BIOLEACHING OF METALS FROM SOILS OR SEDIMENTS USING THE MICROBIAL SULFUR CYCLE

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Cover photo: the background of the cog-wheel elements is formed by a scanning electron microscopic image of an elemental sulfur powder. Artwork 1/2 OC, Harpuna.

PROPOSITIONS

1. Application of the microbial sulfur cycle to control metals pollution will get an increasing attention in the future, when truly sustainable and cost-effective ways of controlling the metals pollution will be searched for.

   This thesis, Chapter 2

2. The microbially produced elemental sulfur is a better substrate for bioleaching than the elemental sulfur flower both because of its higher specific surface area and its higher hydrophillicity.

   This thesis, Chapters 3 and 4

3. In leaching of toxic metals from soils or sediments, two different strategies can be applied: intensive and extensive one. The increasing relative importance of the diffused sources pollution nowadays suggests that the extensive approach will have much higher importance in the future, compared to the present.

   This thesis, Chapters 1, 5 and 7

4. Equilibrium pH of an aerated sediment slurry upon addition of acid is controlled by two different processes: 1. scavenging of protons by the adsorption and diffusion, and 2. release of acid by oxidation of reduced components. The combined effect of the two processes makes more feasible chemical extraction at high addition of sulfuric acid and short extraction times, and bioleaching processes at no or low acid addition and long extraction times.

   This thesis, Chapter 7

5. Throughout the literature, the wastewater treatment using wetlands is usually considered as a cheap technique. In some cases, however. this may be a big mistake: when heavy metals are present in the wastewater and trapped by the wetland bed, the dismantling costs of a wetland can far exceed the initial investments.

6. Very high costs of soil sanitation techniques are often paid because the cheaper, extensive, or preventive measures fall into another budgetary category.

7. The European East-West cooperation is often mislead by a false presumption that there is one uniform development path for the society and that the difference is only in the distance, which various nations managed to pass.

8. Healing of the Czech society from its historic burdens does not depend on foreign investments, enlighted politicians, or alignment with European Union. The true hope is in current generations of teenagers riding skateboards, listening strange music, using English and computers, and paying no attention to whether you were a communist, what colour is your skin and how much money do you earn.

9. The only ultimate resource for civilization growth is human imagination, a resource that knows no limits.

J.L. Simon: The Ultimate Resource. Princeton University Press, Princeton, NJ, 1981

10. Major challenge for present science and many scientists is to realize that they will never be able to fully understand the world with scientific methods: if only for the reason of limited funding.

11. In recuperation after a serious illness, the crucial question is not how, but why to return back to the worldly matters.

12. Statements like "I don't want to bother about software, I want just to be a simple user of a computer" can be compared to a painter addressing people with his art and not bothering about harmony of colours, brushes, or a composition. However, we can always decide not be artists.

13. Snowboarding, riding motorbike, or using Linux operation system have frequently common background: it is a strive for freedom and self-expression, escaping the press of general habits like skiing, driving car or using Windows, respectively.

Propositions to the PhD. thesis '**Bioleaching of Metals from Soils or Sediments Using the Microbial Sulfur Cycle**'

Richard Tichý
Wageningen, November 24, 1998

ABSTRACT:

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Bioleaching is a microbial process which can be applied for the removal of heavy metals from polluted soils or sediments. It is driven by oxidation of reduced sulfur compounds resulting in acidification and solubilization of heavy metals. This thesis evaluates the bioleaching of heavy metals from soils or sediments using the microbially recycled elemental sulfur and possible reactor configurations for practical application of the process. An extensive literature survey is provided concerning solid-state reduced sulfur compounds, their transformations in the environment and their influence on metals mobility. Two substrates for bioleaching were compared experimentally, i.e. the orthorhombic sulfur flower and microbially recycled sulfur, suggesting the latter sulphur type as an excellent substrate. The application domain of bioleaching process was identified by a series of leaching studies with soil slurries, unmixed potted soil, and freshwater sediment slurries. Generally, two different application domains of the leaching processes can be identified: (1) intensive extraction at extremely low pH ($<$2-3), and (2) extensive extraction at higher pH ($>$3-4). A substantial benefit of bioleaching using microbially recycled sulfur is in its possible combination with other two processes of the microbial sulfur cycle, i.e. (1) sulfate reduction leading to sulfate removal and separation of metals from the spent liquor after bioleaching, and (2) partial sulfide oxidation which removes excess sulfide after sulfate reduction and results in microbial production of elemental sulfur.

I wish to thank to many people who assisted me in proceeding this work. Tim Grotenhuis, thank you very much for your enthusiasm, interest, all the hours we spent together above data, manuscripts, during discussions about various topics, and for your excellent being who you are. Albert Janssen: after our suspicious encounter, we made a lot together, and I will always recall a joyful time of working with you. Wim Rulkens, your compassion and cordial approach was gratefully appreciated, and without your efforts, this work could not have been finished. Gatze Lettinga, I enjoyed your views of science and life, as well as your interests in international students. Nice and pleasant cooperation with Piet Lens resulted also in an important part of this thesis. The whole staff of the Sub-department of Environmental Technology at Wageningen Agricultural University created an excellent and stimulative atmosphere for my work, and I wish to express my deep feelings about the fact that I could have been working there. My special thanks go to Katja Grolle: Katja, thanks a lot for your openness, care, help, offering shelters, translating *Samenvatting*, and many, many other things.

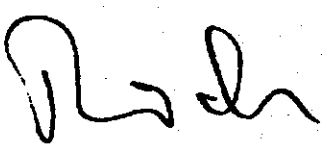
I wish to express my thanks to the Institute of Landscape Ecology, Academy of Sciences of the Czech Republic, for providing me sufficient space and freedom to finalize this work. I also appreciated a technical assistance and openness to collaboration from the Department of General Plant Nutrition, Faculty of Agronomy of the University of South Bohemia in České Budějovice.

Václav Nýdl, my teacher and friend from České Budějovice, made a significant contribution to this work not only via excellent mathematical and statistical work, but, more important, via sharing his world outlook and providing so valuable visions for future. I am honoured that I collaborated with an oustanding expert and personality, Václav Mejstřík: I enjoyed a lot his views of science, life, universe and everything. The two Václavs enabled my comeback from The Netherlands to the Czech Republic as smooth as possible and I am glad that I had a chance to work with them.

I am thankful to several students, who made a substantial contribution to this work. They are (in alphabetical order): Remco van Abswoude, Chiel Cuypers, Jiří Fajtl, Martin IJspeert, Jasper Kieboom, Richard Wallet.

Furthermore, my thanks are dedicated to people who were not involved directly in my work, yet also contributed. I wish to thank my beloved parents Josef and Paula and my sister Petra, and I am sorry that my father can not see the ending of my work. Míra Oborník, Jirka Kiprý, Ivo Olda Machač, Petr Vopálka: you were and are a good companion and reliable friends, and I gained a lot from your friendship. Radan Harpooner Běhoun, thanks a lot for your technical assistance in printing this booklet and designing the cover.

Finally, Šárka, life with you has become a pleasant and marvellous adventure. Thank you for your being.



'A cold coming we had of it,
Just the worst time of the year
For a journey, and such a long journey:
The ways deep and the weather sharp,
The very dead of winter.'
And the camels galled, sore-footed, refractory,
Lying down in the melting snow.
There were times we regretted
The summer palaces on slopes, the terraces,
And the silken girls bringing sherbet.
Then the camel men cursing and grumbling
And running away, and wanting their liquor and women,
And the night-fires going out, and the lack of shelters,
And the cities hostile and the towns unfriendly
And the villages dirty and charging high prices:
A hard time we had of it.
At the end we preferred to travel all night,
Sleeping in snatches,
With the voices singing in our ears, saying
That this was all folly.

T.S. Eliot: Journey of the Magi

Contents:

CHAPTER 1

GENERAL INTRODUCTION

Soils and sediments contaminated with toxic heavy metals are a serious problem both in highly industrialized Western countries, as well as in transition countries of Central and Eastern Europe. Most of standard treatment technologies for these materials are costly or not efficient. The limitations of many treatment technologies are pronounced in moderately-contaminated sites, since the treatment efficiency of standard techniques usually decreases with lowering the overall concentration of metals. This brings about an increasing need to apply innovative technological configurations which are less costly and can be operated at milder treatment conditions and for prolonged treatment times. Examples of these innovative techniques are e.g. heap-leaching, in-situ extraction, or phytoremediation.

One of the investigated options for the removal of heavy metals from soil or sediments is a possible use of bioleaching. Bioleaching is a process mediated by specific acidophillic (acid-loving) bacteria capable of acid production. Since many of these microbes belong to the genus *Thiobacillus*, they are called thiobacilli or thiobacilli-like organisms. Thiobacilli are able to oxidize reduced sulfur or ferrous iron and thus produce the acidity. Increasing acidity causes solubilization of many cations, including heavy metals, via various processes of solubilization, desorption, ion-exchange etc. Moreover, some thiobacilli are capable of direct metabolic oxidation and solubilization of heavy metal sulfides, e.g. copper, zinc, or lead sulfides.

Oxidation of reduced sulfur containing compounds occurs spontaneously in nature, causing sometimes rather serious environmental problems like acid mine drainage, mine-tailing leachates containing heavy metals, or acid sulfate soils. However, in a more positive way it can be applied to control mobility of heavy metals or sulfur compounds, e.g. for bioleaching of heavy metals from polluted sewage sludge, soils or sediments, for microbial metal mining from low-grade ores or for coal desulfurization.

For the bioleaching of metal-polluted soils or sediments, the crucial parameter is a presence of sulfur or sulfides. These compounds can be expected in anoxic sediments or anaerobically-pretreated wastes, however, acidification of common aerobic soils will likely require either addition of extra reduced sulfur compounds or, more logically, a direct addition of sulfuric acid.

An attractive feature of using bioleaching or leaching with sulfuric acid is a possible coupling of the leaching process with some other biotechnologies (see Figure 1.1). Particularly, the spent liquor after the bioleaching and separation of "clean" soil or sediment can be processed by sulfate reduction. This microbial process occurs in anaerobic conditions in the presence of organic substrates like lactate, acetate or some complex organic compounds. Sulfate reducing bacteria use sulfate as a terminal electron acceptor, which results in a production of sulfide. Sulfide can be applied to remove the toxic heavy metals from the liquor, since cationic metals form readily insoluble metal sulfides. Therefore, an anaerobic suspension reactor can serve for the treatment of the spent bioleaching process water.

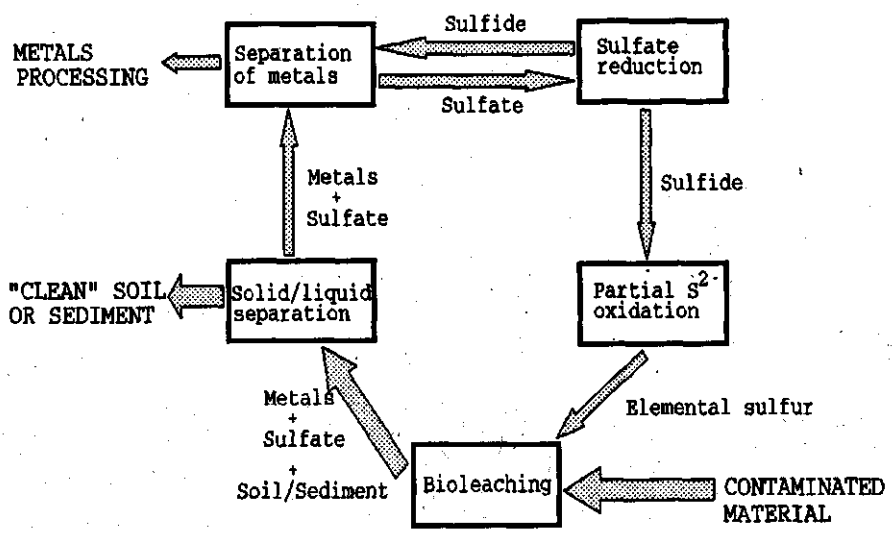


Figure 1.1 Possible use of microbial sulphur cycle for control of toxic metals.

Alternatively, sulfate reduction can be carried out in a more extensive configuration, like wetlands or anaerobic ponds. These systems can collect and purify voluminous aqueous streams containing sulfate, acidity and heavy metals, like acid mine drainage or similar leachates, and their function can be sustained for considerable time periods with only minimum care. However, after reaching their maximum carrying capacity for pollutants, e.g. after saturating the metals binding capacity and/or filling up with precipitates and other sediments, they will have to undergo treatment and regeneration. Here, the bioleaching of the resulting sediments will likely be the most natural way of treatment.

The use of microbial sulfur conversions to control heavy metals pollution can benefit from yet another biotechnological process: a sulfide-rich waters coming from sulfate-reducing reactors can be microbially oxidized under oxygen-limiting conditions, which results in a production of elemental sulfur. Since sulfur is virtually insoluble in water, it precipitates and can be separated from the liquor. This process is applied broadly in practice. When used in the framework of the sulfur cycle (Figure 1.1), at least a part of sulfur may be recovered and re-fed back to the bioleaching step.

In general, the three processes, i.e. bioleaching, sulfate reduction and partial sulfide oxidation, can be coupled in a full biotechnological sulfur cycle (Figure 1.1), leading to a turnover of sulfur. However, in most cases it is unlikely that the full sulfur cycle will be applied in practice. More likely options are just the partial uses: e.g. the use of microbially-produced elemental sulfur as a substrate for bioleaching or a combination of extensive sulfate reduction in wetland or pond and subsequent bioleaching of the resulting sediments

THE SCOPE OF THE THESIS

The aim of this thesis is to evaluate the possible use of bioleaching to remove heavy metals from soils or sediments with a special attention to the microbial sulfur cycle. The work involves the following subtasks:

1. Finding the pH of soil or sediment slurry which is required to achieve satisfactory extraction efficiency for heavy metals.
2. Evaluating the capacity of microbial sulfur oxidation to reach the required pH values.
3. Investigating the possible use of sulfur as a substrate for bioleaching and comparing the two different types of sulfur, i.e. orthorhombic sulfur flower and microbially produced elemental sulfur, in their capacity to oxidize aerated liquor.
4. Determining the feasibility of bioleaching when used within the framework of the microbial sulfur cycle.

STRUCTURE OF THE THESIS

After the general introduction in the Chapter 1, a survey of literature is given in the Chapter 2. This survey addresses both heavy metals and the solid-state reduced sulfur compounds. Processes leading to the oxidation of sulfur and mobilization of heavy metals, their environmental concerns and possible remediation techniques are discussed. The next two chapters deal with the studies on possible use of microbially produced elemental sulfur as a substrate for thiobacilli in batch (Chapter 3) and continuous (Chapter 4) cultivation. Chapter 3 also compares the processes of microbial oxidation of orthorhombic sulfur flower with the microbially produced elemental sulfur. In Chapters 5-7, the leaching processes are assessed in different configurations (soil slurry, in-situ, sediment slurry). Chapter 5 studies a strategy for leaching of artificially zinc-contaminated clay, silt, and sandy soil slurries using additions of varying concentration of sulfuric acid in place of bioleaching. At the same time, possible negative effects of high acid concentrations on the mineral matrix are studied by quantifying the extent of aluminium solubilization. Chapter 6 aims at evaluation of an in-situ process of microbial acidification and subsequent cadmium solubilization in soil using both the microbially produced sulfur and orthorhombic sulfur flower. Finally, Chapter 7 deals with the bioleaching of toxic metals from a wetland sediment which experienced a loading with mine drainage water in the past. Finally, the general discussion and conclusions (Chapter 8) summarizes the results and findings which have been accomplished in the previous chapters.

CHAPTER 2

SOLID-STATE REDUCED SULFUR COMPOUNDS: ENVIRONMENTAL ASPECTS AND BIO-REMEDIATION

Abstract: The paper reviews major risks caused by microbial transformations of solid-state reduced sulfur compounds which are used or affected by anthropogenic activities. These materials like fossil fuels, ores, anaerobic sediments or solid waste may undergo oxidative changes, resulting in a solubilization of sulfur from the solid phase. The risks are generally associated with acidification of the environment and subsequent mobilization of toxic metals. In the second part of the review, current and perspective techniques applicable for the treatment of such materials are presented. Both methods that prevent solubilization and processes of microbial removal of reduced sulfur are discussed. Special interest is paid to techniques operating on long time scales, applicable for the treatment of large areas or highly voluminous wastes.

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2.1. SULFUR FLUXES IN THE GLOBAL S-CYCLE

2.1.1. Formation of solid-state sulfur in the environment

Within a frame of global biogeochemical cycling, sulfur is transformed with respect to its oxidation state, formation of organic and inorganic compounds, and its physical status (Ivanov, 1983). In oxidizing conditions, the most stable sulfur species is sulfate. In reducing environments, elemental sulfur and sulfide are formed. Numerous other sulfur species, e.g. sulfite, polysulfides, polythionates or thiosulfate, are formed in natural conditions, however, they are considered unstable (Kuhn et al., 1983). Sulfide and some organic compounds containing reduced sulfur, e.g. dimethyl sulfide, are volatile and can escape to the atmosphere (Smet et al., 1997). However, chemical reactivity of these compounds in natural environments results in formation of poorly soluble compounds. Most common examples are metal sulfides with very low solubility in water (Table 2.1).

Table 2.1 Solubility of selected metal sulfides (Ellwood et al., 1992).

Metal ion	Solubility product, $mol.L^{-1}$
Hg^{2+}	$4 \cdot 10^{-54}$
Cu^{2+}	$8 \cdot 10^{-45}$
Cd^{2+}	$5 \cdot 10^{-29}$
Zn^{2+}	10^{-20}
Mn^{2+}	$1.4 \cdot 10^{-14}$
Fe^{2+}	10^{-20}

Global planetary sulfur cycling generates an accumulation of solid-state sulfur stocks due to the formation of the above mentioned insoluble reduced sulfur species in most anaerobic environments, like marshes, wetlands, freshwater and sea sediments all over the globe (Giblin and Wieder, 1992). The formation of solid-state sulfur proceeded already from early history of the planetary biogeochemical cycling (Brimblecombe et al., 1989; Howarth and Stewart, 1992). Accumulation of sulfur in anaerobic deposits of biomass created stocks of metal sulfides and organic-bond sulfur in coal (Bouška, 1977; Shennan, 1996). Certain heavy mineral oils contain

substantial amounts of sulfur as well. Another stock of solid-state sulfur are the sulfidic ores. A broad spectrum of sulfidic ores is known, the most abundant being iron and copper sulfides (Ivanov, 1983; Brimblecombe et al., 1989).

Table 2.2 Major fluxes of the continental part of the global biogeochemical cycle of sulfur, in Tg S per year (Ivanov, 1983).

Nature of flux	Natural	Anthropogenic	Total
Emission to the atmosphere from fuel combustion and metal smelting	-	113	113
Volcanic emission	14	-	14
Aeolian emission	20	-	20
Biogenic emission	17.5	-	17.5
Atmospheric transport of oceanic sulfate	20	-	20
Deposition of large particles from the atmosphere	12	-	12
Washout from the atmosphere, surface uptake and dry deposition	25	47	72
Transport to the oceanic atmosphere	34.5	66	110.5
Weathering	114.1	-	114.1
River run-off to the world oceans	108.9*	104	212.9
Underground run-off to the world oceans	9.2	-	9.2
River run-off to continental waterbodies	35	-	35
Marine abrasion of shores and exaration	6.8	-	6.8
Pollution of rivers with fertilizers	-	28	28
Effluents from chemical industry	-	28	28
Acid mine waters	-	1	1

* Including both ionic and particulate sulfur

Table 2.2 (cont.). Major fluxes of the oceanic part of the global biogeochemical cycle of sulfur, in Tg S per year (Ivanov, 1983).

Nature of flux	Natural	Anthropogenic	Total
Volcanic emission	14	-	14
Biogenic emission	23	-	23
Marine sulfate emission	140	-	140
Washout, surface uptake, and dry deposition	258	-	258
Burial of reduced sulfur in sediments	111.4	-	111.4
Burial of sulfate in sediments	27.8	-	27.8

Increasing anthropogenic extraction of sulfur-containing compounds from the lithosphere considerably perturbates the global sulfur cycle. Ivanov (1983) summarized the annual sulfur fluxes on earth (Table 2.2). These data clearly show the relative importance of anthropogenic sulfur emissions into the environment. The most important anthropogenic flux is the emission into the atmosphere (113 Tg S.year^{-1}, i.e. $113 \cdot 10^{12}$ g S.year^{-1}). In the continental part of the sulfur cycle, this flux is comparable only with weathering (114.1 Tg S.year^{-1}) and river run-off to the world oceans (108.9 Tg S.year^{-1}). The second major anthropogenic flux of sulfur is the pollution of rivers, and a subsequent run-off of the river waters (104 Tg S.year^{-1}). The anthropogenic inputs of the sulfur on the globe results in acceleration of the sulfur cycling. This is manifested in elevated levels of sulfate in run-off waters, build-up of sulfide in anaerobic environments, and, after exposure of reduced-sulfur stocks to air, acidification of the environment and leaching of toxic metals (Ivanov, 1983).

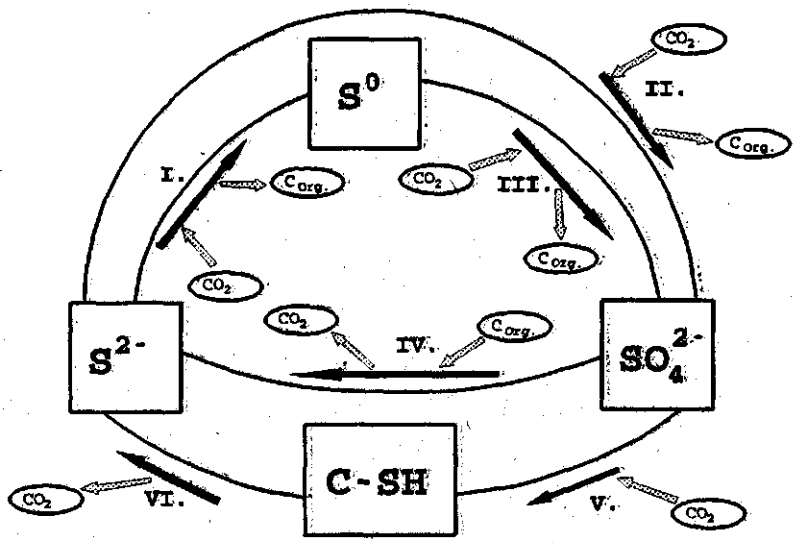

Figure 2.1 Schematic representation of the planetary sulfur cycle. For explanation of the symbols, see text.

2.1.2. The microbial sulfur cycle

The behaviour of sulfur compounds in the environment is highly influenced by the activity of living organisms, particularly microbes (Howarth and Stewart, 1992; Kelly et al., 1997). In Figure 2.1, the stocks of sulfur with different oxidation status (marked by squares) are given: S^{2-} the sulfidic form, S^0 elemental sulfur, SO_4^{2-} sulfate, and C-SH the stock of organic sulfur compounds. Shaded arrows indicate the trophical status of microbes in each process, distinguishing autotrophic (using inorganic CO_2) and heterotrophic (using organic carbon compounds, $C_{org.}$) processes. It should be noted that some processes in Figure 2.1 are not exclusively mediated by microbes, particularly the synthesis of organic sulfur compounds (process V.) and putrefaction (processes of decay) of organic sulfur compounds (process VI. in Figure 2.1).

Sulfate salts are the major stock of mobile sulfur compounds (Ivanov, 1983; Howarth and Stewart, 1992). They are mostly highly soluble in water, and considerable amounts can be transported in the environment. In the microbial sulfur cycle, sulfate is converted into sulfide via microbial sulfate reduction (pathway I. in Figure 2.1). This process of bacterial respiration occurs under strictly anaerobic conditions and uses sulfate as terminal electron acceptor. Electron donors are usually

organic compounds, eventually, hydrogen (Ivanov, 1983; Giblin and Wieder, 1992; van Houten et al., 1994):

$$8H_2 + 2SO_4^{2-} \rightarrow H_2S + HS^- + 5H_2O + 3OH^- \qquad \textbf{2.I}$$

The process of sulfate reduction is extensively applied in commercial technologies (see e.g. section 2.3.2.1). The treatment of sulfate-rich wastewaters (van Houten et al., 1994), polluted groundwater (Scheeren et al., 1991), acid mine drainage (Wildeman and Laudon, 1989; Wieder, 1993) were demonstrated. Also, the biotechnological processing of waste gypsum ($CaSO_4$) via sulfate reduction has been developed (Widdel and Hansen, 1992; Hiligsman et al., 1995; Kaufman et al., 1996).

Oxidation of sulfide into elemental sulfur (pathway II. in Figure 2.1) is performed by autotrophic bacteria. Eq. 2.IIa gives the stoichiometry of the chemoautotrophic process. However, photoautotrophic sulfide oxidation, Eq. 2.IIb, has been observed as well (Ivanov, 1983; Trüper, 1984a).

$$2H_2S + O_2 \rightarrow 2S^0 + 2H_2O \qquad \textbf{2.IIa}$$

$$2H_2S + CO_2 + h\nu \rightarrow 2S^0 + CH_2O + H_2O \qquad \textbf{2.IIb}$$

Sulfide can also be completely oxidized to sulfate (Figure 2.1, pathway III.). Here, a formula for the chemoautotrophic process is given, although photoautotrophic oxidation can also be involved (Ivanov, 1983; Trüper, 1984b).

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \qquad \textbf{2.IIIa}$$

Eventually, oxidation of sulfide may proceed in oxygen-free conditions, using nitrate as electron acceptor (Batchelor and Lawrence, 1978; Driscoll and Bisogni, 1978; Mackintosh, 1978; Claasen et al., 1986):

$$0.422H_2S + 0.422HS^- + NO_3^- + 0.437CO_2 + 0.0865HCO_3^- + 0.0865NH_4^+ \rightarrow$$

$$\rightarrow 1.114SO_4^{2-} + 0.5N_2 + 0.0842C_5H_7O_2N(biomass) + 1.228H^+ \qquad \textbf{2.IIIb}$$

Oxidation of sulfide in anoxic conditions is mostly carried out by bacteria from the genus *Thiobacillus*, like *T. albertis* and *T. neapolitanus*. A technological application of this process is the removal of nitrates from wastewater using *T. denitrificans* (Batchelor and Lawrence, 1978; Driscoll and Bisogni, 1978; Kruithof et al., 1988). Alternatively, other reduced sulfur compounds may be oxidized in this

way, like elemental sulfur or thiosulfate. Particularly elemental sulfur has been used in packed-bed reactors for the microbial removal of nitrates from groundwater (Driscoll and Bisogni, 1978; Kruithof et al., 1988). However, anoxic sulfur oxidation can also create serious environmental risks, when reduced sulfur oxidation is undesirable, e.g. in abandoned mines or mining waste-deposits (Evangelou and Zhang, 1995).

Reduced sulfur is frequently present in the environment as insoluble metal sulfides. Pyrite (FeS_2), chalcopyrite ($CuFeS_2$) and some other minerals are rather common species. These materials are also oxidized to sulfate by thiobacilli. The following reaction equation illustrates this process with the most common sulfidic mineral pyrite (Karavaiko, 1985; Bruynesteyn, 1989; Evangelou and Zhang, 1995):

$$4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4 \qquad \text{2.IIIc}$$

Elemental sulfur can be oxidized to sulfate via chemoautotrophic or photoautotrophic microbes (Figure 2.1, pathway IV.). The stoichiometry of the chemoautotrophic process is given in Eq. 2.IV (Kelly, 1982; Ivanov, 1983; Karavaiko, 1985):

$$2S^0 + 3O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 4H^+ \qquad \text{2.IV}$$

The formation and degradation of organic sulfur (C-SH) are not solely microbial processes, but numerous other organisms participate in them. Particularly, the formation of organic sulfur (Figure 2.1, pathway V) is accomplished by all photosynthesizing organisms, like algae or green plants. Conversion of organic sulfur into sulfide (Figure 2.1, pathway VI.) occurs during the decomposition of organic matter (Ivanov, 1983). Considerable environmental risks are encountered in these processes, especially regarding the volatilization of organic sulfur compounds and associated odour pollution (Smet et al., 1997).

2.1.3. Microbial oxidation of metal sulfides

The biological oxidation of sulfidic minerals is mediated by a special group of bacteria, the so called acidophiles. These bacteria are able to use elemental sulfur and reduced sulfur compounds as their energy source. Others gain energy from a conversion of ferrous (Fe^{2+}) into ferric (Fe^{3+}) iron (Karavaiko, 1985; Johnson et al., 1993; Jensen and Webb, 1995; Nagpal, 1997). The best known acidophile is *Thiobacillus ferrooxidans* which combines the ability to oxidize both sulfur compounds and ferrous iron. The bacterium is able to oxidize at low pH values (pH

1-4) sulfidic minerals such as pyrite according to reaction 2.IIIc (Karavaiko, 1985; Johnson et al., 1993). Acidophilic sulfur and/or iron oxidizers are chemolithoautotrophic bacteria, which means that they use carbon dioxide as their carbon source. *T. ferrooxidans* is one of the first properly described species. It had been isolated from acid mine drainage water in the late forties (Silverman and Lundgren, 1959; Cook, 1964; Agate et al., 1969; Sneath et al., 1989) together with *T. thiooxidans* (Guay and Silver, 1975). The latter species can only oxidize sulfur and reduced sulfur compounds and lacks the ferrous iron oxidizing capacity. In contrast to *T. ferrooxidans*, *T. thiooxidans* cannot attack sulfidic minerals on its own, but it can contribute in their solubilization in a syntrophic relation with ferrous iron oxidizers such as *Leptospirillum ferrooxidans*. The latter can only convert ferrous iron and misses the sulfur oxidizing capacity (Karavaiko, 1985; Sand et al., 1992). All species grow aerobically. However, anaerobic growth has been observed for some species as well (Driscoll and Bisogni, 1978; Pronk et al., 1992)

In the last decades, awareness has grown that in these low pH environments, various other bacterial species are present. Most of them have not been characterized and named properly (Bos and Kuenen, 1990; Karavaiko, 1985). Besides obligate autotrophs, the group of acidophiles also comprises facultative autotrophs and obligate heterotrophs, which utilize organic compounds as carbon and energy source (Johnson and McGinnes, 1991). A representative of the latter subgroup is *T. acidophilus*, a facultative autotrophic sulfur compound oxidizer which can also grow on e.g. sugars (Hazeu et al., 1988; Pronk et al., 1990a). Apart from their role in the solubilization of sulfidic minerals, facultative autotrophic acidophiles are also crucial in cleaning up the environment. They allow the activity of obligate autotrophs, which are otherwise intoxicated by even very low concentrations (5-10 $mg.L^{-1}$) of low-molecular weight organic compounds (Monticello and Finnerty, 1985). These toxic effects are due to the uptake of organic compounds with carboxylic groups, which are undissociated in the outer medium with low pH, but are dissociated in bacterial cytoplasm with circum-neutral pH (Pronk et al., 1990a).

The above mentioned bacterial species are mesophilic (temperature optimum between 20-30°C). In the last two decades, thermophilic acidophiles have been described as well, with temperature optima around 50°C, some up to 70-90°C (Karavaiko, 1985; Larsson et al., 1990; Ghauri and Johnson, 1991). Recently, an extreme thermophilic species has been isolated which can even grow at temperatures above 100°C. These thermophiles have been isolated from "hot" low pH habitats, such as self-heating coal refuse piles and solfateras (Larsson et al., 1990). The phenomenon of self-heating is caused by the exothermic character of the pyrite oxidation, which is the major substrate for acidophiles in such environments (Bos and Kuenen, 1990).

In the literature, two mechanisms by which acidophiles attack the insoluble

metal sulfides are proposed, i.e. the indirect and direct leaching. In the indirect mechanism, the ferric iron acts as a chemical oxidizer of the sulfidic minerals. The ferric iron is a product of bacterial oxidation. In this mechanism, the biological sulfur oxidizing capacity of the acidophiles is of no relevance (Bruynesteyn, 1989; Evangelou and Zhang, 1995; Schippers et al., 1996). In the direct mechanism, sulfidic mineral solubilization involves both the biological ferrous iron and sulfur compound oxidation. For the oxidation of pyrite by *T. ferrooxidans*, evidence was found that both the biological oxidation of ferrous iron and sulfide are important (Arkesteyn, 1980; Hazeu et al. 1987; Walton and Johnson, 1992; Johnson et al., 1993).

It should be noted from Eqs. 2.IIIa-c and 2.IV, that acidity will be produced during elemental sulfur or sulfide oxidation. Consequently, the pH of the system will decrease steadily. This leads to a considerable slow-down of the chemical oxidation rate (Johnson et al., 1993; Evangelou and Zhang, 1995). Around pH 4 the rate of chemical oxidation will be surpassed by the biological oxidation rate, which has a much lower pH optimum. The pH will drop further and pH values lower than 2 can be reached (Plas et al., 1992). At this pH, the rate of the biological pyrite oxidation can be 5-6 orders of magnitude higher than the non-biological oxidation (Smith et al., 1988; Evangelou and Zhang, 1995).

2.2. SOURCES OF SOLID-STATE SULFUR POLLUTION

2.2.1. Atmospheric pollution

Industrial processes utilize large amounts of raw materials, which can contain considerable amounts of sulfur. Generally, industrial activity promotes an oxidation of sulfur to the tetra- or hexavalent states. In most cases, processes like refining, smelting or sintering end up with a sulfur dioxide-contaminated off-gas, which is either emitted to the atmosphere or cleaned using various pollution control techniques (Smet et al., 1997). The total amount of sulfur extracted from the lithosphere is 150 Tg sulfur per anum. From this, 93 Tg sulfur is annually released to the atmosphere (Brimblecombe et al., 1989).

The following types of industrial activities are the major emission sources of sulfur in the atmosphere (Brimblecombe et al., 1989):

a) Processing of sulfidic ores containing non-ferrous metals.
b) Ferrous metal production, including the production and use of coke.
c) Fossil fuel combustion for the production of heat or electricity.
d) Oil refining and processing of oil products.

e) Sulfuric acid production from native sulfur.

On a global scale, pyrometallurgical processes used to produce metals from sulfidic ores are the main source of atmospheric pollution by sulfur dioxide. The sulfur content of the sulfide ores of non-ferrous metals may sometimes even exceed the amount of extractable metal (on a molar basis). During ore-processing, sulfur is oxidized into sulfur dioxide. In some cases, the flue gases contain SO_2 in concentrations high enough to enable a profitable collection and re-use for the production of sulfuric acid (Andreae and Jaeschke, 1992). When SO_2 is not removed from the flue-gases, it dissolves in an aqueous phase in the atmosphere. This results in the formation of sulfuric acid, one of the major compounds responsible for acid-rains. Acidic precipitation promotes the weathering of bedrock and release of toxic metals, increases the sulfur deposition rate in the form of diffuse pollution, damages wildlife and forests, reduces the yield of primary agricultural production and harms constructions at paved areas (Howarth and Stewart, 1992).

Fossil fuels contain varying amounts of sulfur (Shennan, 1996). In solid fuels (like coal or lignite), it appears mostly in mineral and organic forms, eventually, in macromolecular complexes (Bouška, 1977). The major species of mineral sulfur is pyrite (FeS_2), eventually, marcasite, which has the same chemical formula but a different crystalline structure. Elemental sulfur (S^0) is only rarely found in coal. Organic sulfur is incorporated in the coal matrix with various forms of covalent bonds including thiols, mercaptans, sulfide and disulfide linkages and complex thiophene moieties (Monticello and Finnerty, 1985). The sulfur content in coal extracted all over the world is very variable, ranging from 0.05-15.0 % of weight, depending on its extraction site (Morozov, 1971; Bouška, 1977). Macromolecular organic complexes of sulfur are also present in crude-oil. The total sulfur content in petroleum ranges from 0.025-5%. Sulfur species like elemental sulfur, sulfate, sulfite, thiosulfate, and sulfide are present. Moreover, more than 200 different sulfur-containing compounds were isolated from crude oil (Monticello and Finnerty, 1985). The most generally used model substances for these organic-sulfur compounds are thiophenes, e.g. dibenzothiophene (DBT) (Kuenen and Robertson, 1993; van Afferden et al., 1993).

Combustion of fossil fuels leads to emissions of large amounts of sulfur dioxide (gaseous) and sulfur-containing particles. In large-scale industrial combustion, electrostatic precipitators can be applied to remove (partially) the particles in combination with scrubbers to remove gaseous sulfur dioxide (Brimblecombe et al., 1989; Vendrup and Sund, 1994). However, the removal efficiency of such devices is never complete. Moreover, a considerable part of these installations worldwide is not equipped with efficient treatment capacity. This results in a high anthropogenic flux of sulfur into the atmosphere (Table 2.2). Post-

combustion desulfurization presents nevertheless a considerable financial burden. For example, the annual cost of flue-gas desulfurization was estimated to exceed 2.8 billion US dollars by 1990 in the U.S. alone and may impose a 200-300 billion US dollars requirement on electricity consumers over the next 30 years (Monticello and Finnerty, 1985).

2.2.2. Pollution of aqueous environments

Apart from emissions of oxidized sulfur into the atmosphere, a second mechanism of sulfur release from solid-state sulfur-containing compounds can be distinguished. The dominant wastes of this type are mining wastes, spoils (inert soil excavated to get access to the utilizable layers of ore or coal), overburdens and waste resulting from ore enrichments (Bradshaw, 1993; Richards et al., 1993). Ongoing and abandoned mining sites rich in reduced sulfur compounds are traditional sources of pollution. Piles of low-grade ores, technological installations, dust and spoil present considerable sources of pollution (Bouška, 1977). Handling of spoil is a true crisis in the surface (open-pit) mining technology (Richards et al., 1993).

Two types of major negative aspects associated with the presence of solid-state sulfur compounds in aqueous environments may be distinguished:

A) Slow, steady release of sulfate, eventually sulfuric acid, into surface or ground-waters. This sulfur either contributes to the sulfate fluxes in rivers (Brimblecombe et al., 1989) or is retained in a reduced state within anaerobic zones of freshwater and marine sediments (Giblin and Wieder, 1992). Since sulfate wash-out rates are rather low and sulfate concentrations not dramatically higher than legal limits, this is a typical diffuse-source pollution problem.

B) Sudden release of sulfate upon oxidation of reduced sulfur-containing sediments following altered management practices or environmental changes. Sulfides retained within marshes or sediments do not pose significant environmental risks, until they are oxidized. Upon aeration, however, e.g. by dredging or lowering the water level, sudden disintegration of sulfidic precipitates occurs (Maass and Miehlich, 1988).

2.2.2.1. Spoil bank leachates

In open-pit mining, the organic topsoil is removed at first and stored for later re-use (Figure 2.2). In practice, this applies for the maximum top 30-50 cm of the soil surface. Below the topsoil, a mineral soil which covers the coal layers of

interest is encountered (Anonymus, 1993a; Richards et al., 1993). This material, called spoil, consists mainly of an inert mineral matrix. However, inclusions of carbonized organic matter and coal which contain organic sulfur and pyritic inclusions are found when approaching the coal layers (Anonymus, 1993b; Richards et al., 1993). These inclusions are very often disposed off with the spoil. Extraction of coal begins only after the spoil is removed. Since the spoil has no value for direct industrial purposes, it is transported onto permanent disposal sites and heaped into spoil banks. Amounts of produced spoil are usually very high. For example, during open-pit mining of lignite in Northern Bohemia (Czech Republic) in 1989, approximately 200 million m^3 of spoil was excavated and transported to the disposal sites, in order to mine 72 million tons of lignite. Although the coal mining currently declines, still 185 million m^3 of spoil was excavated and transported to produce 52.2 million tons of lignite in 1992 (Anonymus, 1994).

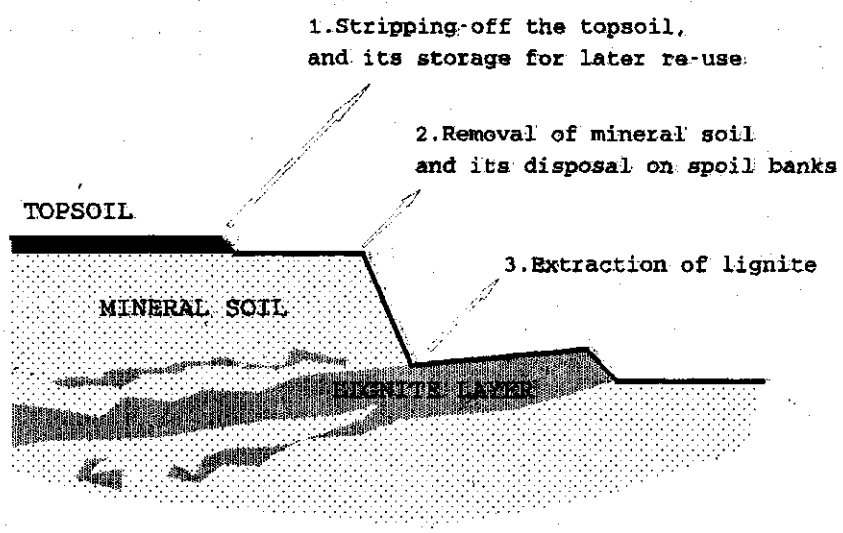

Figure 2.2 Technology of an open-pit extraction of lignite.

Within a relatively short time period (months-years), the surface of the spoil banks is rehabilitated by plants and trees, and a stable hydrological status develops (Bradshaw, 1993). However, due to the presence of reduced sulfur compounds, mainly pyrite, and, eventually, accompanying metals in the inner body of the heap, environmental risks may be encountered. A leachate from the spoil bank is formed following a series of reactions summarized in Eq. 2.IIIc (Richards et al., 1993; Evangelou and Zhang, 1995; Fortin et al., 1996). However, the age of most spoil banks over the world is too short to estimate the long-time scenarios of the development of spoil-bank leachate. Analysis of a leachate from a spoil bank Smolnická (district Karlovy Vary, Czech Republic) revealed elevated concentrations

of sulfate (2210 mg.L^{-1}), iron (18.4 mg.L^{-1}) and manganese (6.1 mg.L^{-1}). The pH values were circum neutral (7.35), i.e. the produced acidity is consumed by pH-buffering systems inside the spoil. At the same time, heavy metal concentrations were not substantially higher than the background levels. However, this spoil bank is relatively new, its heaping was finished in 1991 (data kindly provided by Karlovy Vary Municipal Department for the Environment). A similar study performed with a more matured spoil bank at Litov, near to Sokolov, Northern Bohemia, Czech Republic (finished in 1987), showed a more severe pollution. The leachate had pH of 6.5 and contained elevated levels of Al (170 mg.L^{-1}), As (0.03 mg.L^{-1}), Cd (0.1 mg.L^{-1}), Cu (2.85 mg.L^{-1}), Cr (0.11 mg.L^{-1}), Fe (618 mg.L^{-1}), Ni (2 mg.L^{-1}), Zn (9 mg.L^{-1}) (data kindly provided by Municipal Department for the Environment, Sokolov, Czech Republic).

Similar problems are encountered in landfills used for storage of coal-combustion ash and slag (Bradshaw, 1993). Ash and slag from coal conversion facilities contain considerable portions (0.3-4% of weight) of reduced sulfur compounds (Monticello and Finnerty, 1985). However, such wastes are mostly stored with special concerns, using hydrogeological isolation with central leachate collection and treatment.

2.2.2.2. Acid mine drainage

Acid mine drainage (AMD) is a specific type of wastewater, which arises in mining of sulfidic ores, mining of pyrite-containing coal, ore tailings, or overburdens (Lovell, 1983; Gray, 1996). AMD can be produced in large quantities, which considerably complicate its treatment. In the global planetary sulfur cycling (Table 2.2), the annual planetary flux of sulfur in AMD is 1 Tg, which is 0.6% of the total anthropogenic sulfur flux. In the U.S., the mining industry spends over 1 million US dollars per day to treat AMD (Evangelou and Zhang, 1995).

In the formation of AMD, the same mechanisms are involved as described for spoil bank leachates (see section 2.2.2.1.). Primarily, oxidation of pyrite and other sulfides yields Fe^{3+} and sulfuric acid (Evangelou and Zhang, 1995). Both spontaneous chemical oxidation and microbial processes (see section 2.1.3.) are responsible for the emergence of AMD. As described above, both processes may proceed even at anoxic conditions with Fe^{3+} acting as a chemical oxidizing agent, or with nitrate as an electron acceptor during microbial oxidation (Richards et al., 1993). Evangelou and Zhang (1995) reviewed other factors leading to the production of AMD, like ferrolysis or the presence of manganese and ferrous oxide. The typical composition of AMD is given in Table 2.3.

Table 2.3 Typical composition of acid mine drainage from a coal mine (Richards et al., 1993).

Constituent		Concentration
pH		3.0-5.5
Mg^{2+}	($mg.L^{-1}$)	80
Ca^{2+}	($mg.L^{-1}$)	200
Al_{total}	($mg.L^{-1}$)	50
Fe_{total}	($mg.L^{-1}$)	50-300
Mn^{2+}	($mg.L^{-1}$)	20-300
SO^{2-}_4	($mg.L^{-1}$)	20-2000

Compared to the spoil bank leachates (see section 2.2.2.1), AMD is a more concentrated wastewater (Table 2.3). The pH may even drop below 2. Apart from sulfuric acid, AMD contains considerable amounts of heavy metals. Metals are released as a result of direct solubilization of metal-sulfides and by acidic extraction of metals adsorbed on mineral surfaces (Bruynesteyn, 1989). *Thiobacillus ferrooxidans* was isolated in 90% of the streams in the vicinity of gold mines in Alaska, and concentrations of dissolved arsenic (presumably as a result of arsenic sulfide leaching from mine tailings) higher than 10 $\mu g.L^{-1}$ were found in 80% of these streams (Monticello and Finnerty, 1985). Moreover, AMD can be produced by a single source for long times, sometimes estimated up to hundreds of years (Richards et al., 1993), if no corrective measures are taken.

2.2.2.3. Anaerobic zones in soils and sediments

In natural environments, a considerable part of the soil is permanently or temporarily flooded with water, e.g. wetlands, freshwater, harbour or seawater sediments. The saturated water content in the pores and microbial respiration results in the formation of anaerobic conditions (Sweerts et al., 1989; Giblin and Wieder, 1992). Such zones often serve as a sink for different pollutants, including sulfur compounds. Due to the presence of both organic matter (mostly decaying biomass)

and sulfate, sulfate reduction (Eq. 2.I) occurs and an excessive amount of sulfide is formed. Sulfide precipitates immediately with the cationic species present in the system, like heavy metals and divalent iron (Herlihy and Mills, 1985; Wildeman and Laudon, 1989; Giblin and Wieder, 1992; Eger, 1994). This phenomenon is profound in areas experiencing high sulfur loadings, like in the neighbourhood of the mining industries, power plants using sulfur-containing coal, but also in areas periodically exposed to brackish or sea water (Giblin and Wieder, 1992).

In areas periodically inundated with salt water, accumulation of reduced sulfur compounds leads to the formation of acid sulfate soils (van Breemen, 1973). Considerable amounts of sulfide are accumulated in a sediment under high-tide, i.e. under anaerobic conditions. At low-tide, the insoluble sulfidic compounds are oxidized. This results in a release of sulfate and acidification (Giblin and Wieder, 1992; Marnette, 1993). Subsequently, soil minerals are solubilized by the acid. This yields elevated levels of aluminium in the soil solution, which can reach toxic concentrations for plants or crops (Begheijn et al., 1978; van Breemen, 1973).

Water sediments are vulnerable to changes in the redox-status. After being exposed to oxygen, reduced sulfur compounds oxidize. This is accompanied by the release of heavy metals and sulfuric acid (Maass and Miehlich, 1988). This occurred e.g. with Hamburg-harbour sediments, which were excavated and disposed off on a land surface. Within few days upon exposure to oxygen, the pH started to decrease from circum neutral to acidic ($pH < 4$) values. This was accompanied by increasing concentrations of cadmium (up to 0.1 $mg.L^{-1}$) and zinc (up to 10 $mg.L^{-1}$). The low pH values persisted for several weeks (in extreme cases, several months) and ended only after depletion of the stock of reduced sulfur compounds in the sediment. Afterwards, the pH raised again due to the presence of organic and mineral pH-buffering systems (Maass and Miehlich, 1988). Similar problems were encountered with river Rhine and Elbe sediments, and in many other places worldwide, where nautical reasons enforce a regular dredging of river bottoms (Rulkens et al., 1995).

2.3. BIOLOGICAL TREATMENT TECHNOLOGIES

2.3.1. Preventive measures and technologies

2.3.1.1. Suppression of the activity of thiobacilli

The inhibition of the activity of sulfur-oxidizing bacteria like thiobacilli would directly avoid environmental problems associated with leachates such as AMD. Installment of anaerobic conditions by simple inundation of a site would be an elegant way to deactivate these aerobic microbes (Evangelou and Zhang, 1995).

However, this method proves unsuccessful in practise since the oxygen concentration in a large body of infiltration water is rather complex to control. Surface streams, various terrain heterogeneties and underground cavities can mediate a supply of oxygen to the groundwater which still enables the oxidation of reduced sulfur. Moreover, solubilization of reduced sulfur compounds can also occur in anoxic environments in the presence of nitrate, see Eq. 2.IIIb (Driscoll and Bisogni, 1978). Furthermore, *T. ferrooxidans* is even able to grow anaerobically with sulfur as its electron donor and ferric iron as its terminal electron acceptor (Pronk et al. 1992).

Padival et al. (1995) investigated a possible control of *Thiobacillus sp.* by means of microbial competition. This strategy uses excessive concentrations of nutrients to favour microbes which grow faster than thiobacilli. Experiments carried out with a mixture of thiobacilli and yeasts fed with glucose and thiosulfate have shown a substantial reduction of activity of thiobacilli, and significant lowering of sulfate concentrations in the effluent. However, this method can be applied for prevention of corrosion of technological equipment, but its large-scale application for treatment of overburdens or abandoned mines is not realistic.

Another strategy is to inactivate acid-producing bacteria by biocides. Suppression of leaching was demonstrated using the bactericides Fluorspar and Kathon (Ondruschka and Glombitza, 1993). Fluorspar contains fluorides, which inhibit growth of *Thiobacillus sp.* at concentrations of minimum 400 mg $F^-.L^{-1}$. The isothiazolone Kathon inhibits the activity of thiobacilli at concentrations of 300 mg.L^{-1}. Application of both compounds to a pilot ore heap prevented leaching and no viable thiobacilli were found in the percolate (Ondruschka and Glombitza, 1993). However, the use of biocides cannot be recommended for full-scale applications because of the large amount of biocides required (and associated costs) and the risk of adaptation of the endogenous population.

Acidophillic thiobacilli are inhibited by sodium chloride at concentrations comparable to sea water (Cameron et al., 1984). Therefore, infiltration or pumping of sea water into a mine could inhibit microbial sulfide oxidation. On the other hand, some heavy metal-ions, like cadmium, form stable chloride complexes. This protects readsorption of metals to the spoil or organic matter. Therefore, chloride addition might considerably enhance the leaching process instead of preventing it (Salomons, 1993). An inhibition of growth and activity of thiobacilli fed with pyrite was also demonstrated with activated carbon (Loi et al. 1993). This effect is likely caused by sequestering most of the cells from the suspension. Subsequently, microbes could not attach to the pyrite surface, and the oxidation could not initiate.

Bioleaching can also be suppressed by preventing the bacteria to attach to the surface of sulfidic minerals by dosing surface-active chemicals. Attachment was found to be necessary for initiation of the microbial oxidation (Bryant et al., 1983; Karavaiko, 1985; Devasia et al., 1993; Evangelou and Zhang, 1995). Addition of

20 mg.L^{-1} of the tenside sodium paraffinsulfonat (E30) to the percolate of a pilot ore dump successfully inhibited the growth and activity of thiobacilli (Ondrushka and Glombitza, 1993). This strategy requires the quantification of the specific surface area of the sulfidic mineral, since the applied tenside concentrations should ensure full coverage of the mineral surface. In the reported paper, the specific concentration of E30 was experimentally determined at 0.016 mg per cm^2 of the ore-surface (Ondruschka and Glombitza, 1993).

At large scale, the use of bactericides or surface active agents is not realistic because of costs and possible malfunctioning of the treatment. Moreover, metals and sulfates can also leach out from non-sulfidic deposits, like jarosite precipitates, i.e. basic ferric sulfates $X_3Fe(SO_4)_2(OH)_6$, where $X=K^+$, Na^+, NH_4^+, H_3O^+ (Carlson et al., 1992), or can be desorbed (Scheeren et al., 1991; Richards et al., 1993). This means that in many cases the selective inhibition of thiobacilli will not yield successful results.

2.3.1.2. Microbial desulfurization

Sulfur emissions can be avoided by desulfurization of the raw materials. This approach has been studied in detail for the removal of sulfur from coal. Such a process may be an alternative to the desulfurization of the waste-gases from coal incineration. Numerous physico-chemical processes have been described for the removal of sulfur-containing inclusions from coal (Beddow, 1981; Bos and Kuenen, 1990). Density separation is most frequently proposed, since pyrite, the most commonly observed sulfur inclusion in coal, has a specific weight of 4.8-5.3 kg.dm^{-3}, which is considerably higher than the specific weight of coal (1.1.-1.3 kg.dm^{-3}) (Monticello and Finnerty, 1985). Eventually, for finely distributed sulfidic minerals, physical separation is performed using heavy-media sedimentation, high gradient magnetic separation or froth flotation. However, these processes require a very fine grinding of the material, sometimes down to particles of 10 μm (Beddow, 1981). Chemical desulfurization techniques are often energy intensive. Both oxidative (i.e. selective oxidation of sulfur species predominantly to SO_2), and reductive (i.e. reduction of the sulfur into sulfide-gas) methods can be applied. Other chemical desulfurization processes involve solvent extraction and chemical reactions at high temperature and pressure with carbonates, hydroxides of alkali metals or ferric iron (Monticello and Finnerty, 1985).

Microbial removal of sulfur from coal has been evaluated as a treatment alternative (Andrews and Maczuga, 1984; Bos and Kuenen, 1990; Stevens et al., 1993; Scott et al., 1993). Pyritic sulfur in coal is extracted via the activity of acidophillic thiobacilli, as described in Eq. 2.IIIc. Advantages of the microbial

desulfurization process are:

-Selectivity. The technique focuses fully on inorganic minerals present in the coal. No damage on the carbon skeleton of the coal, and thus no losses of its caloric value occur (Bos and Kuenen, 1990). Such losses are, however, frequently encountered during chemical desulfurization processes.

-Removal of heavy metals. Metal sulfides present in coal are solubilized by microbial action and low pH of the process liquor. Treatment of the process liquor containing heavy metals is more feasible than that of furnace slag or fly-ash, which are otherwise heavily contaminated.

-Ash reduction. Numerous minerals in coal are dissolved due to the low pH of the process liquor.

-Suitability for all types of coal (Bos et al., 1986; Bos and Kuenen, 1990).

On the other hand, the technique has certain disadvantages:

-Slow kinetics. A residence time of at least 4 days is required when mesophillic thiobacilli are used. Moreover, a substantial part of the biomass is adhered to the coal surface and is withdrawn from the reactor (Solari et al., 1992; Bailey and Hansford, 1993; Loi et al., 1993). This means that a configuration with a pre-cultivation of biomass may be required.

-Doubtful removal of organic sulfur. Certain types of coal may contain substantial amounts of organically bound sulfur, up to 50% of the total sulfur content (Bouška 1977; Friedman, 1990). This sulfur is not conceivably solubilized by acidophillic thiobacilli (Bos and Kuenen, 1990). This requires other bacterial species (Kertesz et al., 1994; van Afferden et al., 1993) including bacterial species fromn the genera *Pseudomonas*, *Rhizobium*, *Acinetobacter*, *Arthrobacter*, *Beijerinkia* or *Sulfolobus* (Monticello and Finnerty, 1985).

-Waste production. The acidic process liquor, formed during the desulfurization, requires further processing. This liquor contains sulfuric acid and extracted metals like iron, zinc, copper and cadmium. Biomass and other suspended matter can be present as well. The composition of this wastewater is strongly case specific. Specific techniques for the treatment of this water are mentioned in 3.2.1.

-Jarosite precipitation. At low pH and higher ionic strength, jarosites are formed (Carlson et al., 1992). Due to their insolubility, they may precipitate in the reactor system, thus reducing the sulfur removal efficiency. Therefore, the process control should avoid conditions of $pH < 2$ or elevated salt concentrations in the process liquor (Carlson et al., 1992; Bos and Kuenen, 1990).

-Heat production. The oxidation of pyrite is an exothermic process, yielding $\triangle H^0_r = -1481$ kJ per mol FeS_2. Therefore, high content of pyrite in the desulfurized coal may increase the reactor temperature, thus inhibiting the activity and growth of mesophillic thiobacilli. Therefore, reactors have to be cooled down. An alternative solution is the use of thermophillic process conditions and thermophillic thiobacilli

(Bos and Kuenen, 1990; Peeples and Kelly, 1993).

To obtain more information on the practical feasibility of microbial coal desulfurization, a pilot plant has been built at the premises of EniChem, Porto Torres (Sardinia, Italy) (Rossi, 1993; Loi et al., 1994). The pilot reactor consisted of a cascade of 7 mechanically agitated tanks with a working volume of 7 m^3 each (Figure 2.3). The capacity was about 1 tonne of pulverized (average diameter of 100 μm) coal per day at an operating temperature of 30°C. About 90% of the inorganic sulfur compounds was removed from the coal at a residence time of 5 days. Pulp densities up to almost 40% coal (w/v) appeared to be possible without interfering with the observed first order kinetics of the pyrite removal. However, the energy required for proper mixing of the coal slurry in the tanks, especially at higher pulp densities, appears to be an economical bottleneck limiting full-scale applications (Bos and Kuenen, 1990; Rossi, 1993; Loi et al., 1994).

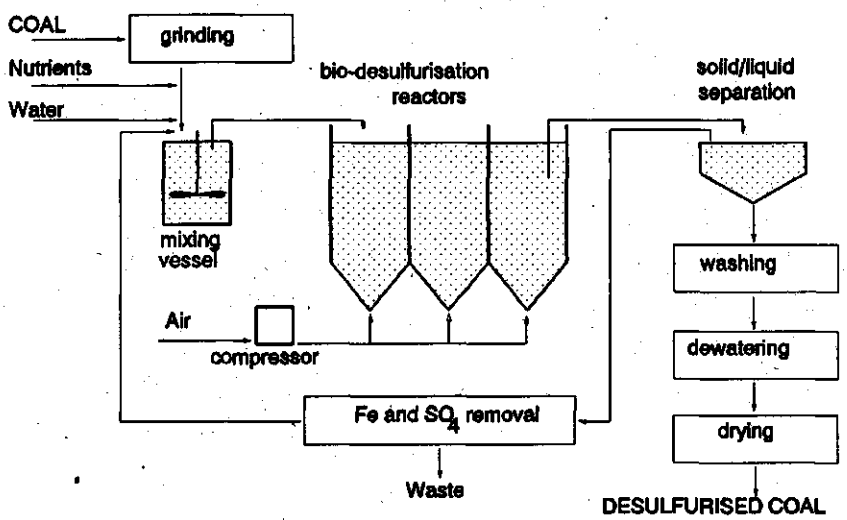

Figure 2.3 Reactor configuration of microbial coal desulfurization (Bos and Kuenen, 1990).

Besides the desulfurization of coal, also the microbial treatment of organo-sulfur containing gas and petroleum oil using *Thiobacillus ferrooxidans* can be applied. However, this process has not yet been used on industrial scale (Das et al., 1993). Most sulfur in crude oil and petroleum is present in a form of organic compounds, especially in aromatic thiophene linkages, e.g. dibenzothiophene (DBT). Microbial oxidation of DBT by *Pseudomonas* species with plasmid-encoded oxidative capacity is feasible using a reactor with permeable membranes to separate the oil and aqueous phases (Monticello and Finnerty, 1985).

2.3.1.3. Restoration/rehabilitation of overburdens and polluted landscapes

In areas which experience, or have experienced sulfur loadings, proper management of the landscape is necessary to control the sulfur stocks and flows. The first aim of landscape management is to prevent the lowering of the water level in sites which retain reduced sulfur compounds, like wetlands, river sediments, spoil banks, etc. In certain regions, atmospheric deposition and leaching of sulfur is unlikely to decline to zero in the near future. There, a permanent control and eventually the creation of new sinks is unavoidable (Bradshaw, 1993; Richards et al., 1993; Anonymus, 1993b).

Recultivation of overburdens or spoil banks is extensively applied to immobilize sulfur and metals, as well as to re-incorporate the polluted sites into the landscape (Cartwright, 1983; Richards et al., 1993). In the treatment of lignite-mining spoil banks, the first measure is the appropriate piling of the spoil material. Once the designed height of the spoil bank is nearly reached, fine minerals like clay or silt are used to finish the heaping. Afterwards, a layer of 20-30 cm of originally screened topsoil is spread on the surface of the heap (Richards et al., 1993). Subsequently, plants or trees are introduced to initiate the landscape recovery (Bradshaw, 1993). This way of rehabilitation disconnects the inner body of the spoil bank from its surface. Depending on the annual precipitation, the water regime of such sites recovers within a few months to several years (Anonymus, 1993a; Richards et al., 1993). However, a considerable pollution of the leachates can still be expected on a long time scale, since the hydraulic disconnection of the surface from the inner body is not permanent. Moreover, the ground water level rises due to hydraulic pressures in the spoil bank, creating further risks of sulfate and/or metals seepage (Richards et al., 1993). Therefore, proper management of such sites in order to reduce the leaching is still required and warrants further research towards effective treatment systems.

2.3.2. Biological remediation techniques

2.3.2.1. Treatment of spoil bank leachates and acid mine drainage

Anaerobic zones, i.e. wetlands and marshes, were already mentioned as a potential source of pollution , when exposed to aeration (see section 2.2.2.3). However, when these environments are maintained in anaerobic status, they offer one of the few available alternatives for the treatment of spoil bank leachates and AMD. Almost no energy and labour is required for its proper functioning, and it provides, apart from water-treatment, also other functions in the landscape, like

adding patchiness and diversity, or creating aesthetic units in the landscape (Reed et al., 1988). This applies especially in the areas heavily degraded by mining, which have to be reconstructed/rehabilitated.

Several pilot projects using wetlands for the treatment of AMD and similar leachates were successfully demonstrated worldwide (Wildeman and Laudon, 1989; Evangelou and Zhang, 1995). Basically, sulfate reduction is the major mechanism purifying the wastewater. The wastewater is neutralized and metals removed by precipitation and adsorption in the wetland sediment (Wildeman and Laudon, 1989). Moreover, interactions of the oxidized and reduced zones in the wetland, e.g. interface of aerobic rhizosphere with anaerobic surroundings, or occurence of an aerobic upper layer on the surface, enhances the removal of species soluble (reduced ferrous iron or manganese) or volatile (sulfide-gas) in strictly anaerobic conditions (Sweerts et al., 1989; Dunbabin and Bowmer, 1992).

In most of the presented studies, no extra organic matter had to be added as a substrate for sulfate reduction, since the wetlands were self-supporting due to the activity of photosynthesizing plants growing on their surfaces (Reynolds et al., 1991). However, after the retention capacity of a wetland is filled-up, the sediment will have to be properly regenerated. As the oxidation of sulfides occurs immediately (Maass and Miehlich, 1988; Eger, 1994), processing of such chemically unstable sediments may cause serious hazards (see section 2.2.2.3). Moreover, the heavy metal content in the sediments can easily trespass the legal standards for hazardous waste.

Eventually, a combination of wetland with passive limestone drains was proposed for the treatment of AMD. Limestone acts as a neutralizing agent (Bosman, 1983; Maree and du Plessis, 1994; Evangelou and Zhang, 1995), which leads to hydrolysis and precipitation of heavy metals. However, limestone does not retain sulfate and a wetland has to be applied consecutively. Alkaline fly ash and topsoil can be used as alternatives to replace the limestone (Jackson et al., 1993). Packed-bed column with manure, compost or similar materials rich in organic matter used as packing material can be used for the treatment of AMD as an alternative to wetlands (Farmer et al., 1995; Wildeman et al., 1995). A column packed with compost was able to treat a low-sulfate (350-550 $mg.L^{-1}$ of sulfate) mine drainage (Farmer et al., 1995). Hammack and Edenborn (1992) described a possible use of mushroom compost for the treatment of simulated AMD containing 2000 mg sulfate and 1000 mg nickel per litre. Basically, two removal mechanisms were distinguished: 1. the initial adsorptive/ion exchange removal, and 2. sulfate reduction. As for the wetlands, the basic mechanism of treatment on a long time scale in such columns is the process of sulfate reduction. However, it should be noted that the spent packing material must be carefully disposed off after its treatment capacity is depleted.

Few other alternatives are availble for the treatment of sulfate- and heavy metal-rich leachates. Since the presence of toxic metals receives the highest environmental attention, most technologies aim at the removal of metals, leaving sulfate out of consideration. Scheeren et al. (1991) described the use of an ion-exchanger, based on the exchange of metal cations with hydrogen ions in a column packed with ion-exchanging resin. However, this packing must be regularly regenerated, and the costs of process operation are usually rather high. As a treatment alternative, the liquid-membrane permeation process has been developed (Draxler and Marr, 1986; Lorbach and Marr, 1987). This process is based on mixing the wastewater with a specific solvent fluid in a counter-current mode. By breaking up the emulsion after the extraction, the metal-containing solvent may be separated from the water and properly regenerated. However, this process is costly and does not remove sulfate from the wastewater.

For combined sulfate and heavy metal removal, the most feasible alternative, besides wetlands, is the use of sulfate reduction in a granular sludge. This process has been applied on industrial scale (Scheeren et al., 1991). In this case, sulfate- and metal-contaminated groundwater is pumped to the surface, and treated in an upflow anaerobic sludge-bed (UASB) reactor, fed with ethanol as an electron donor for sulfate-reducing bacteria (Figure 2.4). In the effluent from the UASB reactor, liquid, gaseous, and solid phases are encountered. The liquid phase contains dissolved sulfide, which is treated in an aerobic fixed-film reactor. This results in formation of elemental sulfur, which can be separated from the main liquid stream by gravity settling (Lens et al., 1997). Fine, not easily settleable particles are removed by a subsequent sand filter. The gaseous phase contains sulfide, carbon dioxide and methane. Sulfide and carbon dioxide are removed in a scrubber by a $ZnSO_4$-solution. The purified methane is combusted in a flare. A compost filter is applied for the treatment of the off-gas from the fixed-film aerobic reactor. The solid phases, i.e. the sludge from the UASB-reactor, the precipitates from the gas-scrubber and the elemental sulfur from the fixed-film reactor, are centrally combusted in a high-temperature roaster. Based on a pilot installation (12 m^3 UASB reactor; 1.5 m^3 fixed-film reactor) operating as summarized in Table 2.4, a full-scale unit treating 300 $m^3.hour^{-1}$ (residence times in UASB and the aerobic fixed-film reactor are 6 and 0.55 hours, respectively) has been built (Scheeren et al., 1991). For voluminous sulfate rich waters, e.g. acid mine drainage, high-rate sulfate reduction systems have to be applied. Therefore, airlift reactors using pumice as a carrier material and hydrogen gas as electron donor for the sulfate reducing bacteria have been developed (van Houten et al., 1996).

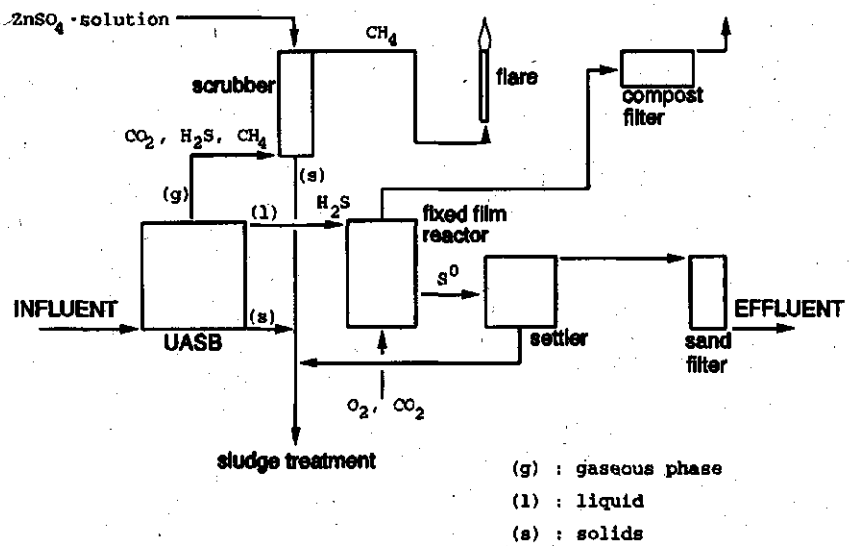

Figure 2.4 Block diagram showing a full-scale sulfate-reducing process for heavy metal removal from wastewater (Scheeren et al., 1991).

Table 2.4 Sulfur mass balance for a pilot plant using UASB and fixed-film reactors for removal of sulfate from ground water (Scheeren et al., 1991).

Component ($mg.L^{-1}$)	UASB influent	Fixed-film reactor influent	Effluent
Sulfate	450	220	150
Sulfide	0	245	5
Sulfur	0	0	290
Total	450	465	445

Process conditions: influent flow 1.5 $m^3.hour^{-1}$; air flow into fixed-film reactor 20 $m^3.hour^{-1}$; UASB off-gas flow 0.24 $m^3.hour^{-1}$.

2.3.2.2. *Treatment of anaerobic sediments and solid waste*

Numerous technologies have been developed for the treatment of contaminated sediments and solid wastes (Rulkens et al., 1995; Overcash, 1996; Tichý and Mejstřík, 1996). However, sulfur is not the major contaminating species of these wastes and the treatment techniques focus primarily on the remediation of heavy metals. They include various physico-chemical treatment processes, aiming either at preventing the contaminants to leach from the solid phase and to store it at a controlled storage place (=immobilization) or at removing the contaminant from the material and eventual re-use of the cleaned material (=clean-up). Immobilization techniques like cementation, solidification or vitrification are applied at large scale in practise (Richards et al., 1993; Rulkens et al., 1995). Clean-up techniques are primarily based on phase separation and treatment of the fine particles with the highest metal content. The separation is done using hydrocyclones, flotation units or fluidized-beds (Spottiswood, 1982; Rulkens et al., 1995). Also extractive clean-up using specific chemicals like mineral and organic acids or chelating agents to extract the metallic contaminants, have been demonstrated (Hinsenveld, 1993).

Despite the relatively broad spectrum of available clean-up technologies, only a few of them are customarily applied in practise. This is mainly due to their high costs, since operations like excavation, transport, extraction, separation, and post-treatment before disposal are costly. The total costs of the process may exceed 500 US dollars per m^3 of treated material (Carrera and Robertiello, 1993). Therefore, innovative remediation techniques are still under investigation.

One of these alternatives is the use of the microbial sulfur cycle (Iske and Glombitza, 1988; Clark and Ehrlich, 1992; van der Steen et al., 1992; Tichý et al., 1993a). Large scale technologies using thiobacilli are applied for bioleaching of non-ferrous ore enrichments and hydrogeological mining. Numerous combinations of bioleaching and physical treatment processes, like gravity-separation have been developed in this field (Bosecker, 1984; Richards et al., 1993; Rulkens et al., 1995). Similarly, bioleaching can be applied for the treatment of metal sulfide containing sediments or soils (van der Steen et al., 1992; Tichý et al., 1993a; Rulkens et al., 1995) and for leaching of metal-sulfide contaminated surplus sludge from wastewater treatment plants (Blais et al., 1992, 1993a, b; Couillard and Chartier, 1991, 1992, 1994; Couillard and Mercier, 1990; Sreekrishnan et al., 1993, 1996; Shreekrishnan and Tyagi, 1996; Tyagi et al., 1990, 1991, 1993). The goals of such a treatment process are:

A) Oxidation of metal sulfides into soluble metal sulfates.

B) Oxidation of excess sulfide, sulfur, thiosulfate, etc., which can substantially decrease the pH. Low pH values will inhibit a possible re-adsorption of ionic metals on mineral and organic binding sites.

C) Oxidation of divalent Fe^{2+} to highly oxidative trivalent Fe^{3+}. Trivalent iron will oxidize other metal sulfides.

During batchwise bioleaching of municipal sludge, a successive shift of two groups of bacterial populations was reported (Blais et al., 1993b). First, less-acidophillic species were abundant, growing at a pH between 4-6. The decrease of pH below 4 lead to the colonization by a more-acidophillic bacterial population, which continued the oxidation until pH 1-2. However, the reduced sulfur content in municipal sludge is mostly insufficient to reach a desirably low pH for optimum growth of thiobacilli and high metal extraction yields. Therefore, additional substrate for bacteria is required. Couillard and Mercier (1990, 1992) proposed reduced iron (Fe^{2+}) as such a substrate for thiobacilli. The oxidation leads to the formation of Fe^{3+}. Trivalent iron has a high oxidative power and mediates indirect leaching of metal sulfides (see section 2.1.3). Moreover, the formation of ferric hydroxide increases the acidity at pH values above 4.5 (Evangelou and Zhang, 1995):

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \qquad 2.V$$

Couillard and Mercier (1990) studied the efficiency of completely-stirred tank reactors (CSTR) and airlift reactors for the leaching of metals from contaminated sewage sludge (Figure 2.5). Two types of sewage sludge from aerobic municipal wastewater treatment were used, i.e. fresh sludge with no pre-treatment and anaerobically digested sludge. The anaerobic pre-treatment was superior because of pathogens removal, sludge volume reduction and the formation of metal sulfides, suitable for subsequent bioleaching. A sludge density of 2.9-3 % (w/v) was used at hydraulic retention times of 1-4 days. Substrate ($FeSO_4$) was supplied at concentrations of 1-3 $g.L^{-1}$. The highest leaching efficiency (62% for copper and 77% for zinc) was obtained at a residence time of 3 days. The effect of the $FeSO_4$ concentration on the leaching efficiency was small compared to the effect of the hydraulic retention time. A continuous system with sludge recycling proved more efficient compared to batch leaching, as the same metal-extraction efficiency was reached after 3 days in continuous systems and only after 8 days during batchwise leaching (Couillard and Mercier, 1990). An economic evaluation of the process of microbial heavy metal solubilization from sludge has shown that the costs of the technique are comparable to standard processes of polluted sewage sludge treatment like landfilling, incineration or controlled land disposal. The unit costs of this bioleaching process were estimated at 302-361 US dollars per ton of dry sludge (Couillard and Mercier, 1994).

Bioleaching of metal sulfides containing solid wastes and sediments possesses several advantages:

Excess sulfide is removed. Since metals are predominantly present in the sulfidic

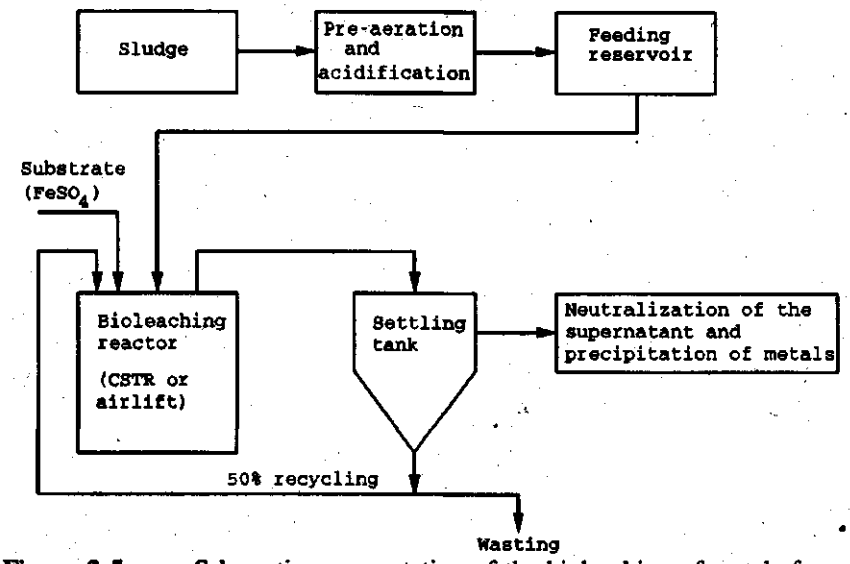

Figure 2.5 Schematic representation of the bioleaching of metals from contaminated sludge (Couillard and Mercier, 1990).

form, these compounds have to be solubilized. When mineral acid is used to dissolve the metal-sulfides, serious problems are encountered. Sulfide is volatilized from such an acidic system, thus creating malodour problems (Smet et al., 1997) and a considerable loss of acidity by volatilization of H_2S (Evangelou and Zhang, 1995). These problems are avoided in the bioleaching process, which oxidizes metal-sulfide and free sulfide, concomittantly, thus avoiding the above mentioned problems.

Thiobacilli are usually present. In most cases, no inoculation by thiobacilli is required. Sediment from a pool receiving AMD usually contains thiobacilli before the bioleaching process starts (Herlihy and Mills, 1985). Even in case of dredged river sediments, the sulfide oxidation initiates spontaneously within a few days of exposure to air (Maass and Miehlich, 1988).

However, bioleaching provides also certain drawbacks:

Insolubility of lead sulfate. Since the solubility product of lead sulfate is very low ($\log K_{sol.} = -7.79$), this metal will not be solubilized (Evans, 1989). This is usually not a problem for AMD recipients, since lead is not present there. However, sediments contaminated by industrial waste-streams may still contain lead after bioleaching. This imposes the need for an additional post-treatment, e.g. using hydrochloric acid or EDTA extraction (Brown and Elliott, 1992; Rulkens et al., 1995; Mercier et al., 1996).

Biomass is attached to the solid phase. In case thiobacilli are not indigenously present in the sediment, a significant portion of newly-grown biomass attached to the solid phase will be withdrawn from the system upon removal of cleaned material

(Solari et al., 1992; Bailey and Hansford, 1993). Therefore, longer residence times will be required or a two stage reactor will be needed: the first reactor for biomass production, followed by the second leaching reactor (Tichý et al., 1993b).

The growth of thiobacilli can be also stimulated by feeding them microbially produced elemental sulfur (Chapter 3). This sulfur is produced during biological sulfide removal from wastewater under oxygen-limiting conditions (Janssen et al., 1995). Microbiologically produced sulfur possesses advantageous properties for the use as a bioleaching substrate (Chapter 3), particularly because of its higher specific surface area and the better dispersability in water compared to the commercially available orthorhombic elemental sulfur (sulfur flower). This is due to the hydrophillic surface characteristics and the small size ($\pm$100 nm) of microbiologically produced sulfur particles (Chapter 3; Janssen et al., 1996). As a result, thiobacilli grow faster on the biologically produced sulfur and convert it at a higher rate. This is demonstrated by the acceleration of the sulfate production during the batchwise cultivation of thiobacilli with both types of sulfur as substrate (see Chapter 3). Thus far, no scale-up of the bioleaching process using microbially-produced sulfur has been demonstrated.

As stated in Chapter 1, the use of microbially-produced elemental sulfur for bioleaching indicates the large possibilities for technological applications using the microbial conversions of the sulfur cycle (Figure 1.1). After the bioleaching process, a solid/liquid separation can be applied. Spent liquor containing metals, acidity and sulfate can be treated using anaerobic sulfate reduction. This results in the production of sulfide, which precipitates heavy metals from the aqueous phase (Cowling et al., 1992; Dvorak et al., 1992, van Houten et al., 1994). Eventually, excess sulfide can be further treated by partial sulfide oxidation to elemental sulfur (Buisman et al., 1990, 1991; Janssen et al., 1995). In this way, sulfur is recycled within the treatment system. Figure 1.1 shows that manipulation of the sulfur fluxes and redox conditions allows a separation and concentration of heavy metals in a solid phase, which can be further processed or disposed off in a controlled way.

2.4. CONCLUSIONS

Solid-state sulfur-containing compounds can present considerable risks in the environment. By numerous mining and industrial activities, man changed the global planetary sulfur cycling. This resulted in accumulation of reduced sulfur compounds in natural wetlands, river sediments and mining overburdens. The main environmental risks of such a reduced sulfur abundance are the release of associated toxic heavy metals and the acidification of the environment upon their oxidation. In this respect, two types of environmental risks can be distinguished: 1. sudden,

unexpected oxidation of sulfur and fast leaching of metals, e.g. in river sediments raised above the water table; and 2. steady release of heavy metals and sulfate at moderate concentrations for a prolonged period of time (up to years-decades), e.g. acid mine drainage and similar leachates.

Two strategies can be applied for the abatement of sulfur and/or heavy metal pollution: 1. reduction of the solubilization of sulfur and subsequent suppression of metal-release; and 2. removal of the contamination from the environment. The solubilization can be minimized by selective inhibition of the microbes responsible for sulfur oxidation, or by proper management of the polluted sites (e.g. recultivation and rehabilitation). The *in-situ* removal of contaminants is rather complicated, since solid-state sulfur pollution is often present in a diffused form. Removal of solid state sulfur from soils or anaerobic sediments is possible using commercially available *ex-situ* treatment techniques, of which bioleaching is an emerging technology. Successful treatment of wastewaters like overburden run-off waters, acid mine drainage or spoil bank leachates can be done in wetland systems, limestone drains combined with wetlands or by anaerobic (UASB, gas lift) reactors optimised for sulfate reduction. The combination of various techniques using microbial sulfur conversions is indispensable for the abatement of the negative effects of pollution due to reduced-sulfur compounds.

CHAPTER 3

POSSIBILITIES FOR USING BIOLOGICALLY-PRODUCED SULFUR FOR CULTIVATION OF THIOBACILLI WITH RESPECT TO BIOLEACHING PROCESSES

Abstract: *The growth of a mixed culture of thiobacilli was evaluated in batch cultivations using two different types of elemental sulfur, i.e. commercially available sulfur flower and a dried biologically produced sulfur. The latter material is produced by a partial oxidation of sulfide under oxygen limitation by a mixed culture of neutrophilic thiobacilli. This process is applied in practice to remove sulfides formed during treatment of sulfate containing wastewater. The biologically produced sulfur can be removed from the process water by sedimentation. Its reuse may become important once the application of the biological sulfur cycle is considered for the removal of heavy metals from a contaminated environment. Biologically produced sulfur oxidized significantly faster than sulfur flower, resulting in higher rates of sulfuric acid production. With biological sulfur pH 1.5 was reached after 65 hours, whereas with sulfur flower this pH was obtained after 160 hours of cultivation. The biological sulfur oxidation was accompanied by a disintegration of sulfur particles, resulting in a high homogeneity of the substrate in a growth medium. The presented findings indicated that the potential of bioleaching techniques may benefit from the reuse of biologically produced sulfur.*

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3.1. INTRODUCTION

Some acidophilic bacteria like thiobacilli can utilise reduced sulfur compounds as energy sources. In practice, this phenomenon is used for biological mining of some non-ferrous metals and for the biological desulfurization of coal (Karavaiko et al., 1988; Martinek et al., 1983). Recently, the use of bioleaching has also been proposed for decontamination of solid wastes or soils (van der Steen et al., 1992; Couillard and Mercier, 1992; Tichý et al., 1993b; Blais et al. 1993b). Particular interest is paid to the possible application of the sulfur cycle, i.e. the integrated use of processes of sulfur oxidation and reduction (Martinek et al., 1983; Tichý et al., 1993a).

The production of acid is a limiting step for the bioleaching process using sulfur as a substrate (Tichý et al., 1993b). The kinetics of acid production was reported to be strongly influenced by the hydrophobic character of elemental sulfur flower (Agate et al., 1969). Since the proper dispersion of the sulfur flower in a cultivation reactor is difficult, insufficient contact between bacteria in suspension and the substrate reduces the sulfur oxidation rate (Solari et al., 1992). Although thiobacilli can produce surface-active agents which enhance the sulfur oxidation (Jones and Starkey, 1960; Takakuwa et al., 1979; Bryant et al., 1983), the acid production rates are still low and industrial applications of this process are doubtful.

For these reasons, the application of biologically produced elemental sulfur might offer distinct advantages. This material can be produced by microbially mediated partial oxidation of sulfide in conditions of sulfide overloading or oxygen limitation (Buisman et al., 1990). A mixed culture of neutrophilic thiobacilli is capable to oxidise the sulfide into sulfur without the formation of any sulfate. This process is applied for the removal of sulfide formed during the anaerobic treatment of sulfate containing waste water (Buisman et al., 1989). The reuse of biologically produced sulfur should be stimulated to avoid its deposition as a chemical waste.

The physico-chemical characteristics of the biological sulfur are still not completely understood but it is believed to be predominantly in the zero oxidation state (Steudel, 1989). Adsorption of polythionates or microbial surfactants may result in the sulfur particles becoming hydrophilic (Moriarty and Nicolas, 1970; Steudel et al., 1989, Janssen et al., 1994). Moreover, formation of aggregates in a continuously-stirred reactor (Janssen et al., 1994; 1995) contributes to the complex character of this material. In the present work, the use of such biologically produced sulfur ("biological sulfur") for feeding acidophilic thiobacilli was compared with the commercially-available, orthorhombic sulfur, flower.

3.2. MATERIALS AND METHODS

3.2.1. Organisms and cultivation media

A mixed culture of acidophilic *Thiobacillus spp.* D_2 was obtained from the Department of Microbiology and Enzymology, Delft University of Technology, The Netherlands. It comprises small Gram-negative rods growing singly or in short chains on pyrite. No further attempts to characterize this culture have been done. The acidophilic culture D_2 is not further characterised. All cultivations were performed in a modified 9K medium (Silverman and Lundgren, 1959), containing per litre of demineralized water: $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 0.6 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.1 g; $Ca(NO_3)_2.4H_2O$, 0.06 g; $FeSO_4.7H_2O$, 11.0 mg; $ZnSO_4.H_2O$, 0.7 mg; $MnCl_2.2H_2O$, 2.0 mg; $CoCl_2.6H_2O$, 0.6 mg; $CuSO_4.5H_2O$, 0.6 mg; $NaMoO_4$, 0.8 mg; H_3BO_3, 2.0 mg; KI, 0.2 mg. The medium was acidified with H_2SO_4, 0.1 mmol per litre. The growth medium was autoclaved at 120°C for 20 min. and substrate was added aseptically afterwards. Solid substrates, i.e. pyrite or sulfur, were sterilized in 96% ethanol (Karavaiko et al., 1988).

The mixed culture (D_2) was maintained on pyrite, 2 $g.L^{-1}$ (Karavaiko et al., 1988), at 30°C. Before the culture was used for the experiments, it was cultivated on the sterilized sulfur flower (2 $g.L^{-1}$, i.e. 62.31 $mmol.L^{-1}$) for 4 days to adapt the thiobacilli to elemental sulfur.

3.2.2. Culture

Experiments were carried out in sterilized batch columns, 10 cm diameter, 1000 mL working volume, in the dark at 30°C. Aeration and mixing were performed by sparging sterile pre-wetted air at a flow rate of 3000 $mL.min^{-1}$ through a sinter disc. Ten grams of sulfur flower or dry biological sulfur was used per 1000 mL of modified 9K medium. The columns were inoculated with a sulfur-adapted suspension of D_2 culture (S^0 content in the inoculum below 0.03 $mmol.L^{-1}$). To prepare an inoculum suspension free from sulfur particles, the culture was grown on the sulfur flower for 120 hours. Subsequently, the suspension was filtered using sterilized paper filter (Schleicher & Scheuell $595^{1/2}$, Germany). The filtrate was further cultivated for 24 hours. The inoculation was performed at a volumetric ratio 1:50. Cultivation and sampling were done under aseptic conditions. All data are presented as means from three independent experiments.

3.2.3. Sulfur source

Sulfur flower (analytical grade) was obtained from Fluka, Germany. Biological sulfur was taken from the effluent of a continuous, oxygen-limited, CSTR-reactor fed with Na_2S, as described previously (Buisman et al., 1990). At a pH value of 8, sulfide is partially oxidized into elementary sulfur under conditions of oxygen limitation by a mixed culture of autotrophic, neutrophillic thiobacilli. As a result, insoluble particles are formed in the medium which can be separated by sedimentation. Hereafter the sulfur particles were separated from the effluent by centrifugation (20 min., 2000 rpm) and washed twice with a demineralized water (100 mL per 1 gram) and dried at 45°C, and sterilized by 96% ethanol (100 mL ethanol per 10 g of biological sulfur). A nylon sieve was used to exclude particles bigger than 0.5 mm in diameter. Biological sulfur contained 91.2% (± 3.3) of sulfur, determined by incineration at 620°C, as described by (Hordijk et al. 1989). The composition of the remaining mass is unclear yet. It is believed to comprise mineral salts and fractions of microbial biomass.

3.2.4. Chemical analyses

The pH was measured using a combined glass electrode (Schott-Geräte H32A). The titrimetric evaluation of acidity was performed as well, although these data did not provide any additional information.

The concentration of sulfate in the medium was determined by ion-exchange HPLC after a high-speed centrifugation (5 minutes, 15000g) to remove colloidal and suspended solids. The samples were frozen (-18°C) and stored before analysis. A Chrompack column was used, filled with Vydac 302-IC (25 cm). Potassium biphtalate (0.027 M) was used as an eluent at a flow of 1.2 $mL.min^{-1}$. A Knauer differential refractometer was used as detector. The injection volume was 20 μl. This method could have detected thiosulfate as well, however, it was never encountered.

Samples for the analysis of elemental sulfur were centrifuged (5 minutes, 15000g) followed by careful decantation and drying overnight at 30°C. The residues obtained were extracted with acetone for 3 days, after which the extracts were analyzed by reversed-phase chromatography using the procedure of Möckel (1984). The samples were diluted to give a maximum sulfur concentration of 200 $mg.L^{-1}$. The HPLC equipment contained a C18 column (2*10 cm), 96:4 methanol/water as a mobile phase (flow was 1.00 $mL.min^{-1}$) and UV detector at 254 nm. Standard solutions of S_8 (orthorhombic sulfur) in acetone were used for calibration. To

improve the solubility of the standard sulfur flower a few drops of CS_2 were added before the measuring flask was filled up with acetone.

Since the presence of sulfur particles interferes with most of the standard methods for biomass determination the amount of biomass was expressed in terms of organic nitrogen after (Novozamsky et al., 1983). Twenty five millilitres of suspension from the cultivation column was centrifuged (10 minutes, 15000g), and washed twice with aliquots of demineralized water. The resulting pellets were frozen (-18°C) and treated as described below. After adding 2.5 mL (96%) sulfuric acid/selenium mixture to the pellet in a destruction tube, the volume was reduced by evaporation at 110°C. Samples were afterwards treated by 6 times repeated addition of 1 mL of H_2O_2 (30%) to remove the easily-oxidisable organic matter. Subsequently, the heat destruction took place at 330°C for three hours. After cooling down, the ammonium-nitrogen content was determined spectrophotometrically.

3.2.5. Physical analyses

Non-filterable sulfur was determined after the filtration of a sample through paper filters (Schleicher & Scheuell No. 595$^{1/2}$, Germany). Single particle optical sizing (SPOS) was used to evaluate changes in the distribution of fine particles. The equipment was home-made and described earlier by (Pelssers et al. 1990). Sulfur particles pass one-by-one through a pulsing laser beam while the reflections are measured under a small angle (5°). The intensity of the scattered light depends on the particle size. Due to the methodological uncertainties, the size of particles is expressed in reading channels. Higher reading channels correspond to bigger particles (Pelssers et al., 1990). Truth calibration for our system was not available because the refractive index of biological sulfur solids is not yet known. The range of sulfur particles size was about 1.2 μm (diameter) at the reading channel 60, and 4-6 μm at the reading channel 150-200. The relative diameter was estimated using light microscopy (data not shown). However, this size range can serve only for a tentative estimation, since the surface and shape of the sulfur particles make the calibration uncertain.

Only fine sulfur particles can be determined by the SPOS method, particles which settle within 3-5 minutes were not recorded by the apparatus. To include bigger particles into the measurements, the sedimentation velocity was measured. Sedimentation kinetics was determined gravimetrically, using a one-litre cylinder with a hanging scale (see Figure 3.1). Kinetics of particles sedimentation was recorded from the weight increase. After 60 minutes of measurement, the suspension was transferred back to the aerated cultivation column and the cultivation continued.

Although the equipment was thoroughly washed each time before the measurement, sterility could not have been ensured during these experiments. We supposed that rather extreme growth conditions of the cultivation limited the risk of contamination. Light microscopic observations using Gram-staining did not reveal the presence of other morphological types of cells. To perform the sedimentation analysis without bacteria, demineralized water at pH 1.5 (H_2SO_4) was used instead of modified 9K medium. Preliminary tests showed that the mineral salts of the modified 9K medium did not affect the sedimentation characteristics of the biological sulfur suspensions (data not shown).

Hydrophobicity of the biological sulfur particles was evaluated using a two-phase system of water and hexadecane, as described by Rosenberg (1984).

A comparison of specific area of chemical and biological sulfur was performed after freeze-drying for 12 hours using nitrogen adsorption (Quanta Chrome, 1000). The result presented is an average of three independent measurements.

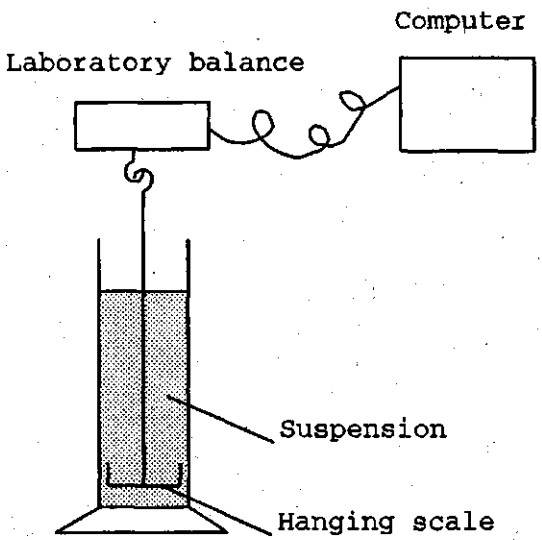

Figure 3.1 Sedimentation velocity measuring device.

3.3. RESULTS AND DISCUSSION

Results of the batch cultivations using commercially-available sulfur flower and biological sulfur are summarized in Figures 3.2a-c. The observed acidification of the growth medium was substantially faster with biological sulfur than with the sulfur flower (Figure 3.2a). With biological sulfur pH 1.5 was reached after 65 hours, whereas with sulfur flower this pH was obtained after 160 hours of cultivation. Light microscopic observations at the end of cultivation (200 hours) revealed an almost complete absence of sulfur particles in suspensions of biological sulfur, whereas considerable amounts of sulfur particles were still observed in variants with sulfur flower. To quantify this effect, final levels of elemental sulfur (S^0) in suspensions were determined. Concentrations of S^0 at the end of cultivation were 0.225 mmol.L^{-1} ($\pm$0.083 mmol.L^{-1}) for biological sulfur and 14.424 mmol.L^{-1} ($\pm$0.203 mmol.L^{-1}) for sulfur flower.

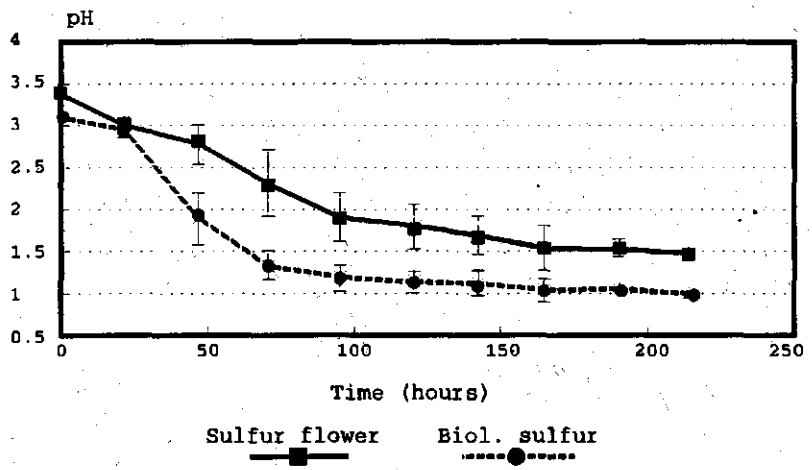

Figure 3.2a pH changes during the batch cultivation of thiobacilli using the two sulfur sources.

The sulfate concentration in the liquid phase showed the opposite trend (Figure 3.2b). As expected from the acidification rates, the measured sulfate levels in medium with biological sulfur were significantly higher. The amount of oxidized elemental sulfur was calculated from the sulfate production, supposing that one mole of SO_4^{2-} is produced from one mole of oxidized S^0. This theoretical amount of oxidized sulfur ($S^0_{theor.}$) was used for the evaluation of growth yield and metabolic activity. However, this assessment might lead to an underestimation of the yield, due to the possible presence of S-compounds with different oxidation states (Steudel et al., 1989; Pronk et al., 1990b).

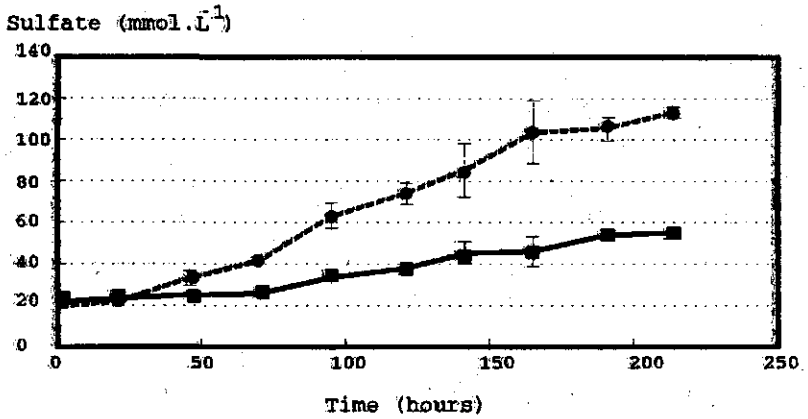

Figure 3.2b Changes in sulfate concentrations in medium during the batch cultivation of thiobacilli using the two sulfur sources (symbols are explained in Figure 3.2a).

The biomass growth in terms of the increase of biomass nitrogen is shown in Figure 3.2c. Although the final concentrations of $N_{biomass}$ in the case of biological sulfur reached 45.22±1.15 mg.L^{-1}, i.e. exceeding twice the amount of $N_{biomass}$ achieved with sulfur flower (20.59±2.01 mg.L^{-1}), the maximum growth rates did not differ substantially. The maximum growth rates achieved with biological sulfur and sulfur flower were 0.082±0.005 h^{-1} and 0.080±0.002 h^{-1}, respectively. This growth is in good agreement with findings published by other authors. Guay and Silver (1975) and Pronk et al. (1990a) reported a maximum growth rate for thiobacilli on thiosulfate of 0.084 h^{-1}, Blais et al. (1993b) found a maximum growth rate of thiobacilli on sulfur flower in the range of 0.067-0.104 h^{-1}. Maximum growth rates were achieved with both sulfur sources within 40-48 hours of cultivation. After this period, the growth rates dropped substantially; possibly being affected by substrate depletion and pH drop (Sreekrishnan et al. 1993) at the same time. However, the decrease of growth rate with biological sulfur was slower than that with sulfur flower. Within 90-140 hours of cultivation, growth rates of thiobacilli using biological sulfur were 0.012-0.017 h^{-1}, and only 0.004-0.007 h^{-1} using sulfur flower. Final growth yields achieved at the end of the batch cultivation were similar: 0.459±0.009 mg $N_{biomass}$ per mmol of oxidized sulfur in the case of biological sulfur and 0.590±0.005 mg $N_{biomass}$ per mmol S_{ox} in the case of sulfur flower. At the beginning of the cultivation, the specific rate of sulfur oxidation, expressed in millimoles of oxidized elemental sulfur per hour and per mg of biomass $N_{biomass}$, was

higher with biological sulfur than with sulfur flower. The maximum specific oxidation rate found with biological sulfur was 0.498 mmol $S_{ox.}$ .mg^{-1} $N_{biomass}$ .h^{-1}, which is almost twice as high as the maximum value found with sulfur flower, i.e. 0.274 mmol $S_{ox.}$ .mg^{-1} $N_{biomass}$ .h^{-1}. The specific sulfur oxidation rate decreased after 100 hours for biological sulfur and 70 hours for the sulfur flower, reaching about equal plateau values of 0.012-0.013 mmol $S_{ox.}$ .mg^{-1} $N_{biomass}$ .h^{-1}.

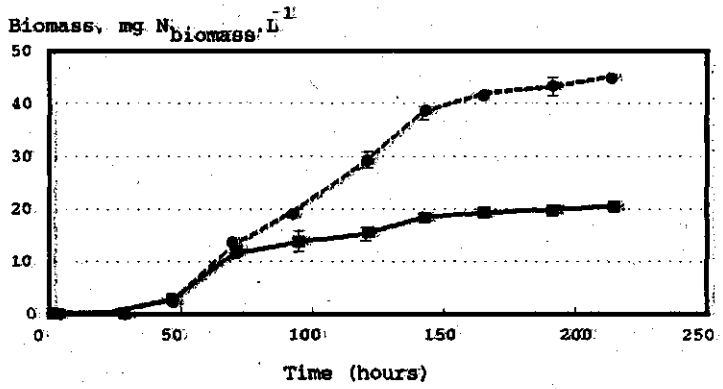

Figure 3.2c Changes in biomass concentrations during the batch cultivation of thiobacilli using the two sulfur sources (symbols are explained in Figure 3.2a).

The observed faster initial oxidation of biological sulfur relative to the chemical sulfur flower can be explained as follows:

1) The higher hydrophilicity of the biological sulfur surface allows for a better contact with water and bacteria and a better homogeneity of the suspension. This effect was evaluated using a two-phase partition test in hexane and water (see Figure 3.3). The lighter hexadecane forms the upper phase above the heavier water. After intensive shaking and separation of the phases, particles of sulfur flower (left) were present in the (hydrophobic) hexadecane phase, sinking to its lower part, whereas all the particles of biological sulfur (right) remained as a suspension in water, or at the phase interface. Several authors have reported on the crucial role of adhesion of bacteria in the sulfur oxidation process (Jones and Starkey, 1960; Agate et al., 1969; Takakuwa et al., 1979). The adhesion of thiobacilli may be accompanied by the formation of exopolysaccharides or wetting agents to make the hydrophobic surface hydrophilic (Bryant et al., 1983; Cook,

1964). Since biological sulfur is already hydrophilic, a rapid adhesion might be allowed, resulting in faster acidification.

2) The particles of biological sulfur may not merely consist of elemental sulfur rings (S_8), but probably also of other reduced sulfur compounds, like polysulfides. These compounds may contribute to the faster oxidation rates at the beginning of cultivation since they are oxidized faster by thiobacilli (Pronk et al., 1990b).

3) The difference in the accessibility of biological and chemical sulfur may also be attributed to different physical and mechanical properties of the sulfur particles. The process of sulfur oxidation by thiobacilli depends on the adhesion of bacterial cells to the sulfur surface (Solari et al. 1990). Therefore, the differences in specific surface, and the particles' architecture, may mediate different availability of the two sulfur types. Determination of the specific area by nitrogen adsorption indeed showed that biological sulfur has a higher specific area (S.A.) than chemical sulfur flower. S.A. of 2.5 $m^2.g^{-1}$ was determined for biological sulfur, whereas the S.A. for sulfur flower was below the detection limit (0.01 $m^2.g^{-1}$). The significantly larger surface of biological sulfur may, next to its higher hydrophilicity, be another explanation for its better bio-availability.

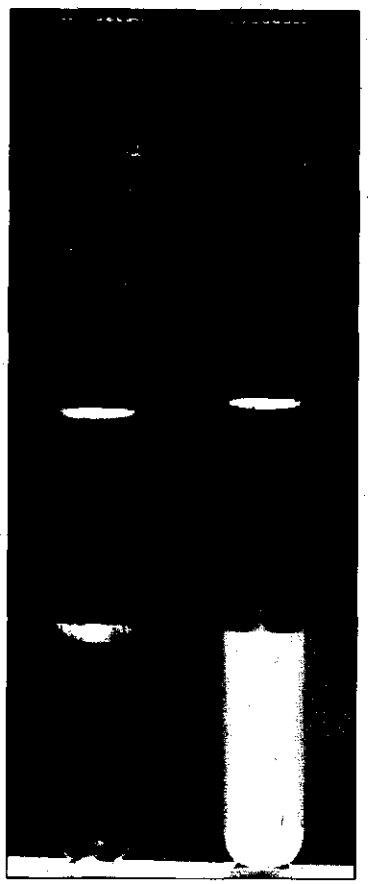

Figure 3.3 Hexadecane/water partition test of sulfur flower (left) and biological sulfur (right).

However, no information is available about the structure of the biological sulfur. During the process of biological sulfur production, agglomeration of sulfur particles was observed (Janssen et al., 1994). A lower mechanical rigidity of such agglomerates might contribute to the higher oxidation rates. The shear forces during

aeration/agitation in a reactor can break unstable particles or their aggregates, creating thus finer particles with higher specific surface area and thus enabling further sulfur oxidation. This phenomenon was manifested by the occurrence of very fine, non-filterable elemental sulfur particles in the medium (Figure 3.4). A substantial increase in this fine fraction of S^0, expressed as the sulfur content passing through the paper filter, was found with biological sulfur within the first 60-70 hours of cultivation. It corresponded with the above mentioned changes in specific rate of sulfur oxidation. These data suggest that fine particles were being formed during the initial stages of the cultivation, either by shear forces due to gas mixing, or by destruction of the particle aggregates by biological/chemical oxidation. After 60-70 hours, the production rate of these very fine particles was most probably exceeded by the consumption due to the biological oxidation, resulting in a rapid decrease of the very fine sulfur fraction. Such a production of very fine sulfur was not observed in the experiments with sulfur flower. Here the concentration of very fine sulfur increased slowly, reaching its final value after 70-80 hours (Figure 3.4).

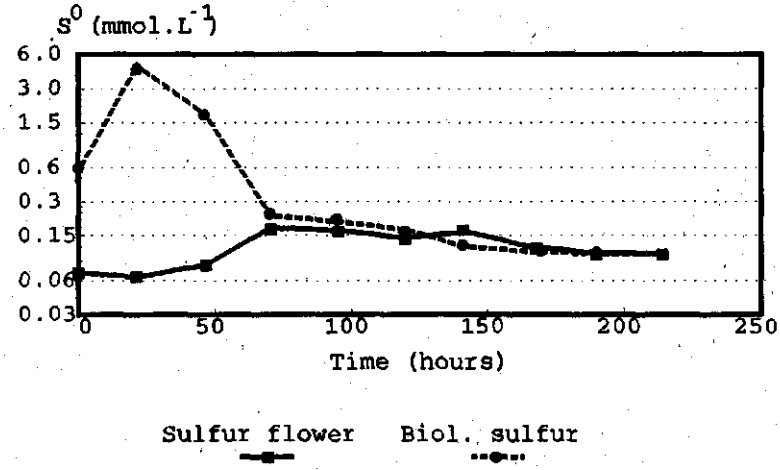

Figure 3.4 *Concentrations of non-filterable elemental sulf in a medium.*

These findings were confirmed by using the single-particle optical sizing (SPOS) method. The results of measurements using biological sulfur with and without thiobacilli are presented in Figure 3.5. The total concentration of particles increased both in the bacterial suspension and in the non-inoculated control within the first 50 hours of the experiment, although for the sterile control no change was observed for the amount of the finest fraction channel (0-50). This means that the fraction larger than 6 μm, which could not be detected by SPOS, was mechanically broken into smaller aggregates. The process was virtually independent of the

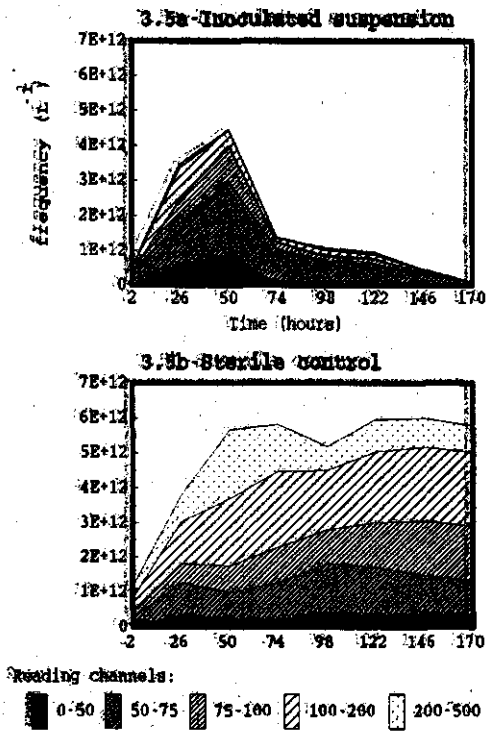

Figure 3.5 SPOS analysis of a distribution of biological sulfur particles with (top) and without bacteria (bottom). Size of particles is increasing with the increasing reading channel.

presence of thiobacilli. Although the process was accompanied by a rapid drop in pH in the presence of thiobacilli and constant pH in the sterile conditions, pH within the range 1-4 did not have any significant effect on the distribution of particles, as was proved by adding sulfuric acid to the sterile control samples (data not shown).

Sedimentation analysis confirmed the suggested rapid decrease in concentration of easily settleable particles during the initial stages of air mixing of the biological sulfur suspension (Figure 3.6). In the sterile control a rapid sedimentation within the first ten minutes was observed at the beginning of the cultivation. The sedimentation velocity subsequently slowed down, and ultimately followed nearly a linear increase in time. After 1 day of cultivation, the amount of particles settleable within the first 10 minutes decreased: i.e. big particles were split into smaller ones. After more than 3 days of cultivation, no further difference in sedimentation velocity of the suspension without bacteria (Figure 3.6b) was observed. This indicated a merely constant particle size in the suspension. With thiobacilli, on the other hand (Figure 3.6a), the sulfur particles were constantly

becoming smaller. The slope of the sedimentation curve became less steep during the whole cultivation and eventually approached zero. At the same time, the total amount of sulfur in suspension diminished.

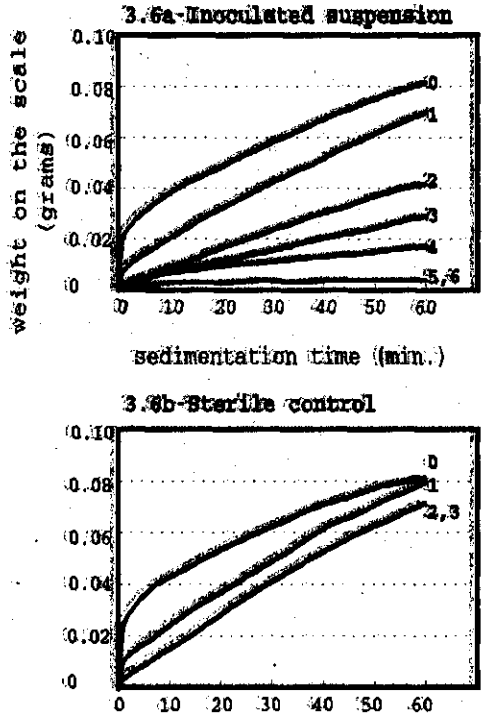

Figure 3.6 Analysis of a sedimentation velocity of biological sulfur. Bold numbers in the plot indicate the time [day] of cultivation.

3.4. CONCLUSIONS

Biologically-produced sulfur showed a faster conversion into sulfuric acid than the commercially-available sulfur flower. Among others, the process of biological sulfur oxidation by thiobacilli is promoted by the hydrophilic character of the sulfur, higher specific surface, and a breaking of particles during aeration. These parameters resulted in the higher accessibility of the sulfur particles for thiobacilli.

A higher rate of biological sulfur oxidation can have a great impact on the development of bioleaching techniques for the removal of heavy metals from solid materials like sludge, soil, low-grade ores etc.. So far, the use of biological sulfur for the processes of biotechnological extraction of heavy metals has not been

reported. Most of the research efforts in this field use sulfur flower or ferrous iron for feeding of thiobacilli. However, since the acidification of the growth medium proceeds significantly faster with biological sulfur, a development of bioleaching technologies using higher rates of sulfuric acid production can be expected.

Moreover, the enhanced oxidation of biological sulfur by thiobacilli is an important fact for the application of the sulfur cycle (Tichý et al., 1993a). In principle, the bioleaching process, after the separation of solids, can be followed by a sulfate reduction step. This process leads to the formation of sulfide, which can remove heavy metals from the liquor by precipitation. Eventually, other techniques may be used to remove metals from the spent extractant. After separation of metals from the process water, the re-use of sulfide is possible by a partial oxidation, leading to the new production of biological sulfur. The future application of such integrated processes might become beneficial. Therefore an evaluation of the biological sulfur oxidation process in a continuous set-up, the effects of pH and heavy metals on the process and some other parameters should be determined.

CHAPTER 4

OXIDATION OF BIOLOGICALLY-PRODUCED SULFUR IN A CONTINUOUS MIXED-SUSPENSION REACTOR

Abstract: *Microbial oxidation of biologically produced elemental sulfur was studied in a continuous bubble-column reactor. This sulfur was supplied from a sulfide oxidizing bioreactor as a suspension or as a suspended sulfur powder. The work focused on two aspects: 1) investigation of the bio-chemical stability of a biologically-produced sulfur suspension when being exposed to aeration at pH 8 in a bioreactor, and 2) evaluating the production of sulfuric acid by acidophillic thiobacilli using the biologically produced sulfur as a substrate. At pH 8, biomass growth showed an apparent Monod kinetics. At dilution rates of 0.1 up to 0.35 h^{-1}, 35-40% of easily available sulfur was oxidized. At $D < 0.1\ h^{-1}$, the sulfur conversion at pH=8 steeply increased, reaching ultimately values of 80-90%. In acidophillic conditions in the bioreactor, the maximum sulfuric acid production rate was searched for. The lowest pH value reached in the bioreactor was 1.7, whilst a maximum sulfuric acid production rate of 1 mmol $H_2SO_4 \cdot L^{-1}h^{-1}$ was found. The possible application of the acidification process for bioleaching is discussed.*

Published in *Water Research* **32/3** (1998), 701-710, authors: R. Tichý, A. Janssen, J. T. C. Grotenhuis, R. van Abswoude and G. Lettinga.

4.1. INTRODUCTION

Colourless *Thiobacillus*-like bacteria are capable of oxidation of elemental sulfur, as reported by numerous authors (Cook, 1964; Kelly, 1982; Trüper, 1984a; Hazeu et al., 1988). The most common form of sulfur, sulfur flower, has a highly hydrophobic surface with a physical structure of rather compact orthorhombic crystals. These properties are not favourable for microbial oxidation (Schaeffer et al., 1963). A considerable lag phase in the batchwise growth of thiobacilli on sulfur flower was recorded, being attributed mainly to the slow adhesion of bacteria on sulfur (Kelly, 1982). The adhesion is mediated by the intrinsic production of bacterial surface active agents (Agate et al., 1969; Bryant et al., 1983). Also, a substantial acceleration of the microbial sulfur oxidation has been reported when surface active agents were artificially introduced into the system (Bryant et al., 1983; Bailey and Hansford, 1993). Moreover, orthorhombic crystals possess a relatively small specific surface, at which the bacteria may attach. These effects determine the low rates of elemental sulfur oxidation (Kelly, 1982; Pronk et al., 1990b).

The enhancement of the process of sulfur oxidation is important for the development of a biotechnologically-mediated extraction of heavy metals from ores, solid waste, soil, or sediment, denoted as bioleaching (Tichý et al., 1993a). During bioleaching, microbially produced acid, or direct microbial attack of metal sulfides, solubilize metals from a solid phase. This phenomenon has been applied extensively in biohydrometalurgical mining of metals (Trüper, 1984a; Bailey and Hansford, 1993). Its use for the extraction of heavy metals from contaminated solid materials like sludge, soil or sediment is still in the developmental phase (Blais et al., 1992; Tichý et al., 1993a). However, in most bioleaching processes, elemental sulfur is not applied, and the use of soluble substrates or metal sulfides is preferred (Trüper, 1984a; Blais et al., 1992).

In our preceding work (Chapter 3) we studied the possible use of biologically produced elemental sulfur for feeding acidophillic thiobacilli. This sulfur, referred to as *biological sulfur*, is an end-product from the biotechnological removal of sulfide from anaerobically-treated waste water or from the biological desulfurization of flue-gases from coal-fired power plants (Buisman et al., 1991). Under oxygen-limiting conditions, sulfide is converted into elemental sulfur by a community of neutrophillic thiobacilli-like microorganisms (Janssen et al., 1995). Such produced elemental sulfur forms aggregates of fine crystalline and colloidal sulfur globules with bacterial biomass (Janssen et al., 1995; 1996). These particles are favourable for bioleaching because:

-Compared to sulfur flower, the biological sulfur is less hydrophobic and, consequently, a more homogeneous suspension is obtained. This provides the organisms a better chance to adhere onto the surface, which is a necessity to initiate its bio-oxidation.

-Biological sulfur particles have a higher specific surface area than those of sulfur flower. Particles are formed as fine particle-agglomerates, which are easily disintegrated by shear forces..

-Currently, the biologically-produced sulfur is an interesting waste product from a sulfide-removing wastewater treatment. Therefore, its price is marginal, compared to the costs of other substrates often used in bioleaching (Blais et al., 1992).

-The spent liquor after bioleaching contains considerable amounts of acidity, sulfate, and heavy metals. Therefore, its treatment is required. For the treatment of these waste streams, microbial sulfate reduction was proposed alternative (Trüper 1984a; Dvorak et al., 1991; van Houten et al., 1994). This process leads to production of the sulfide, which readily reacts with dissolved cationic metals. Resulting metal sulfides are generally insoluble in water, and are easily removed from the solution via precipitation (Dvorak et al., 1992). Excess sulfide is further removed from the wastewater using the partial sulfide oxidation process mentioned above. This last process results in a production of the biological sulfur, which can eventually be re-fed to the bioleaching process. In such a way, microbial conversions of sulfur can be used as environmental techniques in all steps of the sulfur cycle.

The possible application of biological sulfur for bioleaching involves different pH-conditions in the process of sulfur formation and its oxidation. In practise, sulfide conversion into elemental sulfur proceeds at a pH range of 7-8. This process has been successfully applied by Paques B.V., The Netherlands. A 7000 $m^3.day^{-1}$ of ground water stream heavily polluted with sulfate and heavy metals is treated in the forementioned manner (Scheeren et al., 1991). In practical applications for e.g. flue-gas desulfurization or the removal of H_2S from biogas, elemental sulfur has to be separated from the effluent of the sulfur-producing reactor. (Janssen et al., 1996) discussed the possible use of sedimentation, accelerated by addition of flocculants. However, this sedimentation still takes at least several hours. Meanwhile, the freshly produced elemental sulfur may be converted into sulfate (Janssen et al., 1995). Therefore, studying the bio-chemical stability of such elemental sulfur is of importance.

Subsequently, the rate of oxidation of biological sulfur by an acidophillic biomass has to be determined. Acidophillic thiobacilli showed much higher growth and sulfuric acid production rates when fed with biological sulfur in a batch

cultivation, compared to the sulfur flower (Chapter 3). Before scaling-up the continuous process, it is necessary to investigate the following aspects, which are discussed in the presented paper:

A. The bio-chemical stability of the biologically produced elemental sulfur suspension towards any further oxidation after leaving the sulfur producing reactor. Here, a worst-case analysis is applied with respect to the undesirable oxidation of sulfur, i.e. conditions of excessive aeration.

B. The conversion rate of the biologically produced sulfur particles in a continuous reactor under acidic conditions, accompanied by the production of sulfuric acid.

4.2. MATERIALS AND METHODS

4.2.1. Organisms and growth conditions

Sulfur oxidation at neutrophillic conditions (pH=8). Two reactors in series were used for experiments at pH 8. Reactor I was a CSTR tank, with a working volume of 5.9 litres, as described previously (Buisman et al., 1991). Sodium sulfide was used as energy source, and sodium hydrogen carbonate was provided as a source of carbon. The stock solution contained 50 $g.L^{-1}$ of $NaHCO_3$ and 200 $g.L^{-1}$ of Na_2S (i.e. 2.58 $mol.L^{-1}$ of sulfur). This solution was diluted with tap water to obtain a sulfide loading of the biological sulfur producing reactor (I) between 4.19-5.01 $mmol.L^{-1}.h^{-1}$ of sulfide, at a constant hydraulic retention time of 5.9 hours resulting in a dilution rate of 0.17 h^{-1}. The schematic presentation of the experimental setup is given in Figure 4.1. The effluent from reactor I was pumped at various flow rates into a sulfur-oxidizing reactor (II), using a peristaltic pump (Watson Marlow 505S). The dilution rate of the reactor II was regulated by adjustment of flow rate.

Reactor II was an aerated bubble-column with a volume of 1.35 litres and an internal diameter of 6 cm. A constant flow of air was maintained at 3 litres per minute, to supply oxygen in excess and to ensure the turbulent mixing inside of the vessel. Reactor II operated at a constant pH of 8, which was measured by a WTW Type E50 pH electrode, and regulated with a custom made pH-controller (liquisis-p) coupled to the dosing of sodium hydroxide. Both reactors were supplied with nutrients solution containing: NH_4Cl, 4 g; KH_2PO_4, 2 g; $MgCl_2 \cdot 6H_2O$, 0.8 g; Na_2EDTA, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 22 mg; $CaCl_2 \cdot 2H_2O$, 55.4 mg; $MnCl_2 \cdot 4H_2O$, 50.6 mg; $FeSO_4 \cdot 7H_2O$, 50.0 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 11.0 mg; $CuSO_4 \cdot 5H_2O$, 15.1 mg; $CoCl_2 \cdot 6H_2O$, 16.1 mg; per one litre of demineralised water. This nutrient solution solution was supplied to the reactor at a flow rate of 0.01 $L.h^{-1}$ (Buisman et al., 1991). The flow of nutrients into the reactor II was 0.02 $L.h^{-1}$ in order to

prevent limitations of bacterial growth by other nutrients than S^0.

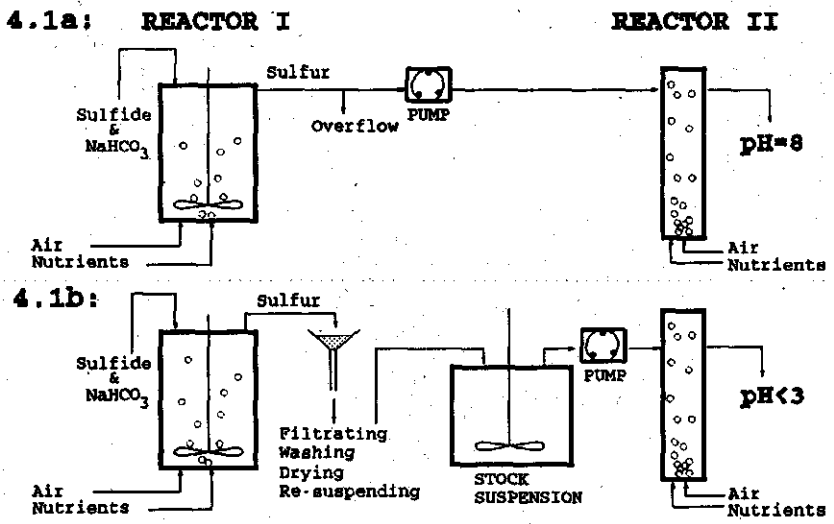

Figure 4.1 Experimental set-up for studying chemical stability (a) and oxidation at acidophillic conditions (b) of biologically-produced sulfur.

Concentrations of sulfate resulting from the addition of the nutrient solution into reactor II were subtracted from the total sulfate concentration determined in the reactors; therefore, they do not appear in the sulfur balance calculations in the text. Since the carbonate was added into the reactor I in excessive amounts, we assumed that carbon was not a limiting factor in our experiments.

In all series of experiments, no inoculation of reactor II was done, and biological activity can be attributed solely to the microbial population from the sulfur-producing reactor (I).

Sulfur oxidation at acidic conditions. The sulfur for experiments at acidic conditions was produced in reactor I analogously to the previous part, and at the same sulfide loading and dilution rate. To separate the sulfur from the liquor, the effluent from reactor I was filtered on a paper filter, whereafter the sulfur particles were washed twice by aliquot volumes of demineralized water. Then, the sulfur was dried at 60°C overnight. The dried material was sieved using a 200 um sieve. The sulfur suspension for feeding reactor II was obtained by adding 1.5 g of dry sulfur per litre of demineralized water, and kept in a stock vessel. This vessel was constantly stirred to prevent any sedimentation of sulfur particles. The sulfur suspension was pumped into the sulfur oxidizing reactor II by a peristaltic pump (Watson Marlow 505S). The input concentration of sulfur averaged 44 ± 4 mmoles

of S^0 per litre. A constant flow of 3 L of air per minute was maintained and no external addition of acid or alkali was applied. Fresh sulfur suspensions were made every two days, which was within the time interval when no significant sulfur oxidation occured in the suspension (data not shown). The experimental setup is given in Figure 4.1. A modified nutrient solution for autotrophic growth of acidophillic thiobacilli (Silverman and Lundgren, 1959) was pumped into the reactor at a flow of 0.02 $L.h^{-1}$, containing per litre of demineralised water: $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.1 g; $Ca(NO_3)_2 \cdot 4H_2O$, 60 mg; $MnCl_2 \cdot 2H_2O$, 2.0 mg; $FeSO_4 \cdot 7H_2O$, 11.0 mg; $ZnSO_4 \cdot H_2O$, 0.7 mg; $NaMoO_4$, 0.8 mg; $CuSO_4 \cdot 5H_2O$, 0.6 mg; $CoCl_2 \cdot 6H_2O$, 0.6 mg; H_3BO_3, 2.0 mg; KI, 0.2 mg; and H_2SO_4 to reach pH of 4.

Atmospheric carbon dioxide was the sole carbon source for the acidophillic thiobacilli. Although several authors reported on substantial improvement of acidophillic thiobacilli growth at higher concentrations of CO_2 in a gas-phase (Bailey and Hansford, 1993), opposite results are known as well (Haddadin et al., 1993; Nagpal et al., 1993). Here it was assumed that carbon was not limiting.

The acidophillic reactor was inoculated by a mixed culture of acidophillic *Thiobacillus* spp. D_2, obtained from the Department of Microbiology and Enzymology, Delft University of Technology, The Netherlands. The culture consisted of small, gram-negative rods growing single or in short chains on pyrite. Prior to inoculation, the culture was adapted to elemental sulfur by cultivation on sulfur flower, 2 $g.L^{-1}$, for one week. The inoculation was performed only at the beginning of the experiment by adding 100 mL of the bacterial suspension containing 10^5-10^6 cells mL^{-1}. No further addition of thiobacilli was done when the dilution rate was changed.

4.2.2. Analyses

The methylene blue photometric method was used to determine the sulfide in reactor I (Trüper and Schlegel, 1964). Elemental sulfur was removed from the medium by centrifugation (5 minutes, 15000g), followed by decantation and drying of the residue for 24 h at 30°C. After dissolving in acetone, the sulfur was determined by reverse-phase high-pressure liquid chromatography, as described elsewhere (Chapter 3). The sulfate and thiosulfate concentrations in the supernatant after centrifugation (5 minutes, 15000g) were determined using HPLC. Biomass was determined as organically-bound (Kjeldahl) nitrogen in a solid phase after centrifugation, as described previously (Chapter 3). We are aware of the fact that $N_{biomass}$ does not always represent the total biomass concentration, however, the presence of particulate sulfur in the suspension disabled a use of standard methods

for estimation of bacterial biomass. Sedimentation analysis was performed using a 1-L column with hanging scale, connected with digital data acquisition, as described previously (Chapter 3).

4.2.3. Calculations

The neutrophillic biomass growth rate was calculated using the mass-balance equation (Siebel, 1992):

$$\frac{dX_{II}}{dt} = D \cdot X_I + \mu \cdot X_{II} - D \cdot X_{II} \qquad \textbf{4.I}$$

Here, dX_{II}/dt is the change of biomass concentration with time, D is the dilution rate (h^{-1}), X_I the concentration of biomass in reactor I (in mg $N_{biomass}.L^{-1}$), and X_{II} is the concentration of biomass in reactor II. Assuming that steady state was achieved, dX_{II}/dt equals to zero, and the growth rate may be expressed as:

$$\mu_{app.} = D \cdot (1 - \frac{X_I}{X_{II}}) \qquad \textbf{4.II}$$

Here, $\mu_{app.}$ stands for apparent growth rate.

In case of experiments with acidophillic thiobacilli, the stock sulfur suspension did not contain any vital acidophillic biomass. This is due to the drying and storage of the sulfur prior to its resuspension, and due to the fact that acidophillic biomass does not participate in the given process of elemental sulfur formation. Therefore, dilution rate D is directly equal to μ as in general chemostat experiments:

$$\mu = D \qquad \textbf{4.III}$$

Sulfur conversion was expressed using the sulfur balance. For this, S^0, $S_2O_3^{2-}$, and SO_4^{2-} concentrations were measured in the effluents of both reactors. For neutrophillic conditions, the sulfur conversion efficiency was obtained as follows:

$$S^0_{converted} = (1 - \frac{[S^0]_{II}}{[S^0]_{II} + [SO_4^{2-}])_{II} - [SO_4^{2-}]_I - 2 \cdot [S_2O_3^{2-}]_I}) \cdot 100\,\% \qquad \textbf{4.IV}$$

In the acidification experiments, no sulfate or thiosulfate were present in the feeding suspension of the reactor II. Therefore, formula (4) could be reduced to:

$$S^0_{converted} = (1 - \frac{[S^0]_{II}}{[S^0]_{II} + [SO_4^{2-}]_{II}}) \cdot 100\% \qquad 4.V$$

4.3. RESULTS AND DISCUSSION

4.3.1. Stability of a biological sulfur

Microbial oxidation of elemental sulfur by the indigenous microflora from the sulfur producing reactor (I) at pH 8 was studied to investigate the rate of sulfate formation. The biomass concentration, measured as $N_{biomass}$ at different dilution rates, is given in Figure 4.2.

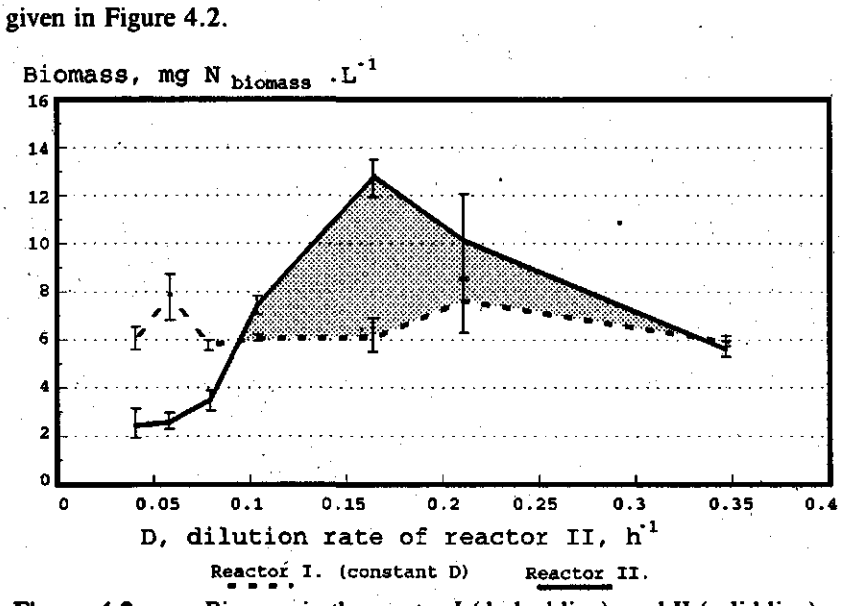

Figure 4.2 Biomass in the reactor I (dashed line), and II (solid line), at varying D at pH=8.

The sulfur-producing reactor (I) was maintained at constant dilution rate, being D=0.17 h^{-1}. Dilution rates between 0.03-0.35 h^{-1} were applied in reactor II. Concentrations of biomass in reactor I apparently varied, its maximum and minimum values being 7.8 mg $N_{biomass}.L^{-1}$ and 5.9 mg $N_{biomass}.L^{-1}$, respectively. Such variations are hardly avoidable in the given system (Buisman et al., 1991). The shaded area

indicates a net biomass formation in reactor II. Higher biomass concentrations in reactor II, compared to reactor I, were observed at dilution rates higher than 0.1 h^{-1}. The maximum biomass concentration was found at D=0.16 h^{-1}, which corresponds to the maximum reported growth rate of neutrophillic thiobacilli (Buisman et al., 1991; Stefess, 1993). At D=0.35 h^{-1}, the concentration of biomass in the reactor II was similar to reactor I.

The decrease of the biomass concentration at dilution rates below 0.16 h^{-1} was closely associated with the substrate depletion. This statement is supported by growth kinetics plot (Figure 4.3). The apparent growth rate followed a Monod kinetics with constant decay rate:

$$\mu = \mu_{max.} \cdot \frac{S}{K_S + S} - d \qquad \textbf{4.VI}$$

Here, $\mu_{max.}$, maximum growth rate, yielded 0.395 h^{-1}, substrate affinity constant K_S was 6.647 mmol.L^{-1} of elemental sulfur, and decay rate d was 0.151 h^{-1}. The correlation coefficient r^2 was 0.89 for 19 data points, which means the correlation was relatively close.

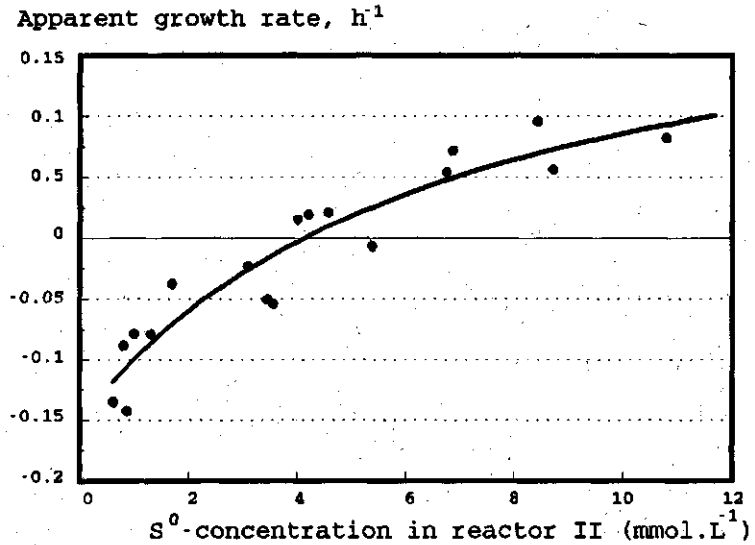

Figure 4.3 Growth kinetics of neutrophillic thiobacilli in the reactor II.

Apparent growth in reactor II was observed at elemental sulfur concentrations higher than 4 mmol $S^0.L^{-1}$. At lower sulfur concentrations, the decay of biomass overruled the growth. The existence of both growth and die-off in the bacterial culture was caused by a selective availability of substrate for bacteria. As

was demonstrated previously (Janssen et al., 1996), biological sulfur is excreted from neutrophillic thiobacilli in the form of particles with size of ca. 100 nm. These particles agglomerate into larger flocs containing bacteria and sulfur. The suspension of individual bacteria, bacteria with small sulfur particles, and abovementioned flocs, is entering the reactor II. The oxidation of biological sulfur suspension affected firstly the fine, easily available sulfur particles (Janssen et al., 1995). After the fine particles are depleted, free bacteria have to adhere and inhabitate the coarse fraction, as was described previously (Chapter 3). This brings increased requirements for maintenance energy, and higher decay rate.

Changes in substrate availability can be deduced from the specific sulfur conversion rate. This is shown in Figure 4.4 as a dependence of specific sulfate production rate, in mmol sulfate per mg $N_{biomass}$ per hour, on dilution rate.

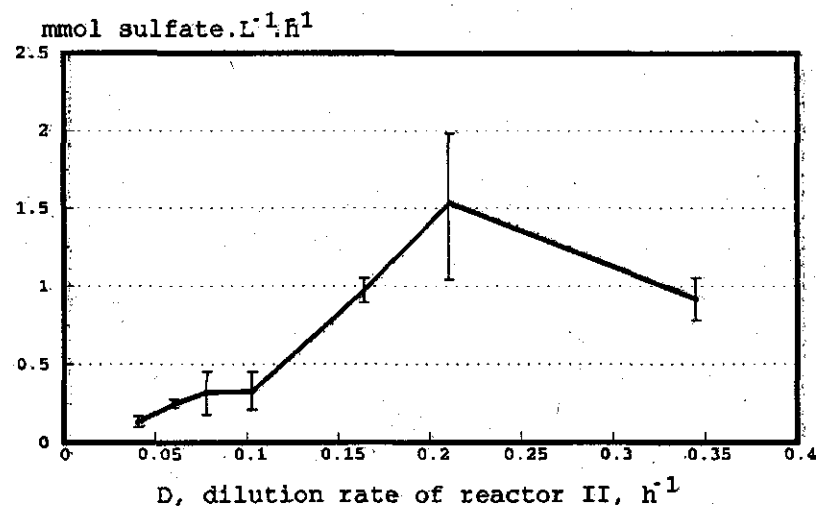

Figure 4.4 Specific sulfate production rate in the sulfur oxidizing reactor (II).

At high dilution rates ($D > 0.2\ h^{-1}$), the specific sulfate production was high, reaching values higher than 0.15 $mmol.L^{-1}.h^{-1}$. With decreasing D, the sulfate production rate decreased, reaching a minimum at $D = 0.1\ h^{-1}$ (0.04 $mmol.L^{-1}.h^{-1}$). With a further decrease of dilution rate, the specific sulfate production increased, with a local maximum at $D = 0.06\ h^{-1}$ (0.11 $mmol.L^{-1}.h^{-1}$). There is no explanation sofar for the existence of this local maximum. However, it was likely caused by successive changes in bacterial colonization of the surface of the coarse sulfur particles as discussed above. The oxidation of fine particles occurred in our system at $D > 0.16\ h^{-1}$. Depletion of fine particles at D lower than $0.16\ h^{-1}$ resulted in

decreasing specific sulfate production rate with decreasing D. The presence of sulfur in such coarse, unavailable fraction is proved by the fact that even at $D=0.05\ h^{-1}$, 11% of elemental sulfur remained unoxidized (Figure 4.5).

The substrate availability due to changes in particle-size composition was studied with sedimentation analysis. The calculated distribution of particles among the size-fractions for selected dilution rates is given in Table 4.1. Here, the decrease of particle-diameter at constant frequency classes is clearly seen.

Table 4.1 Particle-size distribution of the biological sulfur calculated from the sedimentation curves, at pH=8.

	Diameter (μm) of particles at cumulative frequency of:	
D (h^{-1})	90%	30%
0.04	<11.9	<5.6
0.08	<17.6	<7.1
0.10	<22.7	<9.2
0.16	<25.7	<10.5

The numbers should be interpreted as e.g. at $D=0.04\ h^{-1}$, 90% of particles were smaller than 11.9 μm, and 30% of particles were smaller than 5.6 μm.

For application of the process in practice, the maximum bio-chemical stability of elemental sulfur should be achieved, i.e. minimum conversion to sulfate. The elemental sulfur conversion efficiency, see Eq. 4.IV, at pH=8 is expressed in Figure 4.5. At $D>0.10\ h^{-1}$, approximately 40% of elemental sulfur is converted in reactor II to sulfate, slowly decreasing to 30-35% at $D=0.35\ h^{-1}$. A significant percentage of sulfur was converted even at a dilution rate of $0.35\ h^{-1}$. This corresponds to the abovementioned high availability of fine sulfur particles. At dilution rates below $0.16\ h^{-1}$, the pool of fine, easily available sulfur particles was depleted, and the bacteria attacked the coarse, less-available sulfur fractions. This resulted in an increasing percentage of converted elemental sulfur, shown in Figure 4.5. At dilution rates below $0.16\ h^{-1}$, the part of converted sulfur increased sharply, reaching maximum of 89% of converted sulfur at $D=0.06\ h^{-1}$. It is likely that the process may proceed down to the 100% conversion efficiency, however, at low rate. This is caused by the presence of remaining sulfur in large particles, with small

specific surface area, however, with a considerable sulfur content.

It may be concluded that the elemental sulfur availability is closely associated with the presence of fine particles. In case of biological sulfur, this available fraction of fine particles forms 31-46.5% of the total elemental sulfur. This fraction was converted even at the maximum dilution rate applied in our experiments. The less-available sulfur fraction was converted only at $D < 0.16$ h^{-1}. The oxidation of easily available sulfur can be hardly overcome: the use of chemicals to ensure a formation of large, easily settleable sulfur flocs is a prerequisite in practise (Janssen et al., 1997).

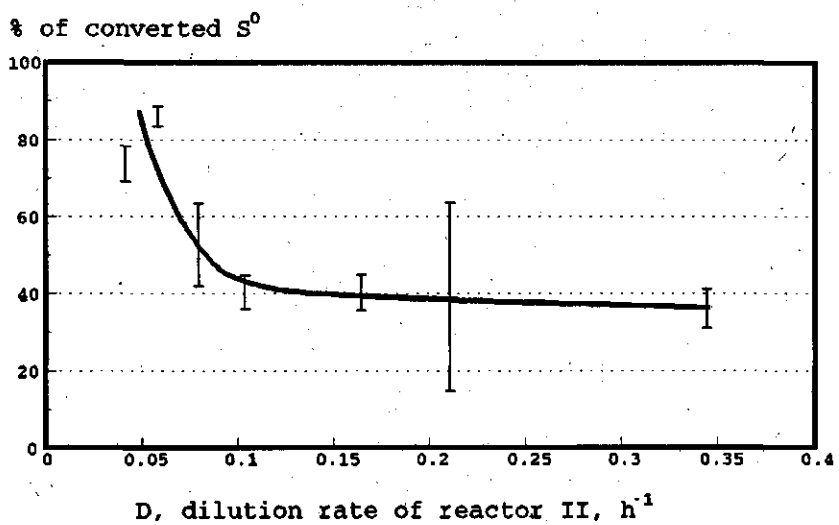

Figure 4.5 Sulfur conversion in the reactor II at pH=8, calculated after Eq. 4.IV.

4.3.2. Biological sulfur oxidation by acidophillic thiobacilli

Oxidation of biological sulfur by acidophillic thiobacilli was studied at dilution rates between 0.04-0.2 h^{-1}. Dilution rates higher then 0.2 h^{-1} were not investigated since the maximum growth rate of acidophillic thiobacilli on elemental sulfur was reported to be in the range of 0.05-0.15 h^{-1} (Kelly, 1982; Pronk et al., 1990b). Substantial changes in biomass concentration were observed at varying D in reactor II (Figure 4.6). A clear decrease of $N_{biomass}$ concentration with increasing D in reactor II was observed within the interval 0.04-0.11 h^{-1}. At dilution rates higher than 0.11 h^{-1}, biomass analysis was disturbed by cellular debris from neutrophillic thiobacilli, which is impounded in the biological sulfur. We speculate

that at dilution rates higher than 0.11 h^{-1}, the mineralization of this debris was negligible and therefore much higher background concentration of $N_{biomass}$ was encountered. Therefore, the data on biomass concentrations at high dilution rates are not relevant, and as such are not presented.

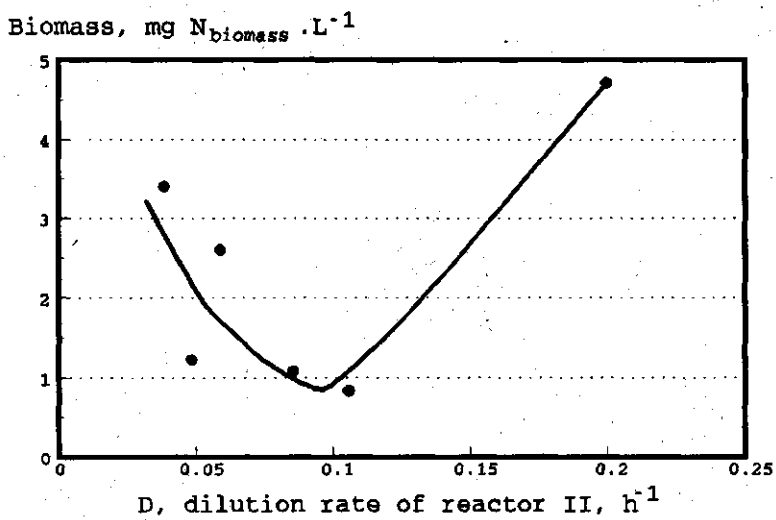

Figure 4.6 Changes in $N_{biomass}$ concentration of acidophillic thiobacilli.

Elemental sulfur concentrations in the influent of the sulfur-oxidizing reactor varied within the range of 40-48 mmol $S^0.L^{-1}$ (data not shown). Analysis of growth kinetics showed a linear relationship between the apparent growth rate and the substrate concentration, S^0, in the medium (data not shown). A correlation coefficient of $r^2=0.84$ for 19 data points was obtained. From this, we assumed the pseudo first-order kinetics of growth for acidophillic thiobacilli.

The effect of selective oxidation of fine sulfur was less pronounced for acidophillic cultivation, when compared to the neutrophillic thiobacilli. In neutrophillic conditions, the most active bacteria were those adhered to fine sulfur particles already in the influent to the bioreactor. In acidophillic conditions, no bacteria were coming with the sulfur suspension. Therefore, all sulfur particles were attacked by thiobacilli, and the availability of substrate was purely controlled by the area of oxidizable surface. Oxidation of large sulfur flocs resulted in their abrasion and breaking to smaller particles. Subsequently, the distribution of sulfur particles among size-fractions was nearly independent of dilution rate (Table 4.2).

Table 4.2 Particle-size distribution of the biological sulfur calculated from the sedimentation curves for acidophillic cultivations. For explanation see Table 4.1.

	Diameter (μm) of particles at cumulative frequency of:	
D (h^{-1})	90%	30%
0.05	<22.1	<7.1
0.06	<22.9	<7.4
0.10	<29.0	N.D.
0.2	<25.6	<7.3

N.D.-not detected

The oxidation of biological sulfur by acidophillic thiobacilli lead to a large decrease of pH. Even at the maximum applied dilution rate of $D=0.2\ h^{-1}$, the culture was able to reach a pH of 2.4 (Figure 4.7). Since this dilution rate is higher than the maximum growth rate of acidophillic thiobacilli on elemental sulfur (Kelly, 1982; Pronk et al., 1990b), we attribute this oxidation to the activity of biomass adhered to the coarse sulfur particles. These particles, due to their high sedimentation velocity, were likely retained within the bubble-column. Immobilization of biomass on these particles resulted in a permanent stock of bacteria within the reactor even at D higher than the maximum growth rate, and therefore prevented the total biomass wash-out. By lowering the dilution rate, a pH decrease down to 1.7 could have been obtained. However, even the highest pH of 2.4 achieved in our experiments with the acidophillic system is satisfactory for bioleaching purposes. Most bioleaching processes operate at pH 2 or higher (Blais et al., 1992; Tichý et al., 1993a).

The production rate of acidity was the highest at a dilution rate of $D=0.09\ h^{-1}$, when it reached 1.7-2.2 mmol $H^+ L^{-1} h^{-1}$, as determined by titration measurements. It corresponded well with the sulfate production rate, which then reached 0.95-1.00 mmol $L^{-1} h^{-1}$, assuming that 1 mole of converted sulfur gave rise to one mole of sulfuric acid. The overall production rate and concentrations of sulfuric acid in the reactor II are given in Figure 4.8. At the dilution rate $D=0.09\ h^{-1}$, when the maximum sulfuric acid production rate was observed, the concentration of acid in the effluent was 11 mmol L^{-1}. At $D<0.04\ h^{-1}$, an even higher molality of

H_2SO_4 was obtained, being maximum 12-14 mmol.L^{-1}. However, the overall acid production rate was lower at these dilution rates due to the sulfur limitations, being only half of the maximum production rate.

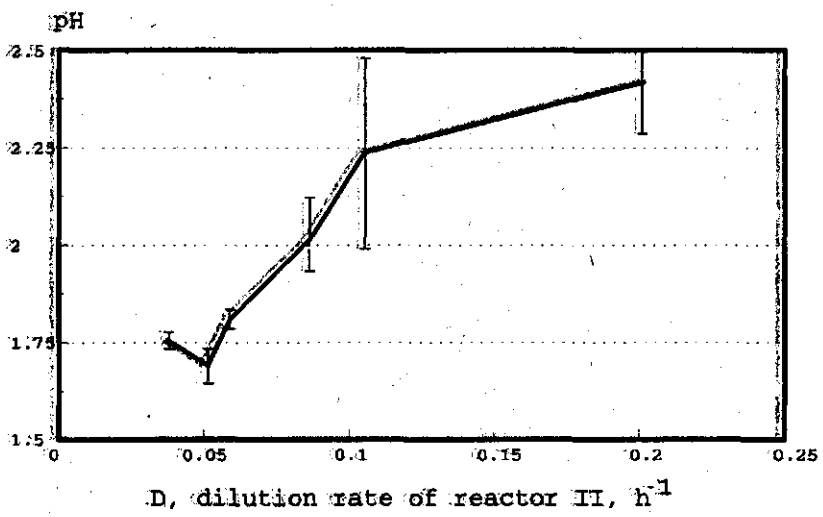

Figure 4.7 pH values of the reactor II effluent at varying D.

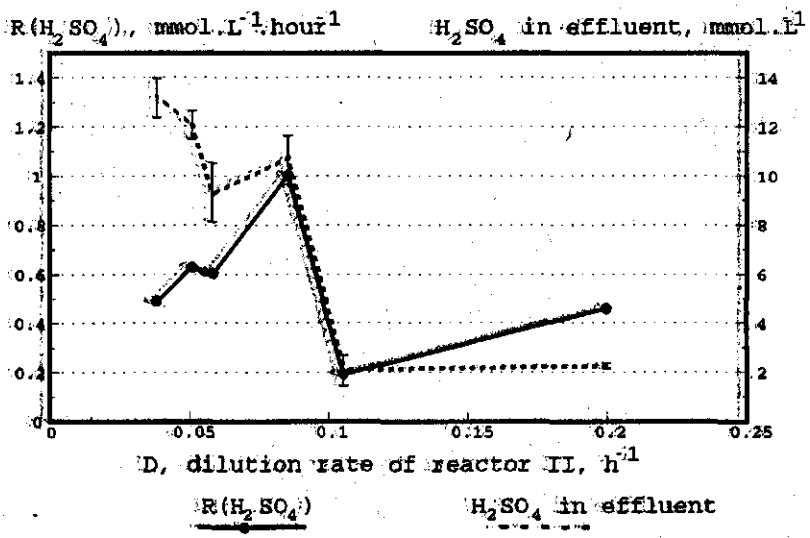

Figure 4.8 H_2SO_4 production rate and concentrations of sulfuric acid in the effluent of reactor II.

4.4. CONCLUSIONS

A freshly-produced suspension of biological sulfur resulted in an immediate biologicl conversion of 35-40% of the easily available elemental sulfur. The same observation has been made in a fed-batch reactor by Janssen et al. (1995). The immediate oxidation of elemental sulfur particles in an aerated bioreactor is hardly avoidable, unless other measures are applied, such as the use of flocculants or specific bactericides. The process of sulfur conversion was substantially enhanced at dilution rates below 0.1 h^{-1}.

In the sulfuric-acid producing reactor with acidophillic thiobacilli, the oxidation of sulfur lead to a substantial acidification of the liquor. The lowest pH value was achieved at D=0.04 h^{-1}, i.e. pH 1.7, while the highest pH value was found to be 2.4 at D=0.21 h^{-1}. The amount of converted sulfur decreased with D, viz. 89% of oxidized elemental sulfur at D=0.04 h^{-1}, 30% at D=0.11 h^{-1}, and minimum 10% at D=0.2 h^{-1}.

When the possible use of biologically-produced elemental sulfur for bioleaching is considered, the crucial parameters are 1) the production rate of sulfuric acid, and 2) the absolute concentration of acid achieved in the effluent. The maximum acid production rate was achieved at D=0.09 h^{-1}. Per 1 L of reactor volume, 2 mmoles H^+ were produced per hour. At a substrate concentration in the influent of 40-48 mmol $S^0.L^{-1}$, the absolute concentration of acid at the maximum production rate was 20-22 mmol $H^+.L^{-1}$, which corresponded to pH of 1.95. Although higher molalities of acid may be achieved at a higher elemental sulfur loading, the concentrations achieved in our study are high enough to be used for a direct bioleaching process. In processes using mineral acids for sanitation of soils or sediments, the pH should not decrease below 3-4 to avoid the solubilization of mineral matrix (Arp and Ouimet, 1986; Zelazny and Jardine, 1989; Chapter 5). When the solid-solution ratio in an extraction slurry is modified with respect to the buffering capacity of the solid phase, concentrations of 20 mmol $H^+.L^{-1}$ in the extracting liquor are satisfactory to achieve the desired pH (Chapter 5; Tichý et al., 1993a; Tuin and Tels, 1990a).

The other two biological processes of the microbial sulfur cycle, sulfate reduction and partial sulfide oxidation, can proceed at considerably higher rates. Sulfate reduction in an anaerobic reactor proceeds at a rate of up to 13 $mmol.L^{-1}.h^{-1}$ of sulfate (van Houten et al., 1994). A maximum sulfide partial oxidation rate of 30 $mmol.L^{-1}.h^{-1}$ was documented (Buisman et al., 1991).

CHAPTER 5

STRATEGY FOR LEACHING OF ZINC FROM ARTIFICIALLY CONTAMINATED SOIL

Abstract: *Extractability of zinc by sulfuric acid was studied using three different artificially contaminated soil types at a pH range of 1.5-6. A simple linear desorption model was modified with respect to pH, resulting in high correlation indices. Aluminium solubilization was studied as an indicator of damage of the soil matrix by extraction. Zinc solubilization increased monotonously with lowering pH within the whole pH-interval, however, only negligible aluminium solubilization was observed at pH above 3-4. Below this pH, Al-concentrations increased exponentially. Therefore, the extraction process for removal of zinc from soil using mineral acids should be optimised with respect to the maximum metal removal at minimum soil damage. The estimated amount of process water needed to clean 1 kg of soil for Dutch standards was 2-10 litres, with concentrations varying from 10 to 50 mmoles of* H_2SO_4 *per litre of extractant.*

Published in *Environmental Technology* **17** (1996), 1181-1192, authors: R. Tichý, J.T.C. Grotenhuis, W.H. Rulkens and V. Nýdl.

5.1. INTRODUCTION

Extraction with mineral acids is frequently proposed to decontaminate soils polluted with heavy metals (Tuin and Tels, 1990a; Rulkens et al., 1995). Sulfuric acid is not broadly considered since the solubility of some metal sulfates is low, particularly $PbSO_4$ (Evans, 1989; Davies, 1995). Therefore, a limited knowledge about its potential use for the extraction of heavy metals from polluted soils is available (Tuin and Tels, 1990b). However, the use of sulfuric acid offers a possibility to use microbial leaching (=bioleaching). This process is mediated by oxidation of sulfur or sulfides by certain groups of acidophillic (=acid loving) bacteria, predominantly from the genus *Thiobacillus*. As a result of this process, sulfuric acid is produced (Tyagi et al., 1990; Trüper, 1984a). At the same time, the use of sulfuric acid might allow further biological treatment of the metals- and sulfate-bearing effluent by microbial sulfate reduction. In this process, heavy metals can be precipitated as metal sulfides and removed from the liquor (Trüper, 1984a; Tichý et al., 1993a).

The technologies for acidic removal of heavy metals from contaminated soils are frequently discussed, however, only few large-scale installations have been demonstrated sofar (Rulkens et al., 1995). Two basic principles are proposed, being a) soil slurry processes, or b) heap leaching. Soil slurry extraction is accomplished in an extracting vessel, using an aqueous solution of acid as extractant. Extensive agitation of the slurry is performed. After the extraction, a solid/liquid separation step follows. Process water is regenerated for further re-use. The requirements of these processes for capital costs, chemicals, energy, and post-treatment of both soil and process water make such techniques rather expensive, and not always affordable (Carrera and Robertiello, 1993). Heap leaching, on the contrary, uses a natural percolation of a process water through a soil heaped homogenously on an isolated bed. The percolate is collected, regenerated, and re-used. Compared to the soil slurry systems, the process requires considerably less energy, however, much longer treatment times are to be expected (Rulkens et al., 1995). Eventually, an in-situ soil extraction may be applied for the removal of heavy metals (Urlings, 1990). Here, the soil is not excavated from its location, and the whole site is flushed by acid by a forced percolation. However, hydrogeological heterogeneities may mediate seepage of metals-bearing acid into the groundwater, and, therefore, an extensive monitoring of the process is required (Urlings, 1990; Rulkens et al., 1995).

Leaching of heavy metals from soils in conditions of low pH was extensively studied (Tuin and Tels, 1990a; Evans, 1989). Numerous models have been proposed for describing adsorption/desorption behaviour of metals in a soil suspension (García-Miragaya and Page, 1977; Chardon, 1984; Sadiq, 1991; de Wit, 1992; Kiekens, 1995). In some cases, metals behaviour may simply be described by

a linear relation between concentration of metal in the solution and in the solid phase. The slope of such model is denoted as K_d, i.e. the distribution coefficient, in $L.kg^{-1}$. Detailed information on linear models using K_d was recently given by Goyette and Lewis (1995). In general, the higher is K_d, the larger part of metal is in the soil. Within this context, extractive removal of metals from soils is always aimed at minimizing K_d. Values of K_d are largely affected by soil type, presence of Ca^{2+} ions, organic matter content, pH of a slurry, and ionic strength (Chardon, 1984; de Wit, 1992; Christensen, 1989; de Haan et al., 1987; Puls et al., 1991). However, effects of environmental parameters on K_d were extensively studied only in native soils and natural soil conditions. Rather extreme pH levels are proposed for extractive removal of metals from contaminated soils in large scale applications (Tuin and Tels, 1990b; Rulkens et al., 1995), and substantially higher levels of heavy metals are initially present in the contaminated soils (van den Berg and Roels, 1991). These pH-values and concentrations of contaminant usually exceed the scope of most of the presented studies on the adsorption/desorption behaviour of heavy metals in soils (Tuin and Tels, 1990b).

Since the proposed treatment conditions during the process of extractive sanitation are rather extreme, the soil matrix may be largely affected. Particularly, strong mineral acids may damage microbial life of the soil, and substantially affect both organic and mineral compounds present in the soil. Such damage may manifest itself in a form of increasing aluminium or silicium concentrations in the solution during extraction (Zelazny and Jardine, 1989). Apart from damages of the soil matrix, the presence of aluminium in spent extractant may substantially complicate the technological set-up because of its harmful properties (Kirk, 1987). Therefore, the post-treatment of the spent extractant will require additional measures (Cushnie, 1984). Parallel, the re-use of soil strongly damaged by drastic pH after extraction may present further complications (Bradshaw, 1993). Therefore, elevated levels of aluminium in the extractant are not desirable.

This study is aimed at an investigation of an extraction of zinc from artificially contaminated soils using sulfuric acid. Zinc was chosen as a model contaminant since it is often observed as a heavy metal pollution in a wide range of concentrations (Tuin and Tels, 1990a; Kiekens, 1995; van den Berg and Roels, 1991). The effect of different levels of pH adjusted with sulfuric acid on the extractability of zinc in a soil slurry was studied. Artificial contamination of soils was chosen in order to reduce the complexity, compared to real contaminated soil. Here, the effects of sulfuric acid on the zinc partition between a solid phase and extractant in a soil slurry at different pH were studied. Parallel, the solubilization of aluminium was followed as an indicator of mineral soil matrix damage by the extraction. Results will be used for the further technological considerations on a possible use of bioleaching for extractive clean-up of contaminated soil.

5.2. ABBREVIATIONS (details given later in text)

A, B		regression parameters describing an empirical relation between equilibrium pH and initial concentration of acid added to a system, $[H_2SO_4]$
Al	$mg.L^{-1}$	total aluminium concentration of in the extraction liquor
Al_{max}	$mg.kg^{-1}$	maximum aluminium concentration in the soil being available for dissolution by the extraction
C	$mg.L^{-1}$	equilibrium concentration of zinc in the extractant
F	%	extractability, a percentage of metal extracted from the soil (Q_{init}-Q), and the initial metal concentration, Q_{init}.
(H^+)	$mol.L^{-1}$	equilibrium concentration of H^+ ions in a solution, as calculated from pH
$[H_2SO_4]$	$mol.L^{-1}$	concentration of sulfuric acid in the extraction liquor before mixing it with soil
K^*	$L.kg^{-1}$	modified K_d with respect to the concentration of H^+ ions
K_{Al}	-	aluminium solubilization constant
K_d	$L.kg^{-1}$	distribution coefficient
M	-	exponent applied for adjustment of K_d to H^+-concentration
N	-	number of observations
O	$kg.L^{-1}$	solid/liquid ratio between the soil and the extractant in a soil slurry
p	-	exponent adjusting aluminium solubilization in respect to (H^+)
Q	$mg.kg^{-1}$	equilibrium concentration of zinc remaining adsorbed to the soil after the desorption
Q_{init}	$mg.kg^{-1}$	initial concentration of zinc in the soil before extraction
r^2	-	non-linear correlation coefficient
Soil-Al	$mg.L^{-1}$	equilibrium concentration of aluminium being available for acid-induced solubilization

5.3. MATERIALS AND METHODS

Soils : Three different types of Dutch soils with a different granular composition were used in this study (Table 5.1). Granular composition, organic matter content (dry combustion), pH-KCl, and content of $CaCO_3$ were analyzed by the Bedrijfslaboratorium voor Grond- en Gewasonderzoek in Oosterbeek, The Netherlands, using methodic packet NEN 5753. Cation exchange capacity was determined via Ba-saturation method followed by Mg quantitative replacement, as

described by Gillman (1979). The soils were sieved using a sieve (<2 mm), air-dried and stored in plastic barrels in the dark at 4°C before use for experiments.

Artificial contamination : 300 g of a soil was mixed up with 300 mL of demineralized water in a 1000 mL serum bottle. Zinc sulfate powder was added in order to achieve the desired Zn-concentration. Bottles with this slurry were mixed in an end-over-end mixer (25 rotations per minute, 50 cm diameter) overnight at 20°C. For the experiments on kinetics of zinc-desorption and aluminium solubilization, one level of zinc was applied, being 3100, 3030, and 2850 $mg.kg^{-1}$ for clay, silt, and sand, respectively. Experiments at one chosen extraction time (100 minutes) covered a broad range of zinc contamination levels. The applied concentrations of zinc in a solution used for artificial contamination of soils were increased by intervals of a factor 10x in a range from 10 to 10,000 mg Zn^{2+} per litre. The concentrations of zinc were chosen in order to match limits for soil contamination defined by the renewed Dutch list (van den Berg and Roels, 1991). Here, the soil type characteristics of the organic matter and clay content determine the values of intervention and reference concentrations of zinc in the soil. The appropriate concentrations of the three studied soils are given in Table 5.2.

Extraction procedure : Samples of 50 mL well-mixed soil slurry after the artificial contamination were diluted by 50 mL demineralized water and transferred into 300 mL serum bottles. The resulting soil/solution ratio in the extraction slurry was $O=0.415\ kg.L^{-1}$ for all three soils. To this mixture, concentrated sulfuric acid was added at varying doses from 0 to 0.1705 $mol.L^{-1}$. Shaking was performed in the above described end-over-end mixer, at 20°C. During extraction, samples 15 mL) of this soil-slurry were taken at chosen times, with a maximum time of 48 hours after the addition of sulfuric acid. Longer extraction times were not considered to be realistic for large scale extraction processes in practice.

Chemical analyses : Samples of a soil slurry were processed within 10 minutes after the sampling. pH of a soil slurry was determined before the solid/liquid separation. Samples were subsequently centrifuged 5 minutes, 15000g). Supernatant was conserved by a concentrated nitric acid volumetric ratio of 1:99) and stored at 4°C in the dark for later analysis. The centrifugate was dried at 105°C and zinc was extracted by boiling with concentrated HCl/HNO_3 mixture in a volumetric ratio 1:3 Tuin and Tels, 1990a). Zinc in the extractant was determined by flame atomic adsorption spectrophotometry Varian Spectra A 300) at 213.9 nm. The concentration of zinc in the soil was corrected with respect to the metal content in the pore water. Therefore, the zinc concentrations in the soil represent always the values after complete soil/solution separation. Aluminium in extracts was determined by the same atomic adsorption spectrophotometer at 309.3 nm, using standard addition of 2000 mg of potassium per litre of extractant to prevent ionization in the flame (Anonymous, 1979).

Table 5.1 Selected parameters of the soil types used in this study. Soil textural classes were identified after Koonevaan et al. (1983).

Soil :	clay	silt	sand
Soil textural class :	silty clay	silty loam	loamy sand
Parameter:			
pH-KCl	5.6	6.2	6.1
organic matter (%)	2.2	2.4	4.1
CEC* (cmol$^+$.kg^{-1})	40.7	9.8	9.6
Ca-carbonate (g.kg^{-1})	0.1	0.1	0.0
	Size fractions weight (%)		
0-2 μm	44.1	8.1	3.9
2-16	32.3	10.1	2.8
16-50	16.8	62.5	9.0
50-105	2.2	7.9	15.2
105-150	1.3	1.5	23.5
150-210	1.3	2.5	22.4
210-2000	2.1	7.4	23.2

* CEC - cation exchange capacity

Table 5.2 Zinc reference and intervention values for the three used soil types, as defined by a Dutch list (van den Berg and Roels, 1991).

	clay	silt	sand
Clay (<2 μm) %)	44.1	8.1	3.9
Organic matter (%)	2.2	2.4	4.1
Reference value (mg.kg^{-1})	186	78	68
Intervention value (mg.kg^{-1})	955	401	349

Numeric data processing. The aim of extraction experiments was to find a relation:

$$C = function(Q_{init}, pH) \qquad 5.I$$

where C is the metal concentration in the solution after extraction, in $mg.L^{-1}$, and Q_{init} is the initial metal concentration in the soil ($mg.kg^{-1}$).

Most current studies use adsorption approach, i.e.:

$$Q = function(C, pH) \qquad 5.II$$

where Q is the equilibrium concentration of metal in the solid phase, in $mg.kg^{-1}$ (García-Miragaya and Page, 1977; Chardon, 1984; de Haan et al., 1987; van der Zee and van Riemsdijk, 1987; Puls et al., 1991; Goyette and Lewis, 1995). Such models express the original relation being of our main interest only in an implicit form. Transformation of this implicit formula into the form as Eq. 5.I is usually rather complicated.

As a basis of our model we used a linear sorption isotherm:

$$Q = K_d \cdot C \qquad 5.III$$

where K_d is a linear partition coefficient (Goyette and Lewis, 1995). This coefficient is highly influenced by the pH of the system. Numerous authors describe the dependence of K_d on pH as follows:

$$K_d = K^* \cdot (H^+)^M \qquad 5.IV$$

where (H^+) is an equilibrium concentration of protons in the solution, and K^*, M are case-specific parameters (de Haan et al., 1987; van der Zee and van Riemsdijk, 1987; Kiekens, 1995). Q_{init} can be calculated on the basis of the sum of metal concentration in the liquor and equilibrium concentration of metal in the soil:

$$Q_{init} = Q + \frac{C}{O} \qquad 5.V$$

where O is a solid/liquid ratio in the extraction slurry, in $kg.L^{-1}$.

Combining equations 5.III, 5.IV and 5.V results in the following equation for the pH-dependent solubilization of zinc as a function of Q_{init}:

$$C = \frac{Q_{init} \cdot O}{K^* \cdot (H^+)^M \cdot O + 1} \qquad \text{5.VI}$$

For predicting the aluminium solubilization, aluminium speciation in the solution was neglected. Therefore, the following stoichiometry was used:

$$Soil\text{-}Al + p \cdot H^+ \rightleftharpoons Al + Soil\text{-}H_p \qquad \text{5.VII}$$

where Soil-Al, Soil-H_p are amounts of aluminium and hydrogen bound to the soil matrix. Here, a reaction in aqueous phase is considered, therefore, units of Soil-Al are in $mg.L^{-1}$. Parameter p is used to express the molar ratio of hydrogen ions needed to release one mol of aluminium. This value may vary, depending on the state of hydrolysation of the aluminium bonds on the surface of soil minerals, and a fraction of exchangeable Al adsorbed in the soil (Stumm and Furrer, 1987; Zelazny and Jardine, 1989). Further, aluminium is subjected to a complex speciation in the solution (Arp and Ouimet, 1986). To simplify the data processing, these effects were considered of minor importance at given pH-range, and as such neglected. Symbol Al stands for the total aluminium dissolved in the solution, as determined by atomic adsorption spectrophotometry, see above.

When assuming that the variations of amount of Soil-H_p is negligible, the equilibrium constant for the aluminium solubilization may be written as:

$$K_{Al} = \frac{Al}{Soil\text{-}Al \cdot (H^+)^p} \qquad \text{5.VIII}$$

Realizing that the total aluminium amount in the system is constant, the maximum possible aluminium concentration in the solution is determined by a maximum desorbable aluminium content in the soil. This is expressed as:

$$Al_{max} \cdot O = Soil\text{-}Al + Al = constant \qquad \text{5.IX}$$

Here, Al_{max} is the total concentration of aluminium in the soil, available for desorption, in $mg.kg^{-1}$. Combination of equations 5.VIII and 5.IX leads to the modified Freundlich-Langmuir equation:

$$Al = Al_{max} \cdot O \cdot \frac{K_{Al} \cdot (H^+)^p}{1 + K_{Al} \cdot (H^+)^p} \qquad \text{5.X}$$

where Al is the equilibrium aluminium concentration, in $mg.L^{-1}$, and (H^+) the

concentration of H^+ ions, in $mol.L^{-1}$, as calculated from pH. Al_{max}, and K_{Al}, and p are parameters of the model, Al_{max} being numerically equal to the maximum amount of aluminium which is desorbable, and K_{Al} is the equilibrium constant.

Since the experimental conditions cover a broad range of pH, different phenomena occur, like surface protonation, dissociation of carboxyl-groups, coagulation of clay, and solubilization of alumo-silicates (Stumm and Furrer, 1987; Bolt and van Riemsdijk, 1987), standard protons-adsorption and ion-exchange models would be too complex. However, a simple 'black-box' regression was applied:

$$pH = A \cdot [H_2SO_4]^B \qquad 5.XI$$

Here, $[H_2SO_4]$ is the concentration of sulfuric acid added into the liquor before mixing it with the soil, in $mol.L^{-1}$, and A,B are regression parameters. This model proved to be valid at conditions in the presented experiments.

Two- or three-variables non-linear regression analysis was implemented using the standard statistical software packet STATGRAPHICS, version 2.6. The parameters' values were calculated using the Marquart method.

5.4. RESULTS AND DISCUSSION

The kinetics of zinc extraction, aluminium solubilization, and eventual pH changes after the addition of a known amount of sulfuric acid was studied to determine the optimum conditions for mobilization of heavy metals from non-contaminated soils. Figures 5.1a-c, respectively, give the results obtained for clay soil at an initial contamination level of 3100 $mg.kg^{-1}$. The equilibrium for zinc was achieved within 30-60 minutes (Figure 5.1a). At prolonged extraction times, the zinc concentration did not exhibit significant changes. Extreme solubilization of aluminium from the soil was observed within the first 10 minutes of extraction (Figure 5.1b). Thereafter, its concentration demonstrated a slow, but steady increase with time, especially at high concentrations of acid. The most likely cause for this phenomenon is a continuous dissolution of alumosilicates in conditions of extremely low pH (Stumm and Furrer, 1987). Similarly, the pH slowly increased with time (Figure 5.1c). Both other soil types behaved similarly, showing differences only in absolute values of zinc extraction, Al-solubilization, and pH (data not shown). From these data it was assumed that at an extraction time of 100 minutes, an equilibrium in distribution of zinc, aluminium, and levels of pH, in the slurry was achieved.

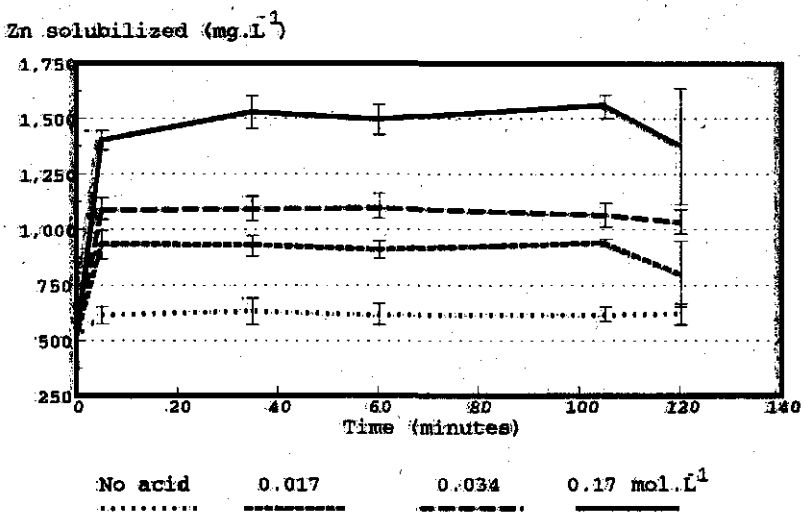

Figure 5.1a Changes in zinc extraction in a clay slurry at Q_{mit}=3100 mg.kg^{-1} with time at different molalities of added sulfuric acid.

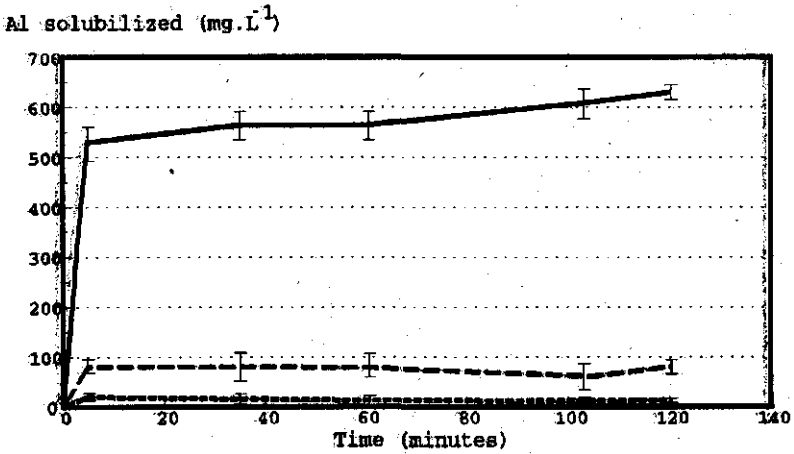

Figure 5.1b Changes in Al-concentrations in a clay slurry. Line styling follows Figure 5.1a.

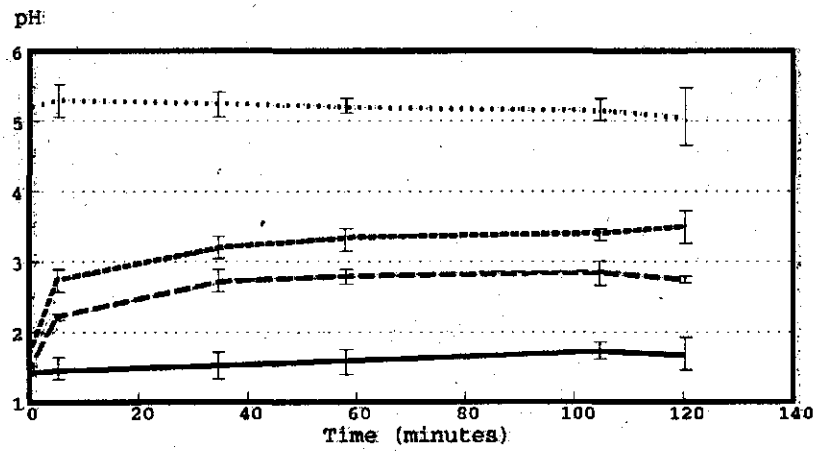

Figure 5.1c Changes of pH with time in a clay slurry. Line styling follows Figure 5.1a.

Uniform extraction times, i.e. 100 minutes, were applied in experiments with varying zinc concentrations in the soil, Q_{init}, and amounts of sulfuric acid added to the system, $[H_2SO_4]$. Non-linear regression of the observed data with the above described equation for extraction, Eq. 5.VI, resulted in correlation coefficient above 0.97 (Table 5.3). The model closeness to original data is further documented using a $C_{observed}$ versus $C_{predicted}$ plot (see Figure 5.2). From this plot it becomes clear that the model properly describes data at concentrations of zinc in the liquor (C) higher than 1 $mg.L^{-1}$. Below this level, predicted concentrations are overestimated when compared to the observed data points. However, the value of C=1 $mg.L^{-1}$ is, at maximum pH achieved in our experiments (pH=6.8), appropriate to Q_{init} of 15.8, 5.2, and 8.5 $mg.kg^{-1}$, for clay, silt, and sand, respectively. These values lie far below the zinc reference levels for the given soil type (Table 5.2). Consequently, we assumed that the presented model can be used for predicting the behaviour of zinc in the extraction slurry.

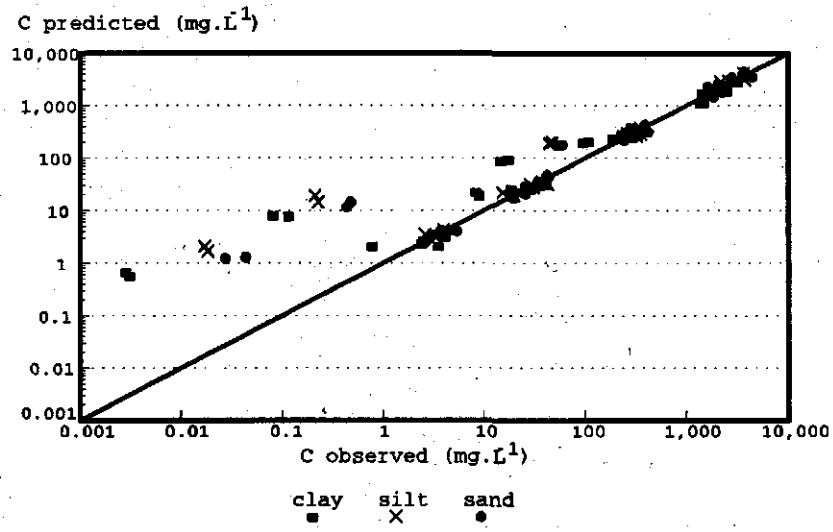

Figure 5.2 Justification of zinc-extraction model, Eq. 5.VI, using a $C_{observed}$ versus $C_{predicted}$ plot.

Table 5.3 Resulting parameters for zinc concentration in extraction liquor versus H^+ and Q_{init} concentrations, with Eq. 5.VI, using a non-linear regression.

	clay	silt	sand
N=	33	33	33
K*	0.3336	0.0830	0.0404
M	-0.2392	-0.2271	-0.3246
r^2	0.98	0.99	0.97

Aluminium solubilization showed considerably increased concentrations with lowering pH, as was expected (Figure 5.3). Since the aluminium concentrations appeared to follow different behaviour when pH values were above 4, we decided to use the equation 5.X only for data with pH lower than 4. The straight regression line in Figure 5.3 depicts the behaviour of aluminium at pH>4. Table 5.4 summarizes results of statistical non-linear regression of the model parameters at pH<4.

Table 5.4 Resulting parameters for aluminium solubilization versus H^+ concentrations in the extraction liquor, using Eq. 5.X. Only data with pH < 4 were used.

	clay	silt	sand
N=	24	24	24
Al_{max}	880.53	704.58	509.41
K_{Al}	6998.9	2801.1	802.8
p	1.770	1.509	1.208
r^2	0.99	0.99	0.95

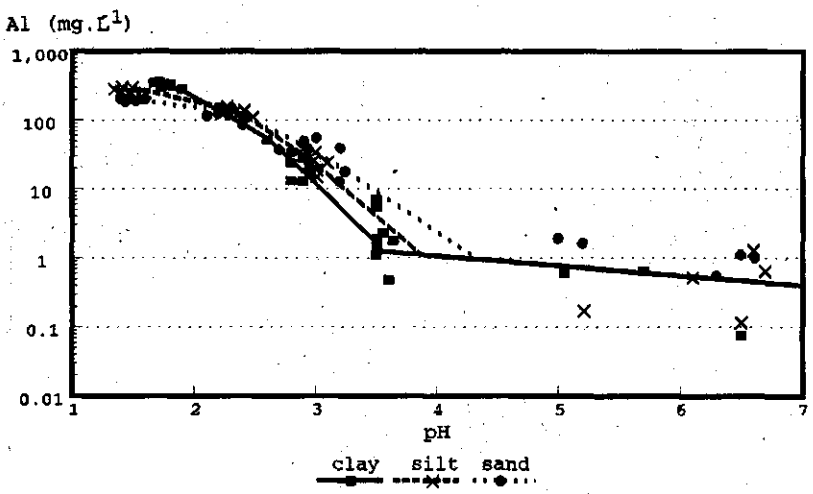

Figure 5.3 Modelling aluminium solubilization at different pH. For pH < 4, the soil-specific Langmuir model, Eq. 5.X, was applied. At pH > 4, a regression Al=3.937*e^(-0.326*pH) was used.

With the above described models for zinc extraction and aluminium solubilization, simulation of the extractive process was performed by using the extractability of zinc, F, defined as:

$$F = \frac{C/O}{Q_{init.}} \cdot 100\ (\%) \qquad 5.XII$$

Silty and sandy soils exhibited at pH=3 an extractability of 85, 86 %, respectively (Figure 5.4). By further lowering pH below 3, these values were only slightly increased, reaching maximum 92%, and 93% at pH=1.5 for silt and sand, respectively. Therefore, lowering pH below 3 is redundant for these two soils. The clayey soil revealed much lower extractability, compared to silt and sand, being at pH 3 only 58%. Maximum extractability achieved in our experiment with clay was 73%.

At pH=3, the aluminium concentrations in the liquor begun to increase in all three soil types (Figure 5.4). This indicates that the mineral matrix of the soil starts to be affected (Bolt and van Riemsdijk, 1987; Zelazny and Jardine, 1989). Therefore, it would not be desirable to lower pH below this value. When pH is further decreased from pH=3, the concentrations of aluminium increase steeply, similarly for all three soils, till pH=2.5. At pH=2.5, aluminium concentrations in sandy slurry start to decline from the clayey and silty soils, following the limit of maximum desorbable aluminium concentration, Al_{max}. This corresponds to the values of Al_{max} given for all three soils in Table 5.4, Al_{max} for sandy soils being 509.41 $mg.kg^{-1}$, i.e. almost 1.7x lower than that for clayey soil (880.53 $mg.kg^{-1}$), and 1.4x lower than that for silt (704.58 $mg.kg^{-1}$). These findings are further in correspondence with the increasing content of clay particles in the three tested soils in order sand<silt<clay. Clay particles are formed from aluminium-silicate oxides, and as such present the most important stock of aluminium in the soil. Arp and Ouimet (1986) studied solubilization of aluminium in soil solution over a broad range of pH (2-10), calculating equilibria of different Al-species. Their results are comparable to our findings. Studies performed with gibbsite and kaolinite revealed a rather constant level of soluble aluminium in the pH range of 7-4, at pH<4 the solubilization from both minerals followed an exponential increase.

To evaluate the actual needs of sulfuric acid in the extracting effluent, we used the regression after Eq. 5.XI, giving the relation between the concentration of sulfuric acid in the extraction liquor and the equilibrium pH of the extraction slurry. Results of this regression are summarized in Table 5.5. We assumed no influence of the ionic strength on such simulation, which was justified by a preliminary experiment with varying sulfuric acid levels in the soil slurry at different ionic strength. Resulting pH of a soil slurry at ionic strengths I=0.1 $mol.L^{-1}$ and I=1 $mol.L^{-1}$ revealed only negligible differences in pH readings (data not shown).

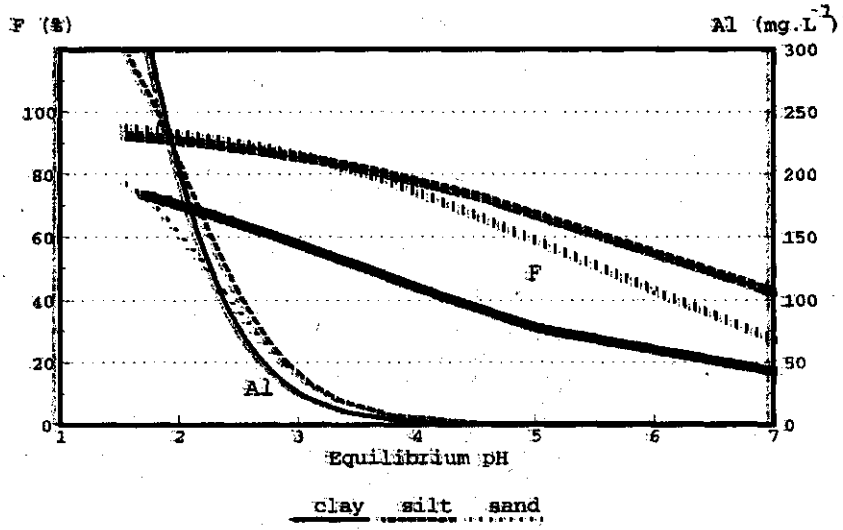

Figure 5.4 Simulated zinc extractability (thick lines) and aluminium concentrations in the liquid phase (thin lines), as function of the soil slurry equilibrium pH.

Table 5.5 Non-linear regression parameters of the equilibrium pH dependence on the concentration of acid in the liquor before extraction, $[H_2SO_4]$, using Eq. 5.XI.

	clay	silt	sand
A	1.067	0.909	0.892
B	-0.305	-0.302	-0.305
N	24	24	24
r^2	0.99	0.98	0.96

Both zinc extraction and aluminium solubilization results show that pH<3 leads to minor, if any, benefit. However, higher extractability is required to meet Dutch reference values. To reach these values by extraction at pH>3, the following two strategies may be applied: a) the extraction should be repeated, or b) the soil/solution ratio, O, could be decreased. Both strategies, however, lead to a larger consumption of process water needed to extract the given amount of soil. Assuming

negligible effects of ionic strength and mixing on the equilibrium, we performed simulations of the extraction process based on the results obtained with the least extractable clayey soil at varying O and pH. Here, we considered 955 $mg.kg^{-1}$ of zinc as an initial zinc contamination level in the soil, Q_{init}. This value corresponds to the appropriate Dutch intervention value for the given soil type (Table 5.2). In Figure 5.5, residual concentrations of zinc in the soil after the extraction (Q) are given as a function of the pH of a soil slurry and the solid/liquid ratio (O). The reference value of zinc, i.e. the required concentration of zinc for the given soil type, is 186 $mg.kg^{-1}$. At pH=3, the solid/liquid ratio can not be higher than 0.14 $kg.L^{-1}$, in order to accomplish the required target values. This means that in this case, 7.14 litres of process water are required to extract 1 kg of clayey soil.

Figure 5.6 gives the results of the simulated extraction process for the three soil types, assuming that the initial zinc concentration was equal to the appropriate Dutch intervention values, and the required target concentrations equal to the Dutch reference values (Table 5.2). The left axis of the Figure 5.6 gives the calculated maximum solid/liquid ratio, O, which has to be applied to reach the required target values. The horizontal axis gives the amount of sulfuric acid, in moles per litre, which has to be present in the extraction liquor before it is mixed with soil. Right vertical axis gives simulated concentrations of aluminium in the solution, calculated from Eq. 5.X using regression parameters given in Table 5.4, and O-values appropriate to the given concentration of acid (as plotted in Figure 5.6).

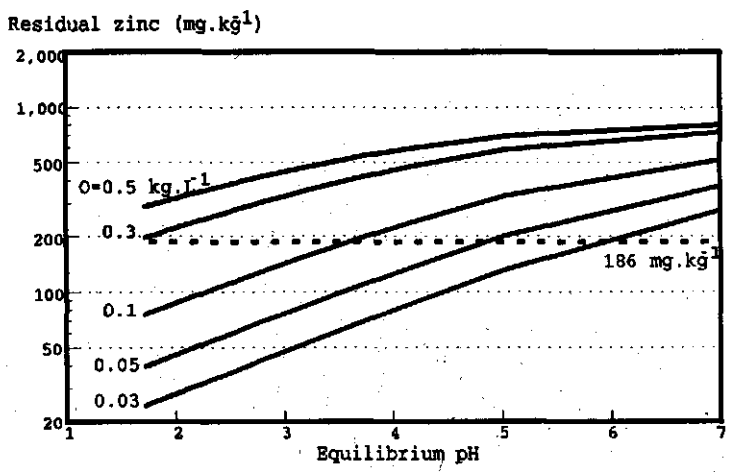

Figure 5.5 Residual Zn-concentration at varying pH and O for clayey soil, Q_{init} being Dutch intervention value (955 $mg.kg^{-1}$).

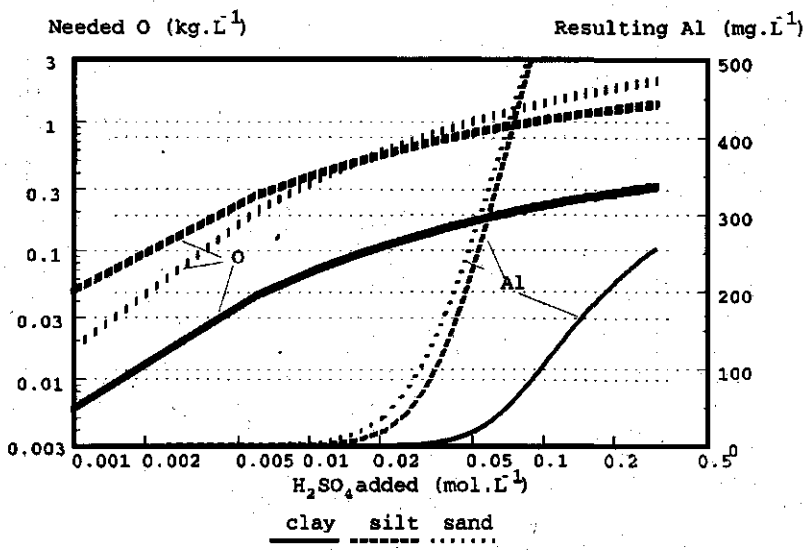

Figure 5.6 Maximum O needed to remove Zn after the Dutch standards at varying [H_2SO_4] and resulting Al levels in the liquor.

From the simulations in Figure 5.6 it becomes clear that the optimum concentration of sulfuric acid for the extraction would be 0.01-0.02 mol.L^{-1} for sandy and silty soil, and 0.02-0.05 mol.L^{-1} for clayey soil. At such conditions, the maximum solid/liquid ratio in the extraction slurry can be 0.4-0.5 kg.L^{-1} for silt and sand, and 0.1-0.2 kg.L^{-1} for clay. These numbers mean that the total consumption of process water per 1 kg of treated soil would be 2-2.5 L.kg^{-1} in case of silt or sand, and 5-10 L.kg^{-1} of the clayey soil. When expressed per amount of sulfuric acid necessary to perform the extraction, the total use of 0.025-0.04 moles of sulfuric acid is predicted per 1 kg of treated sandy and silty soil, whereas 0.2-0.25 moles of sulfuric acid should be needed for treatment of clayey soil. However, much higher use of process water can be foreseen when the concentration of zinc in the soil is higher than the considered intervention value. Such high demand for process water may make a soil-slurry process not feasible, and, eventually, more extensive ways of soil treatment have to be applied. One of the alternatives is heap leaching, or, a combination of the slurry-phase extraction with extensive landfarming (Rulkens et al., 1995).

Apparently, the lower aluminium solubilization in a clay slurry, compared to silt and sand (Figure 5.6), is caused by the lower solid/liquid ratio. The damage of soil, i.e. aluminium solubilization, was considerably higher with clayey soil, compared to the other two types at the same pH level (Figure 5.4). However, since the extractability of zinc from clay was much lower, the desired residual zinc concentrations were accomplished only at much lower soil solution ratio. Aluminium

concentrations in the spent extractant reached maximum 20 mg.kg^{-1} at pH>3 (data not shown). Calculated concentrations of zinc in the liquor at this pH (pH=3) were 80-130 mg.L^{-1} for clayey slurry, and 100-180 mg.L^{-1} for silty or sandy soil-slurries.

5.5. CONCLUSIONS

The results presented above indicate that the models used for zinc and aluminium leaching and sulfuric acid consumption are valid to be used for further considerations on extraction of heavy metals from contaminated soils. Simulations of the extractive process showed that both the K_d model applied for zinc behaviour, and the adapted Freundlich-Langmuir model for aluminium solubilization, performed well within the broad range of pH and zinc levels studied in our contribution. Solubilization of zinc increased with lowering pH within the whole selected pH-range (pH 1.5-6.5). At pH 3 and lower, enhanced aluminium solubilization was observed. Adding acid to the system to decrease pH below 3 did not result in a substantial increase of zinc extractability, however, it strongly increased aluminium dissolution. Therefore, the pH of an extraction soil process should be always optimized with respect to the maximum extraction yield and minimum soil damage.

Appropriate solid/liquid ratio in the extraction slurry was calculated being at least 2-2.5 litres of process water per 1 kg of silty or sandy soil, and 5-10 litres per 1 kg of clayey soil. Concentrations of sulfuric acid in the incoming liquor, needed for successful extraction, were 0.01-0.05 mol.L^{-1}, depending on the soil type, the necessary amount of sulfuric acid needed to clean the soil was calculated as 0.025-0.04 mol.kg^{-1} for silty or sandy soil, and 0.2-0.25 mol.kg^{-1} for clayey soil, provided that Dutch reference and intervention values were considered.

CHAPTER 6

USE OF ELEMENTAL SULFUR TO ENHANCE A CADMIUM SOLUBILIZATION AND ITS VEGETATIVE REMOVAL FROM CONTAMINATED SOIL

Abstract: *To a soil artificially contaminated with cadmium, orthorhombic sulfur flower and a hydrophillic microbially produced elemental sulfur were added to induce the soil acidification. The soil was incubated in pots under open-sky conditions. pH, sulfate, and cadmium solubility were monitored in time. Soil acidification with microbially produced sulfur proceeded without any delay and at considerably higher rates, compared to the sulfur flower. Cadmium solubilization was solely controlled by the soil pH. Similar experiments with cultivation of common mustard (Sinapis alba, cultivar JARA) were performed, evaluating both changes of cadmium solubilization and a possible uptake by biomass. Cadmium concentration in shoots increased with decreasing pH. However, biomass yield was negatively affected by the decreasing pH. Combining these two effects, a pH-optimum for maximum cadmium removal from the soil by plants was found at pH=5-5.5.*

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6.1. INTRODUCTION

Cadmium is one of the most problematic soil contaminants. It exhibits toxic properties at very low concentrations compared to other metals or most organic pollutants (Alloway and Jackson, 1991; Evans, 1989). Moreover, under certain soil chemical conditions such as low pH, its mobility is increased. In such a way, cadmium may become toxic even at low total concentrations in soil (Tichý et al., 1997). This situation occurs with diffuse-source pollution with cadmium, which is characterized by low, yet elevated, levels of contamination affecting large areas of land. This is typical for cadmium originating from agricultural fertilizers or atmospheric deposition (de Boo, 1990). Therefore, the treatment of cadmium-polluted soil often faces the difficult problem of removing relatively low concentrations of pollutant (Rulkens et al., 1995).

Standard techniques aimed at the removal of cadmium from polluted soil are costly (up to 500 U.S. dollars per m^3 of soil), and use rather extreme process conditions, like high molalities of mineral acids, addition of solidification agents, or high temperatures to sinter the soil particles (Rulkens et al., 1995, Sheppard and Thibault, 1992). Such techniques can not be applied to large areas of diffuse-source polluted land. Instead, novel techniques have to be developed using moderate treatment conditions, allowing recovery of the original soil functions, and keeping the costs of treatment feasible (Rulkens et al., 1995).

Several authors have discussed the use of hyperaccumulating plants for the removal of metals from soils (Baker et al., 1991; 1992; Banuelos et al., 1993; Canaruto, 1993). Such plant species can accumulate heavy metals in harvestable biomass in amounts high enough to make the vegetative removal of metals from soil possible. However, Ernst (1992) suggested that this approach would require very long treatment times (i.e. several decades or more, due to the low rate of metal removal achievable in real conditions. In contrast, Canarutto (1993) reported a successful decontamination by alfalfa (*Medicago sativa*) as a result of considerable uptake of metals from the soil and high production of biomass.

Vegetative uptake can be increased by acidification of the soil (Baker et al., 1991; Carillo and Cajuste, 1992; Davies, 1992; Eriksson, 1989; Smilde et al., 1992). Soil pH is the most important parameter controlling mobility of cationic heavy metals in soils (Ervio, 1991). Lowering the soil pH increases the solubilization of cadmium into the pore-water. Acidification can be achieved by the use of acid-producing fertilizers and the reduction of liming (Alloway and Jackson, 1991; Kužel et al., 1994; Merrington and Alloway, 1994; Tichý et al., 1997). Similarly, microbial oxidation of elemental sulfur introduced into the soil leads to a production of sulfuric acid (Germida and Janzen, 1993). Important factors affecting the oxidation of elemental sulfur in soils are: (1) small specific surface area

of the sulfur particles (Watkinson and Blair, 1993), and (2) hydrophobicity of orthorhombic elemental sulfur (Chapter 3). To achieve reasonable oxidation rate, sulfur must be finely ground (Germida and janzen, 1993; Watkinson and Blair, 1993). Moreover, after its application to the soil, a considerable time may be required for the attachment of oxidizing microbes (Watkinson and Blair, 1993), resulting in delayed and uncontrollable soil acidification. Moreover, commercially available orthorhombic elemental sulfur (sulfur flower) is a rather expensive material, particularly at the application rates required to achieve the desired degree of acidification.

In our previous research (Chapter 3; Tichý et al., 1993a) we reported a novel material containing elemental sulfur produced during the treatment of sulfide-containing wastewaters (Buisman et al., 1991; Janssen et al., 1995). This sulfur, when exposed to oxidative conditions and sulfur-oxidizing microbes, is oxidized more rapidly than the orthorhombic sulfur flower (Chapter 3). This phenomenon is caused by: (1) its considerably higher specific surface area, and (2) better resuspendability of the sulfur, due to hydrophillic polymers coating the particles (Chapter 3; Janssen et al., 1996). Currently, this microbially produced elemental sulfur is a waste material and may be economically attractive as an acidifying agent. Moreover, its use would allow the application of other techniques of metal removal. For example, soil percolate containing sulfuric acid and metallic cations can be treated by an anaerobic process of sulfate reduction to sulfide, which subsequently precipitates divalent cationic metals such as cadmium (Tichý et al., 1993a; van Houten et al., 1994). This process can be easily accomplished at the soil-drainage water collectors in an artificial or natural wetland system (Eger, 1994).

Although the high oxidation rates using microbially-produced sulfur have been demonstrated in a suspension reactor (Chapter 4), no information is available on this process occurring in-situ in the soil. Therefore, we conducted the present study to compare the effectiveness of microbially produced elemental sulfur with orthorhombic sulfur flower in the acidification of a Cd-contaminated soil, and the subsequent vegetative removal of Cd using common mustard (*Sinapis alba*).

6.2. MATERIALS AND METHODS:

Source of sulfur. Orthorhombic sulfur flower (Sulfur praecipitatum) was obtained from Lachema Brno, Czech Republic. Microbially-produced elemental sulfur (denoted as biological sulfur further in the text) was produced by a pilot plant oxidizing H_2S in Eerbeek, The Netherlands. Sulfur suspension was decanted, twice washed with aliquot volumes of distilled water, and dried at 60°C. Its content of elemental sulfur was 93 % by weight, the rest being microbial biomass debris and other impurities originating from the sulfide-containing wastewater. Dried sulfur flower and biological sulfur were sterilized by flushing with vapours of ethanol. However, further storage prior to the experiments, and application of the sulfur were not performed aseptically. Before addition to the soil, both sulfur-containing materials were ground manually using a laboratory mortar, and sieved through a 0.5 mm mesh.

Pot experiments without the growth of plants. Medium size Mitscherlich pots were used, each filled with 6 kg of soil. An uncontaminated sandy-loamy soil (brown-gleyic cambisols, collected from the vicinity of Lišov, South Bohemia, Czech Republic) was artificially polluted by an aqueous solution of $CdSO_4.8H_2O$ in order to achieve a uniform contamination level of 25 mg $Cd.kg^{-1}$. Basic nutrients were added at rates of 1.25 g KH_4NO_3, 1.25 g KH_2PO_4, 0.625 g $Mg(NO_3)_2$, all per 1 kg of the soil. Both biological sulfur and sulfur flower were supplied at two different concentrations: medium (5 g, i.e. 155.8 mmol, dry sulfur per kg soil), and high (20 g, i.e. 623.1 mmol, per kg soil). The pots were exposed to ambient atmospheric conditions outdoors in the period of May-September 1993. Initially, 1.5 litre of tap water was added per pot. No additional water was added to the pots during the incubation, with the exception of rain water. Levels of pH, sulfate, total and soluble cadmium were determined weekly as described below.

Pot experiments with the growth of common mustard. Similar conditions were used in experiments with common mustard (*Sinapis alba*, cultivar JARA). Here, orthorhombic sulfur flower addition was not studied, but biological sulfur was applied at similar levels as above. In addition, to exclude the effects of slow kinetics of sulfur oxidation, sulfuric acid was also applied at two levels, i.e. medium (5 mL of 96% H_2SO_4, i.e. 72 mmol per 1 kg of soil), and high (10 mL of 96% H_2SO_4, i.e. 144 mmol per 1 kg of soil). Fifty mustard seeds per pot were sawn to the surface of the soil. Low pH (initially 2.94 ± 0.28) in the high sulfuric acid treatment inhibited the germination. Therefore, these pots were repeatedly re-sawn until the germination was successful. This was achieved after 1.5 months, when the pH of the soil had increased to 3.53 ± 0.31 due to acid removal by the soil pH-buffering systems. The pots were exposed to outdoor conditions in the period May-September 1995. Levels of pH, sulfate, total and soluble cadmium were determined as

described below, on a 1.5 month sampling interval. At the end of the growth period, plant biomass was harvested, and shoot yields and cadmium concentrations were determined as described below.

Chemical analyses. Soil pH was determined in a 1 mol.L^{-1} KCl (1:2.5 w:v) extract using glass electrode. Soil sulfate was extracted with 0.01 mol.L^{-1} $CaCl_2$ (1:10 w:v). The extract was filtered, alkalized, and excess $BaCl_2$ was added. After standing overnight, the resulting barium sulfate precipