_3$ prevented the decrease of pH but did not inhibit the non-biological FeS_2 oxidation (Table 2b). After 14 and 45 days the soil samples were tested for the occurrence of different microorganisms. Viable cells of the various thiobacilli were present in all samples inoculated with the corrresponding pure cultures. No microorganisms were isolated from the inoculated blank.

Table 2a. Pure cultures and mixtures of various thiobacilli added to ethylene-oxide-sterilized FeS_2-containing soil. Fe represents the percentage of total Fe II and Fe III extractable with 6 N HCl. The samples were inoculated with: 1. *T. ferrooxidans*, 2. *T. thiooxidans*, 3. *T. thioparus*, 4. *T. intermedius*, 5. *T. perometabolis* and unsterilized potentially acid sulphate soil (A.s.s.). The blank denotes uninoculated samples.

Inoculum		Time of incubation (days)								
		0	7	14	21	28	38	45	55	68
1, 2, 3, 4, 5	Fe	2.0	2.7	3.5	4.2	5.7	7.8	9.8	13.8	18.1
	pH	5.9	5.3	5.0	4.8	4.1	3.4	3.1	2.9	2.7
1, 2, 3	Fe	1.9	2.6	3.9	4.6	5.6	7.7	9.2	13.5	17.2
	pH	5.8	5.3	5.2	4.2	3.5	3.4	2.9	2.7	2.4
1, 2	Fe	2.1	2.8	3.7	4.7	5.8	7.8	9.7	13.3	17.9
	pH	5.6	5.6	5.1	4.1	3.4	3.1	2.9	2.7	2.6
1, 3	Fe	2.0	2.4	3.9	4.9	5.9	7.8	10.0	13.7	18.2
	pH	5.8	5.6	4.1	3.7	3.3	3.1	2.9	2.7	2.6
1	Fe	1.7	2.3	3.2	4.0	5.7	7.6	9.7	13.3	17.7
	pH	5.8	5.8	4.7	4.0	3.5	3.2	3.0	2.7	2.7
2	Fe	1.6	2.1	3.9	4.9	5.9	6.5	7.1	8.2	9.8
	pH	5.8	5.4	4.3	3.6	3.3	3.0	2.9	2.8	2.7
3	Fe	1.9	2.2	3.1	4.1	5.3	6.7	7.2	8.3	9.7
	pH	5.9	5.6	5.3	4.9	4.1	3.6	3.3	3.1	2.9
4, 5	Fe	2.0	2.7	3.2	4.7	5.2	6.4	7.1	8.0	9.2
	pH	6.0	5.8	5.6	4.6	3.8	3.3	3.2	3.0	2.8
A.s.s	Fe	2.1	2.3	3.3	4.3	5.6	7.8	9.8	13.9	19.1
	pH	6.0	5.9	5.6	4.6	3.4	3.0	3.1	2.7	2.6
Blank	Fe	2.3	2.1	3.1	4.1	5.2	6.3	7.1	8.1	9.3
	pH	6.0	5.7	5.7	4.4	3.7	3.5	3.5	3.1	2.9

Table 2b. Pure cultures and mixtures of various thiobacilli added to ethylene-oxide-sterilized FeS_2-containing soil supplied with 0.5% $CaCO_3$. Fe represents the percentage of total Fe II and Fe III extractable with 6 N HCl. The samples were inoculated with: 1. *T. ferrooxidans*, 2. *T. thiooxidans*, 3. *T. thioparus*, 4. *T. intermedius*, 5. *T. perometabolis* and unsterilized potentially acid sulphate soil (A.s.s.). The blank denotes uninoculated samples.

Inoculum		Time of incubation (days)								
		0	7	14	21	28	38	45	55	68
1, 2, 3, 4, 5.	Fe	2.1	3.0	4.1	4.2	3.6	4.8	7.6	8.1	8.0
	pH	7.4	7.4	7.4	7.5	7.6	7.6	7.5	7.5	7.4
1, 2, 3.	Fe	1.7	3.1	4.5	4.7	4.1	5.9	7.7	8.3	9.4
	pH	7.3	7.2	7.5	7.4	7.4	7.5	7.5	7.4	7.4
1, 2.	Fe	2.3	4.6	4.9	5.0	6.6	7.7	7.9	8.5	9.1
	pH	7.8	7.8	7.7	7.4	7.3	7.7	7.7	7.6	7.6
1, 3.	Fe	1.9	3.4	4.1	5.1	5.7	6.4	8.4	9.3	10.1
	pH	7.6	7.6	7.8	7.8	7.8	7.8	7.8	7.7	7.6
1.	Fe	1.7	3.7	4.8	5.3	5.8	7.5	7.5	8.4	11.0
	pH	7.8	7.8	7.8	7.5	7.6	7.8	7.8	7.6	7.6
2.	Fe	1.4	4.6	5.2	5.7	6.9	7.3	9.3	10.2	11.4
	pH	7.4	7.3	7.2	7.9	7.8	7.1	7.1	7.1	7.1
3.	Fe	2.1	4.4	5.3	5.9	5.9	6.9	10.2	11.1	11.9
	pH	7.3	7.1	7.1	7.4	7.3	7.1	7.1	7.1	7.0
4, 5	Fe	1.9	3.4	4.2	4.2	5.0	6.9	6.8	7.5	9.1
	pH	7.4	7.3	7.2	7.3	7.4	7.2	7.2	7.2	7.1
A.s.s.	Fe	2.3	3.7	4.1	4.9	4.7	5.3	9.3	10.0	11.2
	pH	7.7	7.5	7.6	7.8	7.7	7.6	7.6	7.4	7.5
Blank	Fe	2.1	5.2	6.3	6.1	7.0	7.8	8.0	9.1	10.5
	pH	7.8	7.9	7.8	7.8	7.8	7.8	7.8	7.6	7.7

2.3.5 *Attempts to isolate other bacteria involved in FeS_2 oxidation*

The results of the above-mentioned experiments show that *Thiobacillus ferrooxidans* is the only organism involved directly in the formation of acid sulphate soils. However, an acid-tolerant filamentous bacterium of the genus *Metallogenium*, isolated from different sources, would significantly catalyse iron oxidation in the pH range 5.0 - 3.5 and would directly influence the rate of acid production in coal mine waters (24). Another acidophilic iron-oxidizing organism recently found and described is *Leptospirillum ferrooxidans*. This organism together with a sulphur-oxdizing *Thiobacillus* sp. is able to oxidize FeS_2 (2, 18). Therefore the possible role of these organisms in the formation of acid sulphate soils was examined. Efforts to isolate

Metallogenium-like bacteria were not successful. Attempts to isolate *Leptospirillum ferrooxidans* via enrichment cultures with Fe^{2+} or FeS_2 as energy source failed as well and resulted only in enrichment of *Thiobacillus ferrooxidans*. Therefore enrichment cultures were grown in chemostats. Advantages of using a chemostat included (i) maintaining the pH at a constant value, (ii) the possibility to imitate natural conditions. Diffusion of O_2 into the soil is the rate-limiting step in the formation of acid sulphate soils (8). Therefore O_2 was chosen as the limiting growth factor in the chemostat. The fermentors were inoculated with a filtrate of a freshly sampled acid sulphate soil obtained by filtering through a 2 µm filter to prevent predator pray processes. The dilution rate was 0.005 h^{-1}; after 5 dilutions the bacteria present were microscopically observed and counted according to the MPN technique as described in section 2.2.1. Neither *Metallogenium* nor *Leptospirillum* were observed. The only bacteria present were thiobacilli and heterotrophic micro-organisms (Table 3). About 5% of the heterotrophic flora was able to oxidize thiosulphate to tetrathionate and to decrease the pH.

Table 3. Enrichment cultures in an O_2-limited chemostat with FeS_2 as energy source at different pH values. Figures represent numbers of cells/ml present after 5 dilutions. The dilution rate was 0.005 h^{-1}.

	pH 3.5	pH 4.7	pH 5.7	pH 6.5
T. ferrooxidans	49.10^5	49.10^3	23.10_3	79
T. thiooxidans	23.10	94.10^4	11.10^4	13.10^2
T. thioparus	0	13.10^5	13.10^5	49.10^3
Heterotrophs	41.10^5	74.10^5	54.10^4	96.10^5
Thiosulphate oxidizers	20.10^4	31.10^4	35.10^4	63.10^4

2.4 DISCUSSION

The results of the experiments on pyrite oxidation in acid sulphate soil show that microorganisms were not involved during the initial drop of pH from near neutral to 4.0. Although several sulphur-oxidizing thiobacilli as well as heterotrophic thiosulphate oxidizers were isolated during this initial process, they apparently do not accelerate the oxidation of FeS_2. Little is known about the heterotrophic thiosulphate oxidizers. In a survey of S^o oxidation in 288 Australian soil samples, *Thiobacillus thiooxidans* was undetectable in 1/3 of the soils examined (22). The most numerous sulphur oxidizers were heterotrophic bacteria. However, autoclaved soils reinoculated with autotrophs or heterotrophs or mixtures of both showed the larges amounts of sulphur oxidized when

Thiobacillus thiooxidans was present, less when *Thiobacillus thioparus* occurred alone and least when only heterotrophs were introduced. Consequently, the heterotrophs were probably not very important in sulphur oxidation (22). However, in open ocean areas biological oxidation of reduced sulphur compounds were mainly carried out by facultative autotrophic bacteria whilst chemolitotrophic thiobacilli were rare in the marine environment (23). In the present study on pyritic soils the heterotrophic thiosulphate oxidizers were not important. The autotrophic flora other than *Thiobacillus ferrooxidans* exists apparently on account of oxidizable S compounds, released during the non-biological oxidation of FeS_2. Only a part of the sulphur moiety of FeS_2 was used by the thiobacilli as shown by the following simple calculation. At least 0.5 mM FeS_2 (Fig. 3) was oxidized during 40 days which corresponds to maximally 1 mM S^o formed (Equation 1). Earlier observations showed that for the fixation of 1 mole CO_2 *Thiobacillus ferrooxidans* required 6 to 8 moles of S^o (1, 3). Assuming that with other thiobacilli these figures are of the same order, the following additional calculations can be made. When 1 mmole of S^o is released, about 0.1 mole of CO_2 can be fixed which corresponds to about 1 mg of C or about 10^{10} cells/l, a number many times greater than the 10^8 cells/l actually counted (Fig. 3). This indicates that most of the elemental S, derived from non-biological pyrite oxidation is oxidized non-biologically.

The only microorganism involved in the oxidation of FeS_2 is *Thiobacillus ferrooxidans*. Attempts to isolate other iron oxidizers like *Metallogenium* or *Leptospirillum ferrooxidans* failed. The only microorganisms present in the enrichment cultures were thiobacilli and heterotrophic microorganisms which probably grew on the added yeast extract.

The presence of $CaCO_3$ prevented acidification of the soil (Fig. 1, Table 2). However, $CaCO_3$ did not stop the non-biological oxidation of FeS_2. The initial oxidation of FeS_2 in non-calcareous soil did not differ from the initial oxidation in calcareous soil (Table 2). FeS_2 oxidation in the calcareous soil ceased after about 60 days (Fig. 1). Probably pyrite occurred in the soil in different fractions with different susceptibilities to oxidation. The decrease in the rate of FeS_2 oxidation can be explained by the increased resistance to oxidation of the remaining FeS_2. Preservation of the FeS_2 is probably the result of its relatively large particle size combined with a relatively high pH (7). If this behaviour of pyritic soils would be of general validity, acidification of potentially and sulphate soils could be prevented by the addition of a moderate amount of $CaCO_3$.

Fig. 2 and Fig. 4 and Table 3 show that *Thiobacillus thiooxidans* at pH below 3.5 is present in small numbers. The enhanced mobility of Fe^{3+} ions at lower pH was probably responsible for the rapid non-biological oxidation of the S^{o} so that it was hardly utilized by *Thiobacillus thiooxidans*. At a constant pH of 4.0 the use of the sulphur moiety of FeS_2 by *Thiobacillus thiooxidans* was more efficient than at lower pH. This finding is in fair agreeement with earlier observations concerning the effect of pH on pyrite oxidation (1) (see chapter 3). The lower the pH, the more important is the indirect mechanism in which Fe^{3+} ions represent the primary oxidant of FeS_2. The reduced iron ions are subsequently reoxidized by *Thiobacillus ferrooxidans*.

REFERENCES

1. Arkesteyn, G.J.M.W. 1979 Pyrite oxidation by *Thiobacillus ferrooxidans* with special reference to the sulphur moiety of the mineral. Antonie van Leeuwenhoek J. Microbiol. Serol. (*In press*).
2. Balashova, V.V., Vedenina, I.Ya., Markosyan, G.E. and Zavarzin, G.A. 1974 The auxotrophic growth of *Leptospirillum ferrooxidans*. Mikrobiologiya *43*, 581-585.
3. Beck, J.V. and Shafia, F.M. 1964 Effect of phosphate ion and 2-4 dinitrophenol on the activity of intact cells of *Thiobacillus ferrooxidans*. J. Bacteriol. *88*, 850-857.
4. Begheyn, L.Th., van Breemen, N. and Velthorst, E.J. 1978 Analysis of sulfur compounds in acid sulfate soils and other recent marine soils. Commun. Soil Sci. Plant Anal. *9*, 873-882.
5. Belly, R.T. and Brock, T.D. 1974 Ecology of iron oxidizing bacteria in pyritic materials associated with coal. J. Bacteriol. *117*, 726-732.
6. Bloomfield, C. 1972 The oxidation of iron sulphides in soils in relation to the formation of acid sulphate soils and of ochre deposits in field drains. J. Soil Sci. *23*, 1-16.
7. Breemen, N. van. 1972 Soil forming processes in acid sulphate soils. *In* Acid sulphate soils. Ed. H. Dost. Proceedings of the International Symposium, Wageningen. ILRI Public. *18*, vol. I, 66-130.
8. Breemen, N. van. 1976. Genesis and solution chemistry of acid sulfate soils in Thailand. Thesis Wageningen 1976.
9. Colmer, A.R. and Hinkle, M.E. 1947 The role of microorganisms in acid mine drainage. Science *106*, 253-256.
10. Dugan, P.R. 1975 Bacterial ecology of strip mine areas and its relationship to the production of acidic mine drainage. Ohio J. Sci. *75*, 266-279.
11. Dugan, P.R. and Apel, W.A. 1978 Microbial desulfurization of coal. *In* Metallurgical applications of bacterial leaching and related microbial phenomena. Eds. L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press N.Y.
12. Harmsen, G.W., Quispel, A. and Otzen, D. 1954 Observations on the formation and oxidation of pyrite in the soil. Plant and Soil *5*, 324-348.
13. Hart, M.G.R. 1959 Sulphur oxidation in tidal mangrove soils of Sierra Leone. Plant and Soil *11*, 215-236.
14. Hart, M.G.R. 1963 Observations on the source of acid in empoldered mangrove soils. II Oxidation of soil polysulphides. Plant and Soil *19*, 106-114.

15. Hutchinson, M., Johnstone, K.I. and White, D. 1965 The taxonomy of certain thiobacilli. J. Gen. Microbiol. *41*, 357-366.
16. London, J. 1963 *Thiobacillus intermedius* nov.sp. Arch. Mikrobiol. *46*, 329-337.
17. London, J. and Rittenberg, S.C. 1967 *Thiobacillus perometabolis* nov.sp., A non-autotrophic Thiobacillus. Arch. Mikrobiol. *59*, 218-225.
18. Norris, P.R. and Kelly, D.P. 1978 Dissolution of pyrite (FeS_2) by pure and mixed cultures of some acidophilic bacteria. FEMS Microbiol. Lett. *4*, 143-146.
19. Quispel, A., Harmsen, G.W. and Otzen, D. 1952 Contribution to the chemical and bacteriological oxidation of pyrite in soil. Plant and Soil *4*, 43-55.
20. Roy, A.B. and Trudinger, P.A. 1970 The biochemistry of inorganic compounds of sulphur. Cambridge university press. London.
21. Silverman, M.P. and Lundgren, D.G. 1959 Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. J. Bacteriol. *77*, 642-647.
22. Swaby, R.J. and Vitolins, M.J. 1968 Sulphur oxidation in Australian soils. Trans. 9th Intern. Congress Soil Sci. *4*, 673-681.
23. Tuttle, J.H. and Jannasch, H.W. 1972 Occurrence and types of Thiobacillus-like bacteria in the sea. Limnol. Oceanogr. *17*, 532-543.
24. Walsh, F. and Mitchell, R. 1972 A pH dependent succession of iron bacteria. Environm. Sci. Technol. *6*, 809-812.

3. PYRITE OXIDATION BY *THIOBACILLUS FERROOXIDANS* WITH SPECIAL REFERENCE TO THE SULPHUR MOIETY OF THE MINERAL

ABSTRACT

Available cultures of *Thiobacillus ferrooxidans* were found to be contaminated with bacteria very similar to *Thiobacillus acidophilus*. The experiments described were performed with a homogeneous culture of *Thiobacillus ferrooxidans*.

Pyrite (FeS_2) was oxidized by *Thiobacillus ferrooxidans* grown on iron (Fe^{2+}), elemental sulphur (S^o) or FeS_2.

Evidence for the direct utilization of the sulphur moiety of pyrite by *Thiobacillus ferrooxidans* was derived from the following observations: a. Known inhibitors of Fe^{2+} and S^o oxidation, NaN_3 and NEM respectively, partially abolished FeS_2 oxidation. b. A b-type cytochrome was detectable in FeS_2- and S^o-grown cells but not in Fe^{2+}-grown cells. c. FeS_2 and S^o reduced b-type cytochromes in whole cells grown on S^o. d. CO_2 fixation at pH 4.0 per mole of oxygen consumed was the highest with S^o, lowest with Fe^{2+} and medium with FeS_2 as substrate. e. Bacterial Fe^{2+} oxidation was found to be negligible at pH 5.0 whereas both FeS_2 and S^o oxidation was still appreciable. f. Separation of pyrite and bacteria by means of a dialysis bag caused a pronounced drop of the oxidation rate which was similar to the reduction of pyrite oxidation by NEM; indirect oxidation of the sulphur moiety by Fe^{3+} was not affected by separation of pyrite and bacteria.

Bacterial oxidation and utilization of the sulphur moiety of pyrite were relatively more important with increasing pH.

3.1 INTRODUCTION

Two mechanisms have been proposed for the bacterial oxidation of pyrite (FeS_2) by *Thiobacillus ferrooxidans, viz.* the direct contact mechanism and the indirect contact mechanism (17). In the latter case ferric ions are the primary oxidant of pyrite according to the reaction:

$14\ Fe^{3+} + FeS_2 + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$ (Equation 1). The resulting Fe^{2+} is enzymically oxidized by *Thiobacillus ferrooxidans* to complete a cyclic process. The direct contact mechanism requires intimate physical contact between the organism and pyrite. As the solubility of FeS_2 is low, the organism would oxidize the sulphur moiety of pyrite only via the direct contact mechanism. Few data are available on the bacterial utilization of the sulphur moiety of FeS_2. From the ratio oxygen consumption/CO_2 fixation Beck and Brown (2) concluded that in addition to iron the sulphur moiety of pyrite was most

likely utilized as well. Duncan et al. (8) studied the effect of metabolic inhibitors on pyrite oxidation by *Thiobacillus ferrooxidans* and suggested the bacterial oxidation of the sulphur moiety. The increasing rate of oxygen uptake with time of HCl-washed FeS_2 by *Thiobacillus ferrooxidans* as observed by Silverman (17) does not allow to draw conclusions as to the direct oxidation and consequently the oxidation of the sulphur moiety of FeS_2.

Isolates of *Thiobacillus ferrooxidans* are very often heterogeneous (9, 14, 20, 21). A mixotrophic contaminant was isolated from a strain of *Thiobacillus ferrooxidans* (9). This organism can use S^o as well as organic compounds for energy. So the possibility of co-operation between the two bacteria, in which one oxidizes the iron- and the other the sulphur moiety of pyrite, can not be excluded.

This report contains the results of a study of the oxidation of the sulphur moiety of FeS_2 by *Thiobacillus ferrooxidans*. Special attention has been paid to factors affecting the oxidation of the sulphur moiety such as pH and energy source of the growth medium. Evidence is presented of the possible utilization of the sulphur moiety of pyrite as energy source.

3.2 MATERIALS AND METHODS

3.2.1 *Media and cultures*

Thiobacillus ferrooxidans strain ATCC 13598, isolated by Beck (1), was used throughout this study. It was maintained on 9K Fe^{2+} medium (18). Duplicate cultures were originally obtained from the American Type Culture Collection. Both cultures were found to be heterogeneous and contained *Thiobacillus acidophilus*-type of bacteria when tested by the procedure of Guay and Silver (9). *Thiobacillus ferrooxidans* was purified by single colony isolation from 9K silicagel plates and subsequent routine tests indicated culture homogeneity.

3.2.2 *Growth conditions*

Cultures were grown in a 3.2 litre Biotec fermentor using a working volume of 2.5 litre at 28°C and a dilution rate of 0.03 h^{-1}. The pH was maintained at 3.0 when Fe^{2+} and FeS_2 were used as substrate. With S^o as energy source the pH was kept at 4.5. The medium was composed of A (per litre): Na_2CO_3 3 g, K_2HPO_4 1 g and 1 pellet of KOH, and B: $(NH_4)_2SO_4$ 6 g, KCl 0.2 g, $MgSO_4.7H_2O$

1.0 g, 7 ml of 2 N H_2SO_4 and 2 ml of a trace elements solution (19). When Fe^{2+} was used as energy source, $FeSO_4.7H_2O$ and H_2SO_4 were sterilized together by autoclaving to prevent oxidation of Fe^{2+} and added to the remainder of solution B to a final concentration of 3.6% (w/v) $FeSO_4.7H_2O.FeS_2$ (particles below 100 µm) were sterilized by heating at 160°C under an argon atmosphere and added to solution B to a final concentration of 0.5% (w/v). S^o was sterilized under a layer of 96% ethanol. After 15 h the ethanol was evaporated at 105°C and the remaining S^o added to solution A to a final concentration of 0.5% (w/v); it was kept in suspension by added Tween 80 (10 ppm) and heavy stirring. The two parts of the medium were pumped separately into the culture vessel at the same pumping rate. After three dilutions or more, a 2 litre portion of the culture was harvested and centrifuged at 1000 rpm to remove ferric precipitates or/and the last few FeS_2 or S^o particles. The cells were then collected by centrifuging at 25,000 x g, washed with cold distilled water and suspended in cold water or buffer solution. Only freshly grown cells were used.

3.2.3 *Oxidation rate*

Oxygen uptake rates were measured in a respirometer (Yellow Springs Instrument Co. Ohio). The electrode was connected with a recorder. The electrode vessel contained 3.9 ml of buffer and 1.0 ml of cell suspension (100-300 µg of protein/ml). 0.05 M β-alanine-H_2SO_4 buffer was used over the range of pH 3.0 and 5.0 and 0.05 M K-phosphate buffer was used at pH 6.0 and 7.0. After temperature equilibration at 30°C and saturation with air, the reaction was started by injection of 50 µmoles of $FeSO_4.7H_2O$, 20 mg S^o (homogenized with an ultraturrax), or 20 mg FeS_2 (particles below 20 µm) to make a final volume of 5.0 ml. Oxidation rates were followed for 10 min and were linear with time and with the amount of bacterial protein.

3.2.4 *CO_2 fixation*

Incorporation of $^{14}CO_2$ was measured in 200 ml Erlenmeyer flasks with Suba-seal stoppers containing 8.0 ml of medium, pH 3.0 or 4.0, of the following composition (per litre): $(NH_4)_2SO_4$ 0.1 g, $MgCl_2.6H_2O$ 0.5 g, KH_2PO_4 0.5 g, KCl 0.1 g, 1 ml of trace elements-containing solution and 1.0 ml of 0.1 M $Fe_2SO_4.7H_2O$, or 1 ml suspension of FeS_2 (0.2 g/ml particles below 20 µm), or 1 ml suspension of S^o (0.2 g/ml). The $^{14}CO_2$ content in the gas phase was about

3.5%; it was obtained by injecting 1 ml of 2 N HCl into a tube standing vertically in the flask and containing 1 ml of 0.30 M $NaH^{14}CO_3$ (5μCi/ml). After injection of 1.0 ml cell suspension, the mixtures were incubated at 30°C for 4 h (Fe^{2+} as substrate) and 16 h (FeS_2 and S^o as substrate) in a shaking waterbath. Toluene was added before the bacteria to the chemical control flasks. Oxidation was terminated by injection of 5.0 ml of 6 N HCl. After flushing with non-radioactive CO_2 to remove excess $^{14}CO_2$, the contents of the flasks were centrifuged at 1000 rpm to remove FeS_2 or S^o and then at 25,000 x g for 20 min to collect the cells. The pellet was repeatedly washed with 8.0 ml H_2O. The combined supernatants were examined for Fe^{2+}, Fe^{3+} and SO_4^{2-} in order to calculate the amounts of substrate oxidized.

The pellet was dissolved in 1 ml Soluene and radioactivity measured in a Nuclear Chicago Mark I scintillation counter with Dimilume 30 as scintillation liquid. 1.0 ml of the supernatant was also counted with 10 ml Instafluor Titron x 100 (2:1 v/v) as scintillation liquid. The total radioactivity in sediment and supernatant was considered to represent assimilated $^{14}CO_2$.

3.2.5 *Cytochrome spectra*

Reduced minus oxidized cytochrome spectra were measured in a Beckman double-beam spectrophotometer model 25. The cuvette with oxidized cytochromes contained air-saturated cell-free extract (80-100 μg protein) in 0.05 M Na-acetate buffer of pH 5.5. The cuvette with reduced cytochromes contained the same amount of protein, 1 mM NaN_3 and a few crystals of dithionite.

Cytochrome reduction by Fe^{2+}, S^o and FeS_2 with whole cells was carried out in 1.5 ml anaerobic cuvettes containing 100-200 μg cells (dry weight) 50 μmoles K-phosphate, pH 5.0, a few crystals of NaN_3 and 20 mg S^o, or 20 mg freshly acid-washed FeS_2 (particles below 20 μm) or 20 μmoles $FeSO_4.7H_2O$ or a few crystals of dithionite and water to make a total volume of 1.5 ml. After precipitation of S^o or FeS_2, spectra were recorded against a control cuvette without added reducing agent and NaN_3 in an Aminco Chance double beam spectrophotometer.

3.2.6 *Chemical analysis*

Fe^{2+} and Fe^{3+} were estimated colorimetrically with 1,10-phenantroline according to the method of L.Th. Begheyn (3). Sulphate was determined turbidimetrically by the procedure of Begheyn et al. (4). Protein was assayed by the Folin Ciocalteu method as described by Lowry et al. (12).

3.2.7 *Pyrite*

Pyrite was obtained from Dr Krantz, Mineralien, Versteinerungen und Geogeräte, D53, Bonn, Germany. It contained 41.0% ± 1.0% iron and 53.3 ± 1.0% sulphur (determined as SO_4^{2-}); in addition, 2% $Fe_2(SO_4)_3$ and 4% silicates were determined as impurities. Prior to use in growth and oxidation experiments, FeS_2 was milled in a ball mill, wet-sieved through a 100 µm and 20 µm nylon sieve gauze, centrifuged, washed with 2 N HCl to remove Fe^{3+} salts and finally washed with distilled water until Cl^- free and freeze-dried.

3.3 RESULTS

3.3.1 *Oxidation of Fe^{2+}, FeS_2 and S^o and their inhibition by N-ethylmaleimide (NEM) and NaN_3*

Table 1 shows that Fe^{2+}, S^o and FeS_2 were readily oxidized by cells grown on each substrate. Cells grown on S^o exhibited lower rates of both Fe^{2+} and FeS_2 oxidation than cells grown on either FeS_2 or Fe^{2+}. The S^o oxidation rate was always relatively low, independent of the growth substrate. The biological oxidation of pyrite was inhibited by 10^{-3} M NEM, a known inhibitor of reduced sulphur compounds oxidation (Table 1), indicating that the sulphur moiety was indeed directly oxidized. At the concentration used, NEM did not affect Fe^{2+} oxidation, which confirmed the results of Duncan et al. (8). Inhibition was more pronounced in S^o-than in Fe^{2+}-grown cells (71 and 36% inhibition, respectively). FeS_2 oxidation by cells grown on this substrate was moderately reduced by NEM (55% inhibition). Reverse results were obtained with 10^{-5} M NaN_3, a known inhibitor of iron oxidation. At the concentration, used NaN_3 did not inhibit S^o oxidation, as found previously by Duncan et al. (8). Inhibition of FeS_2 oxidation as observed with Fe^{2+}-grown cells or with S^o-grown cells was 69% and 35%, respectively. Earlier results obtained with the heterogeneous culture of *Thiobacillus ferrooxidans* strain ATCC 13598 hardly differed from the data of Table 1.

Table 1. Oxidation of Fe^{2+}, FeS_2 and S^o by washed cell suspensions of *Thiobacillus ferrooxidans* grown on each substrate. Prior to injection of inhibitors the oxygen uptake of the substrate supplied cells was measured. After addition of the inhibitor the oxygen uptake was followed for another 3-5 min. Oxygen uptake rates were estimated as indicated in section 3.2.3 in a 0.05 M β-alanine buffer of pH 3.0. Results in $\mu l\ O_2.min^{-1}.mg\ protein^{-1}$. In parentheses % inhibition. NEM is an inhibitor of S^o oxidation and NaN an inhibitor of Fe^{2+} oxidation. Figures are averages of 3 determinations.

		Oxidation of		
Growth substrate	Inhibitor added	Fe^{2+}	FeS_2	S^o
Fe^{2+}	-	75	39	4
	1 mM NEM	75 (0)	25 (36)	0 (100)
	0,01 mM NaN_3	0 (100)	12 (69)	4 (0)
FeS_2	-	74	33	6
	1 mM NEM	74 (0)	15 (55)	0 (100)
	0,01 mM NaN_3	0 (100)	18 (45)	6 (0)
S^o	-	17	17	7
	1 mM NEM	17 (0)	5 (71)	0 (100)
	0,01 mM NaN_3	0 (100)	11 (35)	7 (0)

3.3.2 *Oxidation of Fe^{2+}, FeS_2 and S^o as affected by pH*

Since Fe^{2+} and S^o oxidation have different pH optima, it was expected that the oxidation of FeS_2, and especially the oxidation of the sulphur moiety, would be influenced by the pH value of the incubation mixture. Figs 1A and 1B show that above pH 5, Fe^{2+} oxidation by cells grown on either Fe^{2+} or FeS_2 was abolished, whereas pyrite oxidation was still detectable. S^o oxidation was almost unaffected over the pH range 2.8-7.0. Similar results were obtained with cells grown on S^o, be it that the rate of oxidation of Fe^{2+} and FeS_2, below pH 5.0 was much lower than with cells grown on Fe^{2+} or FeS_2 (Fig. 1C). With these cells the FeS_2 oxidation rate exceeded the Fe^{2+} oxidation rate even at a pH above 3.5. It seems that oxidation of pyrite at pH values above 5.0 involves predominantly oxidation of the sulphur moiety of FeS_2. This assumption is confirmed by the data plotted in Fig. 2 from which it can be seen that inhibition of FeS_2 oxidation by NEM was strongly dependent on the pH of the incubation mixture. The oxidation of pyrite was completely abolished by NEM at pH values higher than 5.0.

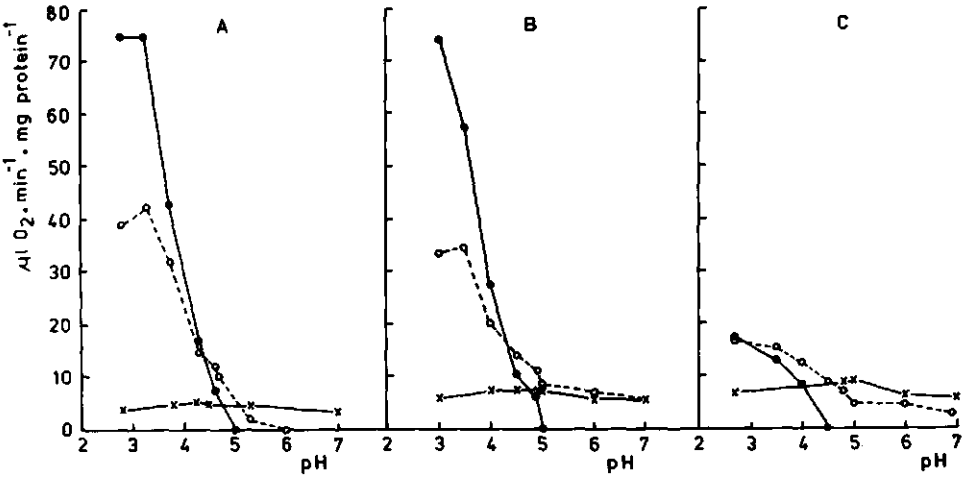

Fig. 1. Biological oxidation of Fe^{2+}, S^o and FeS_2 by *Thiobacillus ferrooxidans* grown on: A, $FeSO_4.7H_2O$; B, FeS_2; C, S^o. Oxidation was measured as indicated in section 3.2.3. ●——● Fe^{2+} oxidation; o-----o FeS_2 oxidation; x——x S^o oxidation.

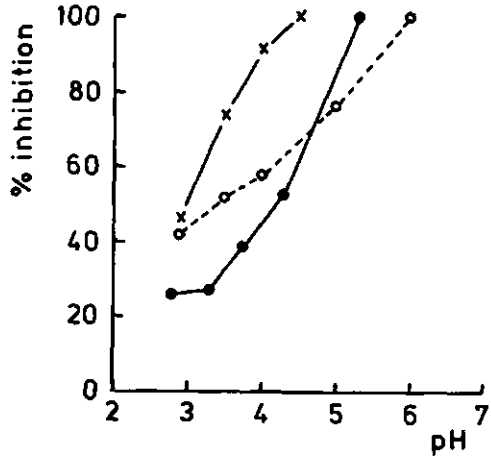

Fig. 2. Inhibition of FeS_2 oxidation by 1 mM NEM in relation to pH. The inhibitor was injected after the oxygen uptake by the FeS_2 supplied bacterial suspension had been recorded as shown in Fig. 1. After addition of NEM the incubation was continued for a further 3-5 min. ●——● Fe^{2+} grown cells; o-----o FeS_2-grown cells; x——x S^o-grown cells.

3.3.3 *Oxidation of FeS_2 in a dialysis bag*

In order to prevent direct contact between bacteria and FeS_2, 1.0 g pyrite (particles below 20 µm) was transferred to a dialysis bag. After temperature equilibration and air saturation of the bacterial suspension, the dialysis bag with pyrite was transferred to the reaction vessel and the oxidation rate measured as described in section 3.2.3. Oxygen uptake started immediately and proceeded linearly demonstrating that Fe^{2+} released from FeS_2 diffused out of the bag and was oxidized by the bacteria. The Fe^{3+} probably diffused into the bag, oxidizing the FeS_2 according to equation (1). Oxidation of FeS_2 in the dialysis bag was less rapid than the oxidation of the free FeS_2 (Table 2).

Table 2. Oxidation of FeS_2 contained in a dialysis bag by washed cell suspensions of *Thiobacillus ferrooxidans* grown on Fe^{2+}, FeS_2 or S^o. Inhibition was measured as described in Table 1. Oxidation was estimated as indicated in section 3.2.3. Results in µl $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. Figs are averages of 3 determinations. In parentheses, the oxidation rate of free FeS_2.

	Inhibitor added	Growth substrate		
		Fe^{2+}	FeS_2	S^o
pH 3.0	-	30 (39)	15 (33)	2 (17)
	1 mM NEM	30 (25)	15 (15)	2 (5)
pH 5.0	-	0 (9)	0 (9)	0 (7)

With S^o-grown cells oxidation of FeS_2 in a dialysis bag was 12% of the oxidation of free FeS_2. With FeS_2-grown cells this value was 45% and with Fe^{2+}-grown cells 75%. For the interpretation of these results it is important to note that the reduction of oxidation rate of pyrite by separation of bacteria and substrate is very similar to the reduction of pyrite oxidation by 1 mM NEM. The sulphur moiety of FeS_2 apparently remained in the dialysis bag and presumably was oxidized indirectly by Fe^{3+}. This was concluded from the fact that; (i) NEM did not inhibit the oxidation of FeS_2 in the dialysis bag at pH 3 whereas it did inhibit FeS_2 oxidation when in direct contact with the cells, (Table 1) and (ii), that Fe^{2+} oxidation and pyrite oxidation in the dialysis bag were completely abolished at pH 5.0 (Fig. 1 cf Table 2).

3.3.4 *CO_2 fixation*

Cells harvested from a continuous culture were 100-fold concentrated and subsequently incubated with the growth medium containing Fe^{2+}, FeS_2 or S^o and radioactive $^{14}CO_2$ as described in section 3.2.4. Table 3 shows that at

Table 3. Moles of Fe^{2+}, FeS_2 or S^o oxidized per mole of CO_2 fixed. In parentheses the numbers of electrons transferred to oxygen per molecule of CO_2 fixed. Figures are the average of the data of 2 experiments in triplicate and their standard deviations.

	Fe^{2+}	FeS_2	S^o
pH 3	82 ± 2 (82 ± 2)	5.0 ± 0.3 (75 ± 5)	6.0 ± 0.2 (36 ± 1)
pH 4	107 ± 4 (107 ± 4)	5.6 ± 0.4 (84 ± 6)	5.9 ± 0.2 (35 ± 1)

pH 3.0, 82 moles of Fe^{2+}, 5.0 moles of FeS_2 and 6.0 moles of S^o were required for the fixation of 1 mole of CO_2. The amount of Fe^{2+} required for CO_2 fixation increased upon elevating the pH from 3.0 to 4.0, whereas the amounts of both FeS_2 and S^o remained almost unchanged.

3.3.5 *Cytochrome spectra*

Cytochrome spectra of cell-free extracts of differently grown cells are shown in Fig. 3. S^o- and FeS_2-grown cells have absorption maxima at 560, 530 nm and 430 nm evidently reflecting the α, β and γ bands of a b-type cytochrome. These bands could not be detected in Fe^{2+}-grown cells. Bacteria grown on all three substrates contained a- and c-type cytochromes as may be deduced from the absorption maxima at 595 and 440 nm (a-type) and 552, 522 and 418 nm (c-type).

It is assumed that cyt. c acts as electron acceptor for the iron-oxidizing system of *Thiobacillus ferrooxidans* (5, 13). Since S^o- and FeS_2-grown cells contained b-type cytochromes and since S^o oxidation is far more efficient than iron oxidation in providing energy and reducing power for carbon dioxide fixation (Table 3), it was expected that cytochromes with lower redox potential are involved as electron acceptors for the S^o-oxidizing system.

Cytochrome reduction by S^o and FeS_2 in differently grown cells is shown in Fig. 4. In S^o-grown cells b-type cytochromes are reduced by S^o and FeS_2 (Fig. 4B) indicating that cyt. b is involved in the oxidation of S^o and the sulphur moiety of pyrite. Cyt. b reduction by FeS_2 is less pronounced than that by S^o and dithionite in S^o-grown cells (Fig. 4B). In pyrite-grown cells cyt. b

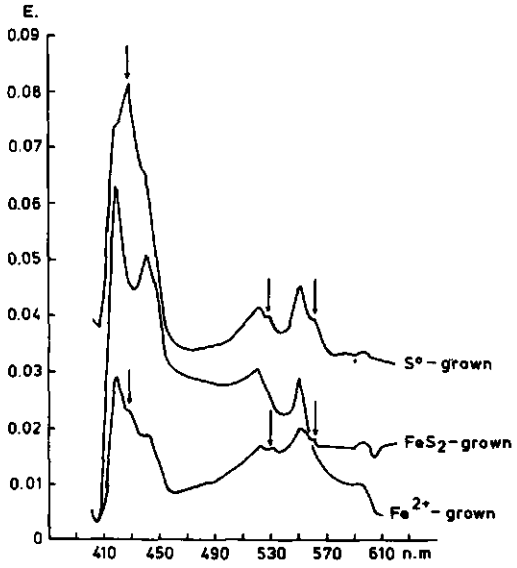

Fig. 3. Cytochrome spectra of cell-free extracts of *Thiobacillus ferrooxidans* grown on different substrates. Arrows indicate absorption maxima of b-type cytochromes.

reduction could not be detected (Fig. 4A). This may be due to the rapid sedimentation of the pyrite particles, so that only part of the cells were in direct contact with the mineral which is necessary for cyt. b reduction. Consequently cyt. b is only partly reduced whereas cyt. c is easily reduced by the Fe^{2+} released from pyrite. As a result the relatively high peaks of cyt. c may mask the reduced cyt. b peaks. In the case S^{O} serves as reductant sedimentation of the sulphur particles is much slower. As a result of this the more intense contact between bacteria and substrate may favour cyt. b reduction. Furhtermore in this case Fe^{2+} is not present and therefore cyt. c may be reduced only by cyt. b which would explain why in this case cyt. c did not mask the cyt. b peaks.

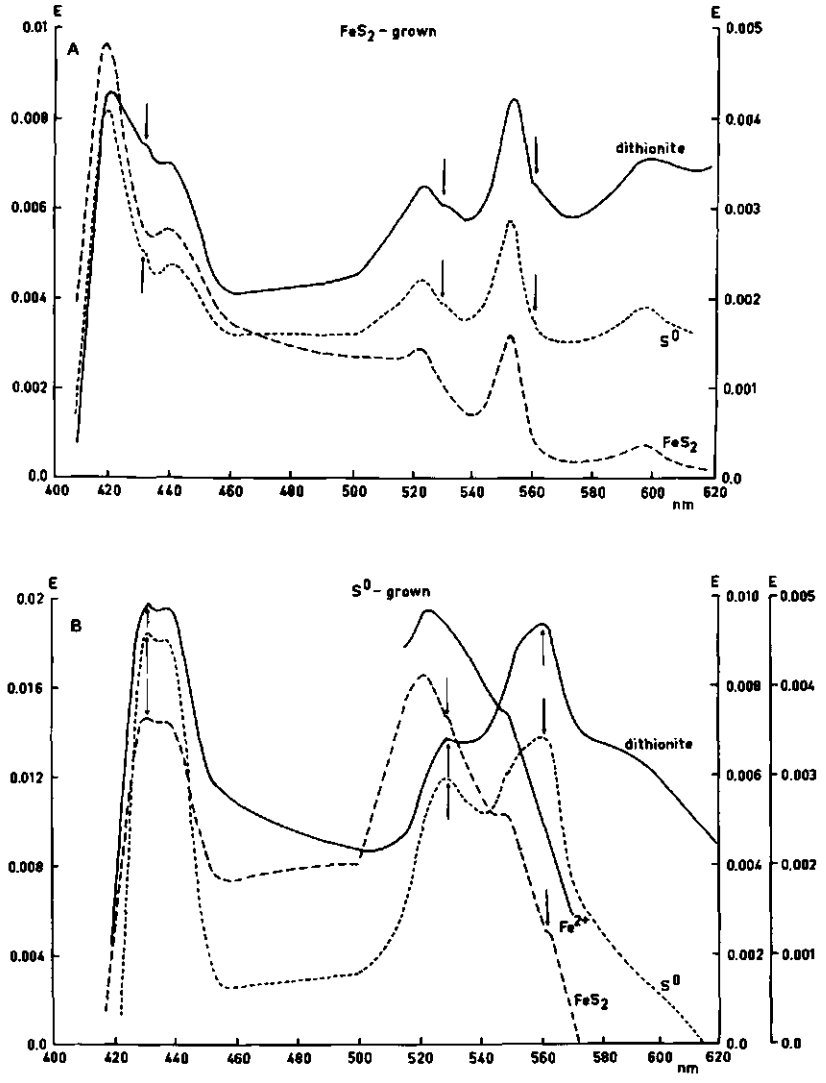

Fig. 4. Cytochrome spectra of cell suspensions of *Thiobacillus ferrooxidans* grown on different substrates. Arrows indicate absorption maxima of b-type cytochromes.

Absorption scales relative to full range

A 400-500 nm; 0.010 E
500-620 nm; 0.005 E

B 400-500 nm; 0.020 E
500-620 nm S^0 and dithionite; 0.010 E
500-620 nm Fe^{2+} and FeS_2 ; 0.005 E

3.4 DISCUSSION

Cultures of *Thiobacillus ferrooxidans* were found to be heterogeneous. Isolates of this organism derived from different sources (ATCC strain 13598, acid sulphate soils from Suriname and the Netherlands) (unpublished results) were contaminated with an organism which probably is identical with *Thiobacillus acidophilus*. Both organisms were separated as described in section 3.2.1. There was no difference between a purified and an unpurified culture of *Thiobacillus ferrooxidans* with respect to Fe^{2+}, FeS_2 and S^o oxidation and to the inhibition of FeS_2 oxidation by NaN_3 and NEM. These observations suggest that the density of the heterotrophic or mixotrophic population in the autotrophic culture of *Thiobacillus ferrooxidans* is low. An explanation for the persistence of the *Thiobacillus acidophilus* type bacteria in cultures of *Thiobacillus ferrooxidans* is not known but the association of the two organisms must be very strong since dilution series in a liquid salts medium do not lead to pure cultures.

The results of the experiments presented in this paper clearly show that a pure culture of *Thiobacillus ferrooxidans* is not only able to oxidize the iron moiety of pyrite but is also able to oxidize enzymically the sulphur moiety of the mineral. Known inhibitors of the Fe^{2+} and S^o oxidation partially abolished FeS_2 oxidation as was found earlier by Duncan et al. (8).

Cytochromes of the b-type were readily detectable in FeS_2 - and S^o-grown cells but not in Fe^{2+}-grown bacteria. Furthermore FeS_2 and S^o reduced cytochrome b in S^o-grown cells. This supports the assumption that cytochromes with lower redox potential are involved in the transport of electrons released by oxidation of S^o and of the sulphur moiety of pyrite (16). It is additional evidence that *Thiobacillus ferrooxidans* can oxidize the sulphur moiety of pyrite directly.

Fixation of 1 molecule of CO_2 at pH 4.0 required the oxidation of 107 ions of Fe^{2+}, 5.6 molecules of FeS_2 or 5.9 molecules of S^o. This is equivalent to 107, 84 and 35 electrons, respectively, transferred to oxygen, showing that the transport of electrons derived from S^o yielded more energy than the transport of electrons from Fe^{2+}. The yield of energy from the transport of electrons released from FeS_2 was intermediate. This finding is in agreement with the observation that b-type cytochromes are reduced by FeS_2 and S^o but not by Fe^{2+}. At pH 3.0 hardly any difference in the efficiency between Fe^{2+} and FeS_2 oxidation was observed, a result deviating from that of Beck and

Brown (2). Our observation that CO_2 fixation during Fe^{2+} oxidation was more efficient at pH 3.0 than at pH 4.0 is in agreement with the model for energy conservation during Fe^{2+} oxidation proposed by Ingledew et al. (10) in which the difference between the internal pH of the cell and the external pH of the growth medium drives the ADP phosphorylation and the setting up of an opposing $\Delta \Psi$.

Further observations supporting the direct oxidation of the sulphur component of pyrite include that biological Fe^{2+} oxidation was negligible at pH values above 5, at which FeS_2 oxidation is still appreciable, and that separation of bacteria from pyrite by means of a dialysis bag caused a pronounced drop of the oxidation rate of the mineral (Table 3).

The oxidation of the iron and sulphur moieties is influenced by the energy source on which the organism was grown. This is concluded from the results from Table 1 and Table 2.

Oxidation of Fe^{2+} and of the iron moiety of pyrite proceeds much more readily with decreasing pH. This is in contrast with the S^o oxidation which is hardly affected by pH (Figs 1A-C). Consequently the oxidation of the S^o component of FeS_2 is relatively more important with increasing pH. At a pH value above 4.5 the oxidation rate of FeS_2 exceeded that of Fe^{2+}. Owing to this effect, FeS_2 oxidation was found to be progressively inhibited by NEM with increasing pH (Fig. 2). Furthermore, higher amounts of Fe^{2+} were required for CO_2 fixation with increasing pH, whereas the amounts of S^o required did not alter with higher pH and those of pyrite only slightly increased.

It is not known whether the direct contact mechanism occurs in nature. During the formation of acid drainage from FeS_2 of coal mines the bacterium may be in direct contact with the mineral. However, in slowly acidifying acid sulphate soils there is a distinct separation between pyritic soil and the ferric precipitates near channels and cracks, through which oxygen is moving inwards. Oxygen is inactivated by dissolved Fe^{2+} before it can reach the pyrite. Diffusion of Fe^{3+} towards pyrite takes place especially at a pH lower than 4.0. This implies that ferric iron must be the main oxidant of pyrite in these soils (7).

The strain of *Thiobacillus ferrooxidans* used in this study had a low iron-oxidizing activity when grown on S^o; cytochrome b was not detectable in Fe^{2+}-grown cells; the rate of simultaneous Fe^{2+} and S^o oxidation was equal to the sum of the oxidation rates of the single substrates (unpublished results). This is in contrast with the results obtained with the strain isolated by

Leathen et al. (11). In this strain the iron oxidizing activity was the same in both Fe^{2+}- and S^{o}-grown cells (15); b-type cytochromes were detected in Fe^{2+}-grown cells (5, 6); the oxidation rate of Fe^{2+} together with S^{o} was lower than the oxidation rate of Fe^{2+} alone (15). The dissimilarity in these two strains with respect to both cytochrome contents and Fe^{2+} or S^{o} oxidizing capacity need not necessarily be explained by assuming qualitative differences. It more likely is a reflection of quantitative differences between the two bacteria, since our strain when grown on Fe^{2+} must have possessed cyt b for reversed electron transport. Furthermore, low activity of Fe^{2+} oxidation was observed when S^{o} served as energy source.

REFERENCES

1. Beck, J.V. 1960 A ferrous iron oxidizing bacterium; I. isolation and some general physiological characteristics. J. Bacteriol. *79*, 502-509.
2. Beck, J.V. and Brown, D.G. 1968 Direct sulfide oxidation in the solubilization of sulfide ores by *Thiobacillus ferrooxidans*. J. Bacteriol. *96*, 1433-1434.
3. Begheyn, L.Th. 1978 Determination of ferrous iron in rock soil and clay. In preparation.
4. Begheyn, L.Th., van Breemen, N. and Velthorst, E.J. 1978 Analysis of sulfur compounds in acid sulfate soils and other recent marine soils. Commun. Soil Sci. Plant Anal. *9*, 873-882.
5. Blaylock, B.A. and Nason, A. 1963 Electron transport systems of the chemoautotroph *Ferrobacillus ferrooxidans*. J. Biol. Chem. *238*, 3453-3462.
6. Bodo, C. and Lundgren, D.G. 1974 Iron oxidation by cell envelopes of *Thiobacillus ferrooxidans*. Can. J. Microbiol. *20*, 1647-1652.
7. Breemen, N. van 1976. Genesis and solution chemistry of acid sulfate soils in Thailand. Thesis Agricultural University, Wageningen.
8. Duncan, D.W., Landesman, J. and Walden, C.C. 1967 Role of *Thiobacillus ferrooxidans* in the oxidation of sulphide minerals. Can. J. Microbiol. *13*, 397-403.
9. Guay, R. and Silver, M. 1975 *Thiobacillus acidophilus* sp. nov.; isolation and some physiological characteristics. Can. J. Microbiol. *21*, 281-288.
10. Ingledew, W.J., Cox, J.C. and Halling, P.J. 1977 A proposed mechanism for energy conservation during Fe^{2+} oxidation by *Thiobacillus ferrooxidans*. Chemiosmotic coupling to net H^{+} influx. FEMS Microbiol. Lett. *2*, 193-197.
11. Leathen, W.W., Kinsel, N.A. and Braley, S.A. Sr. 1956 *Ferrobacillus ferrooxidans*: a chemosynthetic autotrophic bacterium. J. Bacteriol. *72*, 700-704.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 263-275.
13. Lundgren, D.G., Vestal, J.R. and Tabita, F.R. 1974 The iron oxidizing bacteria In Microbial iron metabolism. Ed. J.B. Neilands Academic Press N.Y., London. 457-473.
14. Mackintosh, M.E. 1978 Nitrogen fixation by *Thiobacillus ferrooxidans*. J. Gen. Microbiol. *105*, 215-218.
15. Margalith, P., Silver, M. and Lundgren, D.G. 1966 Sulfur oxidation by the iron bacterium *Ferrobacillus ferrooxidans*. J. Bacteriol. *92*(2), 1706-1709.

16. Peeters, T. and Aleem, M.I.H. 1970 Oxidation of sulfur compounds and electron transport in *Thiobacillus denitrificans*. Arch. Mikrobiol. *71*, 319-330.
17. Silverman, M.P. 1967 Mechanism of bacterial pyrite oxidation. J. Bacteriol. *94* (2), 1046-1051.
18. Silverman, M.P. and Lundgren, D.G. 1959 Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. J. Bacteriol. *77*, 642-647.
19. Takakuwa, S., Nishiwaki, T., Tominaga, N., Iwasaki, H. and Hosada, K. 1977 Promoting effect of molybdate on the growth of a sulfur oxidizing bacterium, *Thiobacillus thiooxidans*. J. Gen. Appl. Microbiol. *23*, 163-173.
20. Tuovinen, O.H., Kelly, D.P., Dow, C.S. and Eccleston, M. 1978 Metabolic transitions in cultures of acidophilic thiobacilli. In Metallurgical applications of bacterial leaching and related microbiological phenomena. Eds. L.E. Murr, A.E. Torma, J.A. Brierley. Academic press, New York. 61-81.
21. Zavarzin, G. 1972 A heterotrophic satellite of *Thiobacillus ferrooxidans*. Mikrobiologiya *41*, 369-370.

4. *THIOBACILLUS ACIDOPHILUS*: A STUDY OF ITS PRESENCE IN *THIOBACILLUS FERROOXIDANS* CULTURES

ABSTRACT

A study has been undertaken to account for the presence of *Thiobacillus acidophilus* in iron-grown cultures of *Thiobacillus ferrooxidans*. Attempts to adapt *Thiobacillus acidophilus* to ferrous iron were not successful but the mixotroph grew to a limited extent in the spent medium of *Thiobacillus ferrooxidans* and was also able to grow oligocarbophilically. Possible oligocarbophilic substrates were methanol, ethanol and hydrogen sulphide. *Thiobacillus ferrooxidans* may benefit from the presence of *Thiobacillus acidophilus* because in mixed cultures some inhibiting organic compounds such as alcohols, organic acids and amino acids were utilized by *Thiobacillus acidophilus*. The number of *Thiobacillus acidophilus* cells in heterogeneous cultures with *Thiobacillus ferrooxidans* was of the same order of magnitude as the number of *Thiobacillus ferrooxidans* cells as revealed by fluorescent-labelled antibodies.

4.1 INTRODUCTION

The ability of *Thiobacillus ferrooxidans* to use glucose as the sole carbon and energy source was reported by Gale and Beck (5), Shafia and Wilkinson (11) and Tabita and Lundgren (14), but more recent work has cast serious doubt on this ability, contaminating organisms being suspected. Margalith *et al.* (9) first suggested that cultures of *Thiobacillus ferrooxidans*, grown on either ferrous iron or on elemental sulphur as energy source, were heterogeneous. A heterotrophic satellite of *Thiobacillus ferrooxidans* was first isolated by Zavarzin (18). This satellite utilized glucose but also required yeast extract for growth. Although the organism needed a high concentration of ferrous iron for its development, it did not oxidize the iron. A different organism was obtained by Guay and Silver (6) who isolated *Thiobacillus acidophilus* from an iron-grown culture of *Thiobacillus ferrooxidans*. *Thiobacillus acidophilus* utilized organic compounds for growth and was also able to grow autotrophically on elemental sulphur (S^o). Like the heterotrophic organism of Zavarzin, it did not oxidize ferrous iron although Tuovinen *et al.* (15) in a later report, based on growth studies, claimed autotrophic growth of *Thiobacillus acidophilus* on ferrous iron.

We aslo investigated this subject because during studies on pyrite oxidation by *Thiobacillus ferrooxidans* the presence of *Thiobacillus acidophilus* was invariably observed (see Chapter 3). Results of a study on factors that are of significance in understanding the association between *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus* are given in this paper.

4.2 MATERIALS AND METHODS

4.2.1 *Cultures and media*

Thiobacillus ferrooxidans strain ATCC 13598, isolated by Beck (3) and *Thiobacillus acidophilus* strain DSM 700, isolated by Guay and Silver (6) were used throughout this study. The bacteria were maintained on 9K Fe^{2+} (*Thiobacillus ferrooxidans*) and 9K glucose (*Thiobacillus acidophilus*) media (13). The basal 9K medium was composed of (per litre): $(NH_4)_2SO_4$, 3 g; KCl, 0.1 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $Ca(NO_3)_2$, 0.01 g; 10 N H_2SO_4, 1.0 ml, and 1.0 ml of a trace element solution consisting of (per litre): $ZnSO_4.7H_2O$, 0.3 g; $MnSO_4.4H_2O$, 2.2 g; $CuSO_4.5H_2O$, 0.3 g; $CoCl_2.6H_2O$, 0.2 g; H_3BO_3, 2.0 g, and $Na_2MoO_4.2H_2O$, 0.75 g. Cells were grown at 30°C while being shaken in Erlenmeyer flasks. Some cultures were grown in a Biotec fermentor of 3.2 l with a working volume of 1.5 l at a constant pH of 3.5 and a temperature of 30°C. The medium was composed of two parts as described earlier (see 3.2.2). Fe^{2+} and glucose were sterilized apart either by autoclaving or by filtration through a 0.2 µm membrane filter. FeS_2 was sterilized in the dry state under an argon atmosphere at 160°C for 2 h. S^o was sterilized under a layer of 96% ethanol. After about 16 h the alcohol was evaporated and the sulphur added to the liquid salts medium.

Cells were counted on 9K agar plates with 0.5% glucose and 0.01% yeast extract. Direct counts were performed in a Bürker-Turk counting chamber.

4.2.2 *Incorporation of* $^{14}CO_2$

Sterile Erlenmeyer flasks of 100 ml were inoculated with 3 ml of either a pure culture of *Thiobacillus ferrooxidans* or a mixed culture of *Thiobacillus ferrooxidans* together with *Thiobacillus acidophilus* in 9K medium. Both pyrite and ferrous sulphate were used as energy sources. After inoculation, the flasks were stoppered with sterile Suba-seals. $NaH^{14}CO_3$ (0.3 M 10 µCi/ml) was sterilized through a 0.2 µm membrane filter and 0.1 ml of the solution was injected into each culture. Assimilation of $^{14}CO_2$ was tested by periodically analysing a pure

as well as a mixed culture for radio-activity. The flasks were flushed with non-radioactive CO_2 for 45 min to remove excess $^{14}CO_2$, whereafter the cultures were filtered through a 0.2 µm membrane filter. One ml of the filtrate was supplied with 10 ml of Instafluor/Titron-X-100 (2:1 v/v) and the radio-activity measured in a Nuclear Chicago scintillation counter type Mark 1. The filter containing the bacteria was washed three times with distilled water, transferred to 5 ml 0.05 M NaOH and boiled for 5 min to dissolve the cells. After chilling, when ferric iron or pyrite had precipiated, 1 ml of the sample was treated as described for the filtrate.

4.2.3 *Preparation of spent medium*

The pH of 6-days-old cultures of *Thiobacillus ferrooxidans* grown on ferrous iron was adjusted to 3.0 with solid $NaHCO_3$. After centrifuging to remove ferric precipitates and cells, the supernatant was sterilized through a 0.2 µm membrane filter and used as growth medium for *Thiobacillus acidophilus*. Spent media of both pure cultures of *Thiobacillus ferrooxidans* and mixed cultures of *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus* were also used for the total organic carbon content (TOC). After adding one ml of 2 N HCL to 10 ml of the spent medium, the samples were boiled under vacuum to remove inorganic carbon. The samples were then analysed with a Beckman total organic-carbon analyser.

4.2.4 *Immunological methods*

Antiserum against *Thiobacillus acidophilus* was made according to the procedure of Apel *et al.* (1). Blood (25 ml) was obtained from the ear of a rabbit 7 days after the last injection with bacterial suspension. The titers of the antisera were between 1:512 and 1:1024. The antiserum was cross-adsorbed with *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* whereafter it was diluted with physiological salt solution to a protein content of about 1%. Both 4 ml 0.1 M Na-phosphate buffer pH 7.0 and 4 ml 0.1 M Na-phosphate buffer pH 8.0 containing 5 mg fluorescein isothiocyanate were added to 10 ml of the diluted sera. Merthiolate (10 mg/l) was added to prevent bacteriological growth. Unreacted dye was removed by dialysis against physiological salt solution after 24 h incubation at 4°C. The fluorescent antiserum thus obtained was divided into portions of 1 ml and stored at -20°C until needed. Thoroughly washed cell suspensions of pure cultures of *Thiobacillus ferooxidans*, *Thiobacillus thio-oxidans* and *Thiobacillus acidophilus* and heterogeneous cultures of *Thiobacillus*

ferrooxidans and *Thiobacillus acidophilus* were stained according to the procedure of Walker *et al.* (17). The fluorescent-labelled cells were examined with a Leitz dialux microscope equipped for Ultraviolet epiilumination.

4.2.5 *Chemical analysis*

Ferric and ferrous iron were determined colorimetrically with 1,10-phenanthroline.HCl. Glucose was estimated with anthrone. Thiosulphate was assayed by by iodometric titration in 5% acetic acid using starch as an indicator. Protein was determined by the procedure of Lowry *et al.* (8). Sulphate was determined according to the procedure of Bergheyn *et al.* (4). Amino acids were analysed in concentrated (400 times) spent medium of both iron-grown pure cultures of *Thiobacillus ferrooxidans* and iron-grown mixed cultures of *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus*. The concentrated samples were desalted on Dowex ion exchanger and analysed on an amino-acid analyser.

4.3 RESULTS

4.3.1 *Isolation of Thiobacillus acidophilus from different sources*

Thiobacillus acidophilus-type bacteria were isolated from each of the three available cultures of *Thiobacillus ferrooxidans* according to the procedure of Guay and Silver (6) and designated *Thiobacillus acidophilus* strains A, D and G. Morphologically these strains resembled *Thiobacillus acidophilus* isolated by the above-mentioned authors. It was observed that ferrous iron had an inhibiting effect on the mixotrophs. When $FeSO_4.7H_2O$ (18 mM) was added together with glucose (27.8 mM) in the last isolation step, according to the procedure of Guay and Silver (6), no growth was observed. The three organisms could also grow autotrophically when S^0 served as energy source. Attempts to isolate *Thiobacillus acidophilus*-type bacteria from other acidophilic thiobacilli *viz.*: *Thiobacillus thiooxidans* strain DSM 504, strain DSM 612, strain DSM 622 and our own isolated strain of *Thiobacillus thiooxidans* were not successful.

4.3.2 *Efforts to grow Thiobacillus acidophilus with ferrous iron as energy source*

Since the *Thiobacillus acidophilus* strains were isolated from cultures of *Thiobacillus ferrooxidans* grown on ferrous iron that had been subcultured repeatedly in a medium containing Fe^{2+} as the sole energy source, it was

difficult to understand why the strain of Guay and Silver (6) and the newly isolated organisms failed to oxidize ferrous iron and to utilize the oxidation energy for growth. Therefore we tried to adapt *Thiobacillus acidophilus* (the strain of Guay and Silver (6)) to ferrous iron via four serial transfers to media with increasing amounts of ferrous iron. For that reason medium 9K glucose was supplemented with increasing amounts of $FeSO_4.7H_2O$ from 0.36 via 1.2 and 3.6 to 18 mM and with concomitant decreasing amounts of glucose from 5.6 via 0.56 and 0.056 mM to 0 and yeast extract from 0.01 via 0.005% to 0.00. The bacteria failed to grow after the last transfer. Therefore we tried to adapt the organism to ferrous iron in a different way.

Thiobacillus acidophilus was grown in a glucose-limited chemostat at an initial dilution rate of 0.05 h^{-1}. Advantages of using a chemostat include: (i) the concentration of glucose in the culture is low and consequently repression of a ferrous iron-oxidizing system or enzymes of the Calvin cycle by glucose is unlikely; (ii) adaptation to ferrous iron can start with high numbers of active cells. Fe^{2+} at an inital concentration of 0.66 mM was pumped into the culture vessel after 10 dilutions with glucose and the dilution rate was then fixed at 0.02 h^{-1}. After every 5 dilutions the ferrous iron concentration was increased stepwise to a final concentration of 66 mM whereas the glucose concentration of the medium was decreased to zero. *Thiobacillus acidophilus* did not adapt to Fe^{2+} under these circumstances as is shown in Table 1. Increasing ferrous iron concentrations resulted in a washout of the culture.

Table 1. Attempt to adapt *T. acidophilus* to ferrous iron in a chemostat

Numbers of dilutions	D. (h^{-1})	S_r (mM)		X (g/l)	S (mM)	
		Glucose	Fe^{2+}		Glucose	Fe^{2+}
10	0.05	55.6	0.00	1.7	0.3	0.00
5	0.02	55.6	0.66	1.7	0.2	0.62
5	0.02	27.8	6.6	0.8	0.2	6.3
5	0.02	27.8	66	0.02	27.8	59.7
5	0.02	0	66	0.00	0	58.3

S_r represents the concentration of substrate in the medium reservoir. S denotes its concentration in the culture vessel. The symbol X represents the cell yield determined as protein

In a similar experiment with thiosulphate instead of Fe^{2+}, similar results were obtained. Thiosulphate did not serve as energy source although high concentrations of the compound were not inhibiting unlike high concentrations of Fe^{2+}.

In another experiment we investigated whether *Thiobacillus acidophilus* needs a growth factor excreted by *Thiobacillus ferrooxidans* for growth on ferrous iron. Spent medium of the latter organism was supplemented with $FeSO_4.7H_2O$ to final concentrations of 10 and 36 mM. The organism was also inoculated into spent and fresh 9K medium without additional ferrous iron and fresh 9K medium supplemented with Fe^{2+} to a final concentration of 10 and 36 mM and into 9K glucose medium. Cells in these media were counted at different time intervals on 9K glucose agar plates and directly in a counting chamber if possible. The results of the experiment as shown in Table 2 indicate that under these circumstances *Thiobacillus acidophilus* cannot oxidize ferrous iron and use the oxidation energy for growth. Cells from spent 9K media with 36 mM Fe^{2+} did not grow readily on 9K glucose yeast extract agar, although the numbers of cells counted in a counting chamber did not differ much from the numbers in the spent media without supplementary ferrous iron. It seems that ferrous iron did not inhibit growth on the spent media but adversely influenced the ability to grow on glucose. It is unlikely that the failure to grow *Thiobacillus acidophilus* on ferrous iron during the experiment described above was due to the inability of the organism to induce the enzymes of the Calvin cycle. This was demonstrated by growing the bacteria autotrophically on 9K S^o medium with and without additional ferrous iron. Good growth and quantitative oxidation of S^o was observed in both media but Fe^{2+} oxidation did not occur.

4.3.3 *Examination of spent media of both a pure culture of Thiobacillus ferrooxidans and a mixed culture of Thiobacillus ferrooxidans and Thiobacillus acidophilus*

Thiobacillus acidophilus is able to grow on the spent medium of *Thiobacillus ferrooxidans*, as can be concluded from the data of Table 2. Therefore we investigated whether the spent medium of a pure culture of *Thiobacillus ferrooxidans* contained more organic material than the spent medium of a mixed culture of the iron oxidizer with *Thiobacillus acidophilus*. Batch cultures of both the pure and the mixed culture were grown on 9K medium with ferrous iron as energy source; the total organic carbon content of the spent media was determined at various times. No significant differences in organic carbon content were observed (Table 3).

Table 2. Numbers[a] of cells of *T. acidophilus* grown on 9K basal medium with various additions.

Medium	Time (days)				
	0	5	7	12	27
9K fresh	23.10^3	30.10^3	21.10^3	43.10^4	91.10^5
9K fresh + 10 mM Fe^{2+}	24.10^3	10.10^3	61.10^3	51.10^4	41.10^5
9K fresh + 36 mM Fe^{2+}	14.10^3	26.10^3	10.10^3	11.10^3	16.10^2
9K spent	14.10^3	22.10^3	77.10^4	37.10^6 (9.10^7)	18.10^6 (11.10^7)
9K spent + 10 mM Fe^{2+}	27.10^3	27.10^3	83.10^3	63.10^3 (8.10^7)	52.10^6 (7.10^7)
9K spent + 36 mM Fe^{2+}	14.10^3	30.10^3	27.10^4	18.10^4 (7.10^7)	13.10^3 (6.10^7)
9K glucose	14.10^3	27.10^7	77.10^9(8.10^{10})	32.10^{10}(5.10^{12})	13.10^{10}) 4.10^{12})

[a]Numbers of cells per ml determined on 9K glucose yeast extract agar plates. In parantheses the numbers of cells per ml obtained by direct counting in a Bürker-Turk counting chmaber are given.

Table 3. Total organic carbon[a] in spent media of pure cultures of *T. ferrooxidans* and of mixed cultures of *T. ferrooxidans* and *T. acidophilus* grown on ferrous iron.

Time (days)	*T. ferrooxidans*	*T. ferrooxidans* and *T. acidophilus*
3	3.9	7.9
6	3.0	5.9
8	4.2	4.3
10	5.9	4.9
13	5.7	4.3

[a]Expressed as mg/l

Mixed and pure cultures were also grown in ferrous-iron-limited continuous cultures at a dilution rate of 0.02 h^{-1}. After every 5 dilutions the concentration of ferrous iron was increased in 4 consecutive steps from 0.9 via 1.8 and 3.6 to 8.9 mM. Samples of 25 ml were periodically taken from the different cultures and treated and analysed as described in "Material and Methods". In each sample both cultures were checked for the presence of *Thiobacillus acidophilus*. The organism was always present in the mixed cultures but did not occur in the cultures of *Thiobacillus ferrooxidans*. The total organic carbon content of the spent-liquid samples was between 1 and 8 mg/l (Tables 3 and 4). The total assimilated CO_2 of both continuous cultures was estimated by determining

Table 4. Total organic carbon[a] in spent media and the bacteria-containing culture liquid of *T. ferrooxidans* and mixed cultures of *T. ferrooxidans* and *T. acidophilus* grown in continuous cultures at various iron concentrations.

Fe^{2+} (g/l)	*T. ferrooxidans*		*T. ferrooxidans* and *T. acidophilus*	
	Spent media	Culture liquids	Spent media	Culture liquids
2.5	5.8	7.2	3.2	8.3
5.0	5.7	10.3	6,4	8.8
10.0	3.5	11.9	2.5	11.5
25.0	4.8	19.5	4.0	19.8

[a]Expressed as mg/l

the total organic carbon of the culture liquids. No significant differences were observed between the two cultures with regard to both the total organic carbon content of the spent medium and the total assimilated CO_2 at the different iron concentrations (Table 4). Progressive increase of the ferrous iron concentration failed to increase the final cell yield proportionally. This phenomenon was first observed by Silverman and Lundgren (13) and may be due to the inhibiting

effect of high concentrations of ferric iron (7).

Excretion of organic compounds by *Thiobacillus ferrooxidans* was further studied in batch culture experiments with $FeSO_4.7H_2O$ and FeS_2 as energy sources and using assimilation of $^{14}CO_2$ as measure for growth and excretion of organic compounds (see 'Materials and Mehods"). Also in this experiment, no differences as to both cell yield and total amount of excreted carbon between the mixed culture and the pure culture were observed (Figs. 1A and B). About 10% of the CO_2 fixed was found in the spent medium of both the ferrous sulphate and FeS_2

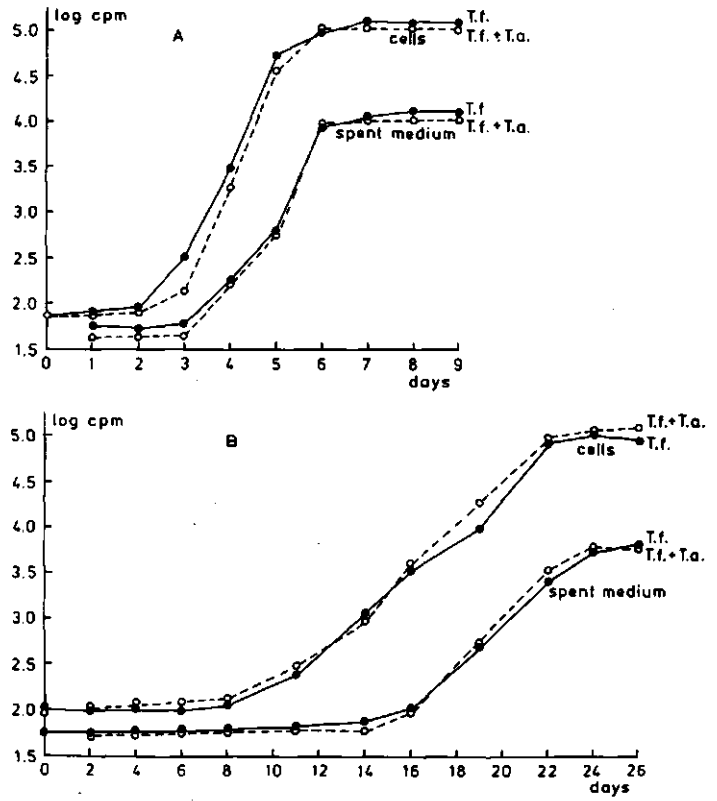

Fig. 1. $^{14}CO_2$ fixation by growing pure cultures of *T. ferrooxidans* (T.f.) and mixed cultures of *T. ferrooxidans* and *T. acidophilus* (T.f. + T.A.) with: A, Fe^{2+} and B, FeS_2 as energy source. Spent medium contains the excreted organic material. o———o, pure cultures of *T. ferrooxidans*; o- - - - -o, mixed cultures of *T. ferrooxidans* and *T. acidophilus* .

grown cells when the stationary phase was reached. From the results shown in Fig. 1 it can also be concluded that a mixed culture of *Thiobacillus acidophilus* and *Thiobacillus ferrooxidans* did not oxidize ferrous iron faster than a pure culture of the iron-oxidizing autotroph.

Although no quantitative differences in carbon content of the spent media between the mixed and the pure cultures were observed, qualitative differences in composition of the excreted products might exist. Therefore, spent media of mixed and pure cultures both grown on ferrous iron were concentrated from 400 ml to 1 ml and the samples analysed for amino acids. From the results obtained (Table 5) it can be seen that the different amino acids occurred in the four

Table 5. Amino acid composition[a] of the spent media of a pure culture of *T. ferrooxidans* and a mixed culture of *T. ferrooxidans* and *T. acidophilus*.

Amino acid	*T. ferrooxidans*		*T. ferrooxidans* + *T. acidophilus*	
	1[b]	2[b]	1[b]	2[b]
Glycine	43	31	34	40
Alanine	12	14	13	19
Serine	20	26	28	21
Threonine	2	4	4	5
Leucine	4	6	6	5
Isoleucine	3	4	4	3
Aspartic acid	4	6	8	5
Glutamic acid	12	9	3	2

[a] Figures denote separate amino acids as % of total amino acids (wt/wt)
[b] 1 and 2 represent the results of different cultures

samples in almost equal proportions with the exception of glutamic acid which was found in clearly higher amounts in the spent medium of *T. ferrooxidans* as compared to that of the mixotroph. Of the relatively few amino acids occurring in detectable amounts in the spent media, glycine and serine were the highest, followed by alanine and glutamic acid. However, total amino acid content formed only about 2% of the total organic matter excreted into the spent medium.

4.3.4 *Oligocarbophilic growth of Thiobacillus acidophilus*

Initial support for oligocarbophilic growth was obtained from the results presented in Table 2, because about 10^7 cells ml were counted in the 9K basal salts medium.

To test oligocarbophilic growth, the four available *Thiobacillus acidophilus* cultures were inoculated into flasks containing 9K basal medium without energy

source. Half the number of the flasks were incubated in a 30°C incubation room, the others were placed in different dessicators to prevent exhange of the air of the incubation room with the air in the flasks. The des ccators contained a gas mixture of 20% O_2, 5% CO_2 and 75% N_2. The oxygen content was controlled periodically and was never below 19%. *Thiobacillus acidophilus* was also inoculated into spent medium of *Thiobacillus ferrooxidans* cultures and incubated in the desiccators to exclude oligocarbophilic growth. At different time intervals one desiccator was opened and cells were counted on 9K glucose agar plates and in a Bürker-Turk counting chamber. The results of this experiment (Table 6) clearly demonstrate that growth in the spent medium was limited to a maximum number of 10^6 cells/ml but oligocarbophilic growth had a much more substantial impact.

Some volatile substrates were tested for their capacity to support growth of *Thiobacillus acidophilus*. It was found that the organisn used Na_2S, methanol, and ethanol as substrates as well as a number of non-volatile compounds *viz.* glucose, malate, arginine, aspartate, asparagine and glutamate. No growth occurred with propanol, formaldehyde, acetaldehyde, acetone, formate, acetate, glycolate, lactate, fumarate, succinate, glyoxylate, pyruvate, α-ketoglutarate, alanine, serine, threonine, valine, leucine, isoleucine, lysine, ammonia and hydrogen.

4.3.5 *Effect of Thiobacillus acidophilus on the growth of Thiobacillus ferrooxidans in ferrous-iron containing medium supplied with organic compounds*

The presence of *Thiobacillus acidophilus* as a scavenger of organic material in *Thiobacillus ferrooxidans* cultures may be significant for the obligate autotroph, because this organism is strongly inhibited by organic compounds like α-keto acids (16). Inhibition by glucose of iron oxidation has also been observed (12) and this phenomenon was further investigated in the present study. Four flasks containing 9K medium with Fe^{2+} (30 mM) were inoculated with a pure culture of *Thiobacillus ferrooxidans* and another four flasks containing the same media were inoculated with a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus*. Glucose (39 mM) was aseptically added to various flasks at different time intervals. The results of this experiment given in Fig. 2 show that addition of glucose after the lag phase of *Thiobacillus ferrooxidans* did not have any effect upon the growth of the iron oxidizer. Glucose added at the start of the experiment inhibted growth of both the pure and the mixed cultures. It was observed earlier that ferrous iron inhibits growth on glucose of *Thiobacillus acidophilus*. The present experiment reveals that Fe^{2+} also inhibits the growth of the mixotroph. Lowering the ferrous iron concentration to 10 mM

Table 6. Numbers[a] of cells of *T. acidophilus* grown oligocarbophilically or on spent medium of *T. ferrooxidans*

Medium	*T. acidophilus* strain	Time (days)			
		0	13	27	48
Fresh 9K	DSM 700	10^2	3.10^3	3.10^5	5.10^7 (10^8)
	A	10^3	2.10^3	7.10^5	7.10^7 (5.10^8)
	D	2.10^2	1.10^3	6.10^5	9.10^7 (3.10^8)
	G	3.10^2	5.10^4	1.10^6	1.10^6 (1.10^7)
Fresh 9K dessicator	DSM 700	10^2	4.10^3	10^3	10^3
	A	10^3	3.10^3	10^3	10^3
	D	2.10^2	10^3	10^3	10^3
	G	3.10^2	4.10^3	10^3	10^3
Spent 9K desiccator	DSM 700	10^2	3.10^4	2.10^5	9.10^5
	A	10^3	1.10^5	3.10^5	4.10^5
	D	2.10^2	1.10^5	7.10^5	3.10^5
	G	3.10^2	4.10^5	7.10^5	3.10^5

[a]Figures represent numbers of cells/ml as counted via 9K glucose yeast extract agar plates. In parantheses numbers of cells/ml as counted in a Bürker-Turk counting chamber.

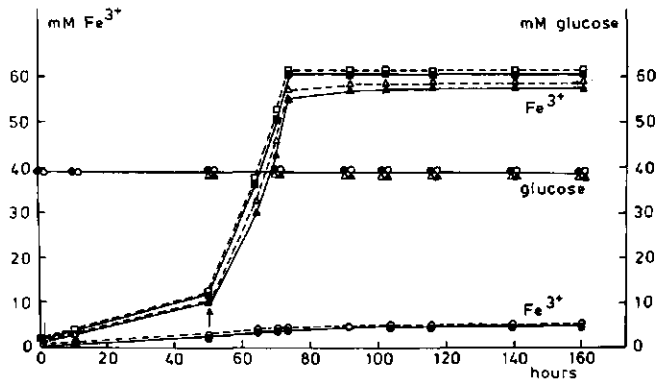

Fig. 2. Growth of pure cultures of *T. ferrooxidans* (closed symbols) and of mixed cultures of *T. ferrooxidans* and *T. acidophilus* (open symbols) as affected by the addition of glucose (39 mM) at different time intervals. □■ , no glucose added; o●, glucose added at the start of the experiment; Δ▲, glucose added after 50 h.

resulted in good growth of *Thiobacillus acidophilus* in the mixed culture whereafter *Thiobacillus ferrooxidans* started to grown on ferrous iron after a lag phase of 5 days when most of the glucose had been utilized by *Thiobacillus acidophilus*. The glucose concentration used in the above-mentioned experiment (Fig. 2) was rather high. Inhibition of growth of *Thiobacillus ferrooxidans* by several other organic compounds was observed at concentrations of 1-10 mM (16). Therefore lower concentrations of various compounds were used in experiments with *Thiobacillus ferrooxidans* and *Thiobacillus ferrooxidans* + *Thiobacillus acidophilus*.

Pure cultures of *Thiobacillus ferrooxidans* and mixed cultures of *Thiobacillus ferrooxidans* and *Thiobacillus acidophilus* were inoculated into 9K medium with Fe^{2+} (30 mM) and supplemented with various organic compounds (1 mM). The contents of the flasks were examined daily for iron oxidation. Alanine and glycine did not inhibit *Thiobacillus ferrooxidans* but glyoxylate, pyruvate and α-ketoglutarate inhibited iron oxidation by the pure culture and the mixed culture. Iron oxidation by *Thiobacillus ferrooxidans* was no longer inhibited by other organic compounds tested when *Thiobacillus acidophilus* was present (Table 7).

Table 7. Inhibition of the growth of *T. ferrooxidans* cultures by different organic compounds, and the effect of *T. acidophilus* upon this inhibition.

Compound added	Growth of the pure culture of *T. ferrooxidans*	Growth of the mixed culture
-	+ (2)	+ (2)
Glucose	-	+ (4)
Methanol	-	+ (6)
Ethanol	-	+ (5)
Glycolate	-	+ (6)
Lactate	-	+ (5)
Fumarate	-	+ (7)
Succinate	-	+ (8)
Malate	-	+ (4)
Glyoxylate	-	-
Pyruvate	-	-
α-Ketoglutarae	-	-
Glycine	+ (4)	+ (3)
Alanine	+ (3)	+ (3)
Serine	-	+ (7)
Threonine	-	+ (9)
Leucine	-	+ (9)
Aspartate	-	+ (4)
Asparagine	-	+ (4)
Glutamate	+ (9)	+ (4)
Lysine	-	+ (4)
Arginine	-	+ (4)

The organisms were grown in 9K medium containing Fe^{2+} (30 mM); the organic compounds were present at concentrations of 1 mM. + denotes growth, - no growth within 15 days. In parentheses: numbers of days after which growth observed.

4.3.6 *Detection of Thiobacillus acidophilus in mixed cultures with fluorescent-labelled antiserum*

A fluorescent antibody stain was obtained as described in "Materials and Methods". Different thiobacilli were tested with this stain. Cells of the four available strains of *Thiobacillus ferrooxidans* grown on either S^0 or glucose were the only organisms that were stained. *Thiobacillus ferrooxidans* grown on Fe^{2+} or S^0 and *Thiobacillus thiooxidans* grown on S^0 were not fluorescent.

This technique has been used to examine the presence of *Thiobacillus acidophilus* in heterogeneous cultures with *Thiobacillus ferrooxidans*. Nine mixed cultures were examined in duplicate. The results shown in Table 8 indicate that about 30% of the cells of the mixed cultures was fluorescent.

Table 8. Percentage of fluorescent cells[a] in mixed cultures of *T. ferrooxidans* and *T. acidophilus*.

Culture type	Substrate	Time (days)	% fluorescent	
			1[b]	2[b]
Batch culture	FeS_2	9	27	36
	FeS_2	21	31	33
	FeS_2	23	26	25
	Fe^{2+}	3	32	38
	Fe^{2+}	5	28	31
Continuous culture	Fe^{2+} (9 mM)		27	33
	Fe^{2+} (18 mM)		35	29
	Fe^{2+} (36 mM)		29	35
	Fe^{2+} (90 mM)		26	37

[a]Cells were stained with fluorescent-labelled *T. acidophilus* antiserum
[b]Duplicate values

4.4 DISCUSSION

Thiobacillus acidophilus was first isolated by Guay and Silver (6) from cultures of *Thiobacillus ferrooxidans* grown on ferrous iron and we also were able to isolate the organism from such a culture. The most straight-forward explanation of the presence of *Thiobacillus acidophilus* in *Thiobacillus ferrooxidans* cultures would be that the organism is also able to oxidize ferrous iron and to use the released energy for growth. However, the results of the experiments described in this paper clearly demonstrate that *Thiobacillus acidophilus* does not possess the capacity to oxidize ferrous iron which confirms the earlier observations of Guay and Silver (6). Tuovinen *et al.* (15), however, were able to adapt *Thiobacillus acidophilus* to growth on both ferrous iron and thiosulphate. However, an analysis of the DNA-base composition showed that the G+C ratio of the ferrous iron-adapted organism was the same as that of *Thiobacillus ferrooxidans*. Those authors suggested that *Thiobacillus acidophilus* would be a heterogeneous culture but no evidence of such a suggestion was obtained in the present investigation.

Ferrous iron did not inhibit autotrophic growth of *Thiobacillus acidophilus* on elemental sulphur but it prevented growth on glucose. In the continuous-culture experiment with glucose as the limiting substrate, a shift from

6.6 to 66 mM Fe^{2+} resulted in a washout (Table 1). When $FeSO_4.7H_2O$ (18 mM) was included in the medium containing glucose (27.8 mM) no growth was observed in batch cultures.

However, *Thiobacillus acidophilus* is not inhibited by ferrous iron, when organic compounds are present in low concentrations, since it grew readily in spent medium of *Thiobacillus ferrooxidans* or in mixed cultures together with this organism (Tables 2 and 6). On the other hand, cells grown in spent 9K medium at 36 mM ferrous iron did not grow readily after transfer to 9K glucose yeast extract agar (Table 2). Counting of *Thiobacillus acidophilus* on plates with glucose yeast extract agar likewise was not possible when the organism had been growing with *Thiobacillus ferrooxidans* in the presence of iron. The need to subculture *Thiobacillus acidophilus* in four consecutive steps, with gradually increasing concentration of glucose and decreasing concentration of ferrous iron, to purify the organism from *Thiobacillus ferrooxidans* cultures may be well a reflection of its inability to grow on media with high concentrations of organic material after it has been growing in the presence of Fe^{2+}. In the present work we have not investigated whether *Thiobacillus acidophilus* requires the presence of high concentrations of Fe^{2+} for growth on glucose after transferring it from Fe^{2+} containing media as it was observed by Zavarzin (18) for the heterotrophic satellite isolated from a *Thiobacillus ferrooxidans* culture.

Since *Thiobacillus acidophilus* does not grow on ferrous iron, there must be another explanation of its presence in heterogeneous cultures of *Thiobacillus ferrooxidans*.

The mixotroph is able to grow in spent media of *Thiobacillus ferrooxidans* cultures although only to a limited limited extent (Table 6). Most of the organic material excreted by *Thiobacillus ferrooxidans* can not be utilized by *Thiobacillus acidophilus* as is illustrated by the small qualitative and quantitative differences in organic carbon compounds between spent medium of a pure culture of *Thiobacillus ferrooxidans* and that of a mixed culture of the two thiobacilli (Tables 3, 4, 5; Fig. 1).

Another parameter in explaining the presence of *Thiobacillus acidophilus* in *Thiobacillus ferrooxidans* cultures is oligocarbophilic growth. Bacterial numbers were substantial when the four strains of *Thiobacillus acidophilus* were allowed to develop in the mineral 9K medium with free exchange of the atmosphere above the cultures with the laboratory atmosphere. After 48 days of incubation, cultures with 10^8 cells/ml had grown without any added carbon

or energy source in the medium (Table 6).

Possible substrates supporting this growth include methanol, ethanol and H_2S. In contrast to Guay and Silver's results (6), growth was obtained with sulphide as energy source.

The fact that the presence of *Thiobacillus acidophilus* prevents inhibition of *Thiobacillus ferrooxidans* by several organic compounds may further account for the close association between the two organisms. However, the protection of *Thiobacillus ferrooxidans* by *Thiobacillus acidophilus* apparently does not apply for the most toxic organic compounds *viz.* α-keto acids.

Several organic compounds which did not support growth of *Thiobacillus acidophilus* did not inhibit the growth of *Thiobacillus ferrooxidans* in the mixed culture of both organisms. It is not known yet what accounts for this phenomenon.

Besides growth on spent media, oligocarbophilic growth, and removal of excreted autotoxic organic compounds, there might be some other factors that account for the presence of the mixotroph in iron-oxidizing cultures because these factors do not explain why until now *Thiobacillus acidophilus* has exclusively been found in iron-oxidizing cultures. The numbers of cells of *Thiobacillus acidophilus* present in heterogeneous *Thiobacillus ferrooxidans* cultures were found to be comparable with the numbers of cells of the iron oxidiser itself (Table 8). This finding is corroborated by the fact that the former organism was isolated from cultures which were repeatedly subcultures in ferrous-iron media by tenfold dilutions. Since the cell yields of mixed and pure cultures did not differ significantly (Table 4; Fig. 1). The mixed culture contained fewer *Thiobacillus ferrooxidans* cells than the pure culture. The only explanation of this phenomenon might be the stimulated excretion by *Thiobacillus ferrooxidans* of organic material in the presence of *Thiobacillus acidophilus*. Without the mixotroph this organic material would have supported growth of *Thiobacillus ferrooxidans* itself. Further evidence to confirm this hypothesis is necessary.

REFERENCES

1. Apel, W.A., Dugan, P.R., Filppi, J.A. and Rheins, M.S. 1976 Detection of *Thiobacillus ferrooxidans* in acid mine environments by indirect fluorescent antibody staining. Appl. Envrinm. Microbiol. *32*, 159-165.
2. Arkesteyn, G.J.M.W. 1979 Pyrite oxidation by *Thiobacillus ferrooxidans* with special reference to the sulphur moiety of the mineral. Antonie v. Leeuwenhoek. J. Microbiol. *45*, 423-435.
3. Beck, J.V. 1960 A ferrous iron oxidizing bacterium. I. isolation and some general physiological characteristics. J. Bacteriol. *79*, 502-509.
4. Begheyn, L.T., van Breemen, N. and Velthorst, E.J. 1978 Analysis of sulfur compounds in acid sulfate soils and other recent marine soils. Commun. Soil Sci. Plant Anal. *9*, 873-882.
5. Gale, N.L. and Beck, J.V. 1967 Evidence for the Calvin cycle and hexose mono phosphate pathway in *Thiobacillus ferrooxidans*. J. Bacteriol. *94*, 1052-1059.
6. Guay, R. and Silver, M. 1975 *Thiobacillus acidophilus* sp.nov.; isolation and some physiological characteristics. Can. J. Microbiol. *21*, 281-288.
7. Kelly, D.P., Eccleston, M. and Jones, C.A. 1977 Evaluation of continuous chemostat cultivation of *Thiobacillus ferrooxidans* on ferrous iron or tetrathionate. In Conference bacterial leaching. Ed. W. Schartz. Verlag Chemie, Weinheim-New York 1-7.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 263-275.
9. Margalith, P., Silver, M. and Lundgren, D.G. 1966 Sulfur oxidation by the iron bacterium *Ferrobacillus ferrooxidans*. J. Bacteriol. *92*, 1706-1709.
10. Shafia, F., Brinson, K.R., Heinzman, M.W. and Brady, J.M. 1972 Transition of chemolithotroph *Ferrobacillus ferrooxidans* to obligate organotrophy and metabolic capabilities of glucose grown cells. J. Bacteriol. *111*, 56-65.
11. Shafia, F. and Wilkinson, R.F. Jr. 1969 Growth of *Ferrobacillus ferrooxidans* on organic matter. J. Bacteriol. *97*, 256-260.
12. Silver, M., Margalith, P. and Lundgren, D.G. 1967 Effect of glucose on carbon dioxide assimilation and substrate oxidation by *Ferrobacillus ferrooxidans*. J. Bacteriol. *93*, 1765-1769.
13. Silverman, M.P. and Lundgren, D.G. 1959 Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J. Bacteriol. *77*, 642-647.
14. Tabita, R. and Lundgren, D.G. 1971 Utilization of glucose and the effect of organic compounds on the chemolithotroph *Thiobacillus ferrooxidans*. J. Bacteriol. *108*, 328-333.
15. Tuovinen, O.H., Kelly, D.P., Dow, C.S. and Eccleston, M. 1978 Metabolic transitions in cultures of acidophilic thiobacilli. In Metallurgical applications of bacterial leaching and related microbiological phenomena. Eds. L.E. Murr, A.E. Torma and J.A. Brierley. Academic Press, New York. 61-81.
16. Tuttle, J.H. and Dugan, P.R. 1976 Inhibition of growth, iron and sulfur oxidation in *Thiobacillus ferrooxidans* by simple organic compounds. Can. J. Microbiol. *22*, 719-730.
17. Walker, P.D., Batty, J. and Thomson, R.O. 1971 The localization of bacterial antigens by the use of the fluorescent and ferritin labelled antibody techniques. In Methods in microbiology 5a. Eds. J.R. Norris and D.W. Ribbons. Academic Press, New York 219-254.

18. Zavarzin, G. 1972 A heterotrophic satellite of *Thiobacillus ferrooxidans*. Mikrobiologiya *41*, 369-370.

SUMMARY

Optimum conditions for the accumulation of substantial amounts of pyrite (FeS_2) in the sediment are found in estuarine areas, especially in the tropics. In such areas anaerobic conditions prevail owing to continuous saturation with water. There is an abundant supply of organic matter, in the tropics mainly derived from extensive mangrove forests. Continuous supply of sulphate takes place from the see by the tidal movement. The complex organic material is decomposed by anaerobic bacteria into low-molecular compounds which in turn can be utilized as energy and/or carbon source by sulphate-reducing bacteria. Sulphate is utilized as electron-acceptor by these bacteria and reduced to hydrogen sulphide. Part of the latter compound is fixed as iron sulphide from which pyrite can be formed. Iron is mostly present in such amounts that accumulation up to 10% pyrite is possible. As a result of the strong tidal movement, partial oxidation of sulphide to elemental sulphur (S^o) is possible and there is a constant removal of alkaline compounds like HCO_3^- which is formed during sulphate reduction. S^o is a necessary intermediate in the formation of pyrite, whilst the continuous removal of the bicarbonate results in a decreased pH which is kinetically favourable for the formation of pyrite,

Drainage of the sediment leads to cracking of the unripened clay through which air can penetrate into the soil. Pyrite is then oxidized partly to sulphuric acid that causes a pH drop of the soil near neutral to 4 or even lower. Such soil is useless for agricultural purposes as a result of the low pH. Besides sulphuric acid, the straw-yellow mineral jarosite, $KFe_3(SO_4)_2(OH)_6$, is formed from which the name "cat clay" originates.

The oxidation of pyrite at a pH below 4, is mainly a microbial process. When starting the experiment it was unknown in what way the initial acidification of the sediment from near neutral to pH 4 proceeds.

Chapter 2 contains the results of a study of microbial processes involved in the formation of acid sulphate soils. Special attention was given to the initial drop of pH from 7 to 4. It appeared that the initial acidification is a non-biologically catalysed process.

Thiobacillus thioparus, *Thiobacillus thiooxidans* and heterotrophic thiosulphate-oxidizing bacteria were isolated from acidifying potentially acid sulphate soil. However, these bacteria were not able to oxidize pyrite. The oxidation of the mineral in synthetic media inoculated with *Thiobacillus thioparus*, *Thiobacillus thiooxidans*, *Thiobacillus intermedius* or *Thiobacillus perometabolis* did not proceed faster than that in sterile media. Nevertheless, the numbers of cells in the inoculated media increased by a factor of 10-100 to a maximum of 10^5 cells/ml medium (2. Fig. 3). The thiobacilli probably oxidize a small amount of the reduced sulphur compounds which are formed during the non-biological oxidation of pyrite. Experiments with sterilized potentially acid sulphate soil to which pure cultures of different thiobacilli or a suspension of unsterilized potentially acid sulphate soil were added aseptically corroborated the above-mentioned findings (2. Table 2). The initial acidification of sterile potentially acid sulphate soils was comparable with that of the inoculated soils. Only after the pH had decreased to values below 4.0 the oxidation of pyrite in the sterile soil was significantly slower than that in the soils inoculated with cultures of *Thiobacillus ferrooxidans*.

Although the addition of lime prevented acidification, the non-biological oxidation of pyrite was not abolished during the 68 days the process was followed (2. Table 2b).

Attempts to isolate other microorganisms that might be involved in the oxidation of pyrite such as *Leptospirillum ferrooxidans* or members of the genus *Metallogenium* were not successful. The only organism which is responsible for the oxidation of pyrite is *Thiobacillus ferrooxidans*.

Two mechanisms of the oxidation of pyrite by *Thiobacillus ferrooxidans* are known *viz.*: "the indirect contact mechanism" and the "direct contact mechanism". According to the former mechanism, the sulphur moiety of pyrite is oxidized to sulphate by ferric ions which in turn are reduced to ferrous iron. *Thiobacillus ferrooxidans* oxidizes the ferrous iron to ferric iron to complete a cyclic process. According to the second mechanism the bacterium is in direct contact with the mineral and it oxidizes the iron as well as the sulphur moiety of pyrite. When starting the experiments it was evident that pyrite can be oxidized by *Thiobacillus ferrooxidans* according to the indirect contact mechanism but there were some indications that the direct contact mechanism can also occur.

Chapter 3 gives the results of a study in which it was clearly shown that *Thiobacillus ferrooxidans* is able to oxidize pyrite according to the direct

contact mechanism. Inhibitors of the oxidation of Fe^{2+} and S^o, NaN_3 and N-ethylmaleimide (NEM), respectively, partially abolished the oxidation of pyrite when added separately but when the inhibitors were added together the oxidation of the mineral completely stopped (3.Table 1).

The electrons released during the oxidation of ferrous iron are transferred to cytochrome c, whilst the electrons derived from the oxidation of elemental sulphur probably reduce acceptors with a lower redox potential. Cytochromes of the b-type were clearly shown to be present in cells grown in media with S^o or FeS_2 as energy source, but they were not observed in cells grown in media with Fe^{2+} as electron donor (3. Fig. 3). The efficiency of the utilization of released electrons at pH 4.0 was highest with S^o, less with FeS_2 and lowest with Fe^{2+} as energy source. This was investigated by measuring the incorporation of radio-active carbon dioxide ($^{14}CO_2$) into the bacteria in media with the three different electron donors (3. Table 3).

Additional evidence for the direct oxidation of the sulphur moiety of pyrite by *Thiobacillus ferrooxidans* was obtained from the fact that the oxidation of Fe^{2+} at pH 5.0 was negligible, whereas the oxidation of S^o and FeS_2 clearly proceeded at this pH. Separation of bacteria and pyrite by means of a dialysis bag to prevent direct contact resulted in a decrease of the rate of oxidation of the mineral which was comparable with the inhibition of pyrite by NEM (3. Table 2). The oxidation of the sulphur moiety of pyrite by *Thiobacillus ferrooxidans* was relatively more important with increasing pH.

During the study of the oxidation of pyrite by *Thiobacillus ferrooxidans* it appeared that cultures of this bacterium grown in media with Fe^{2+} as energy source without exception contained *Thiobacillus acidophilus*. Nothing was known about factors which can explain the close association between the two organisms.

Chapter 4 contains a survey of the results of an investigation into factors responsible for the presence of *Thiobacillus acidophilus* in *Thiobacillus ferrooxidans* cultures. The most obvious explanation would be that *Thiobacillus ferrooxidans* is able to utilize Fe^{2+} as energy source. But attempts to adapt the mixotroph to the oxidation of Fe^{2+} were not successful. *Thiobacillus acidophilus* grew in modest numbers on organic matter excreted by *Thiobacillus ferrooxidans* (4. Table 2). Oligocarbophilic growth also was observed (4. Table 6). Possible substrates for such a growth are: methanol and ethanol whilst hydrogen sulphide also can be utilized as energy source by the organism.

Thiobacillus ferrooxidans may profit from the presence of *Thiobacillus acidophilus* in mixed cultures containing organic compounds such as alcohols, amino acids or organic acids which are toxic to *Thiobacillus ferrooxidans*. Such compounds are utilized by the mixotroph after which the autotrophic organism can grow.

Experiments with fluorescent labelled antibodies against *Thiobacillus acidophilus* showed that in mixed cultures of both organisms the cell numbers of the mixotroph were comparable with cell numbers of the autotroph (4. Table 8). Also the fact that *Thiobacillus ferrooxidans* cultures are hard to purify indicates that both organisms are present in almost equal numbers in heterogeneous cultures. These observations might be an indication that *Thiobacillus ferrooxidans* excretes more assimilable organic matter in the presence of *Thiobacillus acidophilus* than in pure cultures. More research is necessary to prove this assumption.

SAMENVATTING

In estuaria, vooral in de tropen, zijn de omstandigheden optimaal voor de ophoping van aanzienlijke hoeveelheden pyriet in het sediment. Door voortdurende verzadiging met water heersen daar anaërobe condities. Er is een overvloedige aanvoer van organisch materiaal, in de tropen voornamelijk afkomstig van uitgestrekte mangrovebossen. Voortdurende aanvoer van sulfaat vanuit zee vindt plaats door de getijdewerking. Het complexe organische materiaal wordt door anaërobe bacteriën omgezet in laagmoleculaire verbindingen die als energie- en/of koolstofbron gebruikt kunnen worden door sulfaatreducerende bacteriën. Sulfaat wordt door deze bacteriën gebruikt als elektronen-acceptor waarbij het wordt gereduceerd tot zwavelwaterstof. Een deel hiervan wordt vastgelegd als ijzersulfide waaruit pyriet kan ontstaan. IJzer is meestal in zodanige hoeveelheden aanwezig dat ophopingen tot 10% pyriet mogelijk zijn. Door de sterke doorspoeling van het sediment als gevolg van de getijdewerking is partiële oxydatie van sulfide tot elementaire zwavel (S^o) mogelijk en is er een goede afvoer van alkalische produkten, zoals HCO_3^-, dat bij de sulfaatreductie wordt gevormd. S^o is een noodzakelijk intermediair bij de vorming van pyriet, terwijl een goede afvoer van bicarbonaat een verlaging van de pH geeft, hetgeen kinetisch gezien gunstig is voor de pyrietvorming.

Drainage van het sediment leidt tot scheuren van de onrijpe klei waardoor lucht de grond kan binnendringen. Pyriet wordt dan geoxydeerd waarbij o.a. zwavelzuur ontstaat dat de pH van de grond snel doet dalen tot 4 of zelfs 3. Door de lage pH is zulke grond ongeschikt voor landbouwkundige doeleinden. Naast zwavelzuur ontstaat het strogele mineraal jarosiet, $KFe_3(SO_4)_2(OH)_6$, waaraan de grond de naam katteklei ontleent.

De oxydatie van pyriet bij pH-waarden lager dan 4.0 is voornamelijk een microbiologisch proces. Hoe de verzuring van het oorspronkelijke neutrale sediment tot pH 4.0 plaats vindt is onbekend.

Hoofdstuk 2 bevat de resultaten van een studie van de microbiologische processen die een rol spelen bij de vorming van katteklei. Speciale aandacht werd besteed aan de daling van de pH van 7 tot 4. Het bleek dat de aanvankelijke verzuring een niet-biologisch gekatalyseerd proces is. *Thiobacillus thioparus*,

Thiobacillus thiooxidans en heterotrofe thiosulfaatoxyderende bacteriën konden geïsoleerd worden uit verzurende potentiële katteklei. Deze bacteriën waren echter niet in staat pyriet te oxyderen. Oxydatie van het mineraal in synthetische media geënt met *Thiobacillus thioparus*, *Thiobacillus thiooxidans*, *Thiobacillus intermedius* of *Thiobacillus perometabolis* verliep niet sneller dan die in steriele media. Toch namen de bacterieaantallen in de geënte media toe met een factor 10-100 tot maximaal 10^5/ml (2. Fig. 3). De thiobacilli oxyderen waarschijnlijk een gering gedeelte van de gereduceerde zwavelverbindinge die vrijkomen bij de niet-biologische oxydatie van pyriet. Experimenten met gesteriliseerde potentiële katteklei waaraan reincultures van verschillende thiobacilli of een suspensie van ongesteriliseerde potentiële katteklei werden toegevoegd bevestigden bovenstaande bevindingen (2. Tabel 3). De beginverzuring van steriele potentiële katteklei verliep niet langzamer dan die van de geënte gronden. Pas nadat de pH was gedaald tot waarden beneden de 4.0 verliep de oxydatie van pyriet in de steriele grond significant langzamer dan in de gronden geënt met *Thiobacillus ferrooxidans*.

Toevoeging van kalk aan de grond voorkwam verzuring, maar de niet-biologische oxydatie van pyriet stopte niet gedurende de 68 dagen dat het proces vervolgd werd (2. Tabel 2b).

Pogingen om andere micro-organismen te isoleren die betrokken zouden kunnen zijn bij de oxydatie van pyriet, zoals *Leptospirillum ferrooxidans* en vertegenwoordigers van het geslacht *Metallogenium* waren niet succesvol. De enige bacterie die verantwoordelijk is voor de oxydatie van pyriet is *Thiobacillus ferrooxidans*.

Er zijn twee mechanismen bekend van de oxydatie van pyriet door *Thiobacillus ferrooxidans*, nl. het "indirect-contact-mechanisme" en het "direct-contact-mechanisme". Volgens het eerste mechanisme wordt de zwavel-component van pyriet geoxydeerd door ferri-ionen die daarbij gereduceerd worden tot ferro-ijzer. *Thiobacillus ferrooxidans* oxydeert dan de ferro-ionen tot ferri-ijzer, waarmee de kringloop gesloten is. Volgens het tweede mechanisme is de bacterie in direct contact met pyriet en oxydeert dan naast de ijzer- ook de zwavelcomponent van het mineraal. Zeker is dat pyriet geoxydeerd kan worden via het indirect-contact-mechanisme. Er waren aanwijzingen dat het direct-contact-mechanisme ook op kan treden.

Hoofdstuk 3 geeft de resultaten van een onderzoek waarin duidelijk werd aangetoond dat *Thiobacillus ferrooxidans* pyriet kan oxyderen volgens het direct-contact-mechanisme. Remstoffen van de oxydatie van Fe^{2+} en S^o (elementaire zwavel) respectievelijk NaN_3 en N-ethylmaleimide (NEM), waren in staat de oxydatie van pyriet

slechts gedeeltelijk te remmen maar wanneer de remmers gezamenlijk werden toegevoegd werd de oxydatie van pyriet volledig geremd (3. Tabel 1).

De elektronen afkomstig van de ijzeroxydatie worden overgedragen op cytochroom c, terwijl de elektronen afkomstig van de zwavel waarschijnlijk acceptoren met een lagere redoxpotentiaal reduceren. In cellen gegroeid in media met S^o of pyriet als energiebron was cytochroom b duidelijk aantoonbaar hetgeen niet het geval was met cellen die gegroeid waren in media met Fe^{2+} als energiebron (3. Fig. 3). De efficiëntie waarmee de bij de oxydatie vrijgekomen elektronen worden benut bij pH 4.0 was het grootst bij S^o als energiebron, kleiner met FeS_2 en het laagst met Fe^{2+} als substraat. Dit werd onderzocht door de inbouw van radioactief $^{14}CO_2$ in de bacteriën bij gebruik van verschillende elektronendonor na te gaan (3. Tabel 3).

Aanvullende bewijs voor de directe oxydatie van de zwavelcomponent van pyriet door *Thiobacillus ferrooxidans* werd nog verkregen uit het feit dat de oxydatie van Fe^{2+} bij pH 5.0 te verwaarlozen was terwijl de oxydatie van S^o en FeS_2 nog duidelijk plaats vond bij die pH. Scheiding van bacteriën en pyriet door middel vaneen dialysezak ter voorkoming van direct contact veroorzaakte een verlaging van de oxydatiesnelheid die te vergelijken was met de remming van de oxydatie van pyriet door NEM (3. Tabel 2). Verder bleek nog dat de oxydatie van de zwavelcomponent van pyriet door *Thiobacillus ferrooxidans* met stijgende pH relatief steeds belangrijker werd (3. Fig. 1 en 2). Tijdens het onderzoek naar de oxydatie van pyriet door *Thiobacillus ferrooxidans* bleek dat de cultures van deze bacterie gegroeid in media met Fe^{2+} als energiebron zonder uitzondering *Thiobacillus acidophilus* bevatten. Er was niets bekend over factoren die van betekenis zijn voor de nauwe relatie tussen de twee bacteriën.

Inhoofdstuk 4 worden de resultaten besproken van een onderzoek naar factoren die verantwoordelijk zijn voor de aanwezigheid van *Thiobacillus acidophilus* in cultures van *Thiobacillus ferrooxidans*. De meest voor de hand liggende verklaring zou zijn dat *Thiobacillus acidophilus* in staat is Fe^{2+} te gebruiken als energiebron. Maar pogingen om de mixotrofe bacteria te adapteren aan de oxydatie van Fe^{2+} waren niet succesvol. *Thiobacillus acidophilus* groeide in bescheiden aantallen op uitscheidingsprodukten van *Thiobacillus ferrooxidans* (4. Tabel 2). Oligocarbofiele groei werd ook waargenomen (4. Tabel 6). Mogelijke substraten hiervoor zijn methanol en ethanol terwijl de bacterie ook zwavel-waterstof als energiebron kan gebruiken.

In mengcultures van de twee bacteriën kan *Thiobacillus ferrooxidans* voordeel hebben van de aanwezigheid van *Thiobacillus acidophilus* indien organische

verbindingen zoals alcoholen, aminozuren en andere organische zuren in het gebruikte medium van eerstgenoemde bacterie aanwezig zijn. De organische stof, die toxisch is voor *Thiobacillus ferrooxidans*, wordt benut door *Thiobacillus acidophilus* waarna het autotrofe organisme kan groeien.

Experimenten met fluorescerende antilichamen tegen *Thiobacillus acidophilus* wezen uit dat in mengcultures van beide organismen de mixotrofe bacterie in vergelijkbare aantallen aanwezig was als de autotrofe bacteria (4. Tabel 8). Ook het feit dat *Thiobacillus ferrooxidans* zo moeilijk in rein-cultuur is te krijgen wijst er op dat in heterogene cultures de bacterieaantallen van beide soorten van dezelfde orde van grootte zijn. Deze waarnemingen zouden een aanwijzing kunnen zijn dat *Thiobacillus ferrooxidans* door de aanwezigheid van *Thiobacillus acidophilus* gestimuleerd wordt om meer organisch materiaal uit te scheiden dat dan door de laatstgenoemde bacterie kan worden benut. Meer onderzoek is nodig om deze veronderstelling te bewijzen.

CURRICULUM VITAE

De auteur is op 20 december 1951 geboren te Schipluiden. Van 1964 tot 1970 bezocht hij het St. Stanislascollege te Delft. Na het behalen van het diploma HBS-b begon hij zijn studie aan de Landbouwhogeschool te Wageningen. In 1976 behaalde hij het ingenieursdiploma Levensmiddelentechnologie met lof. Het vakkenpakket omvatte de vakken: kennis van levensmiddelen, technische microbiologie en biochemie. Na de studie werd een proefschrift bewerkt in het Laboratorium voor Microbiologie van de Landbouwhogeschool. Sinds 15 augustus 1979 is hij als leraar werkzaam op de Rijks Hogere en Middelbare School voor Levensmiddelentechnologie te Bolsward.

CURRICULUM VITAE

De auteur is op 20 december 1951 geboren te Schipluiden. Van 1964 tot 1970 bezocht hij het St. Stanislascollege te Delft. Na het behalen van het diploma HBS-b begon hij zijn studie aan de Landbouwhogeschool te Wageningen. In 1976 behaalde hij het ingenieursdiploma Levensmiddelentechnologie met lof. Het vakkenpakket omvatte de vakken: kennis van levensmiddelen, technische microbiologie en biochemie. Na de studie werd een proefschrift bewerkt in het Laboratorium voor Microbiologie van de Landbouwhogeschool. Sinds 15 augustus 1977 is hij als leraar werkzaam op de Rijks Hogere en Middelbare School voor Levensmiddelentechnologie te Bolsward.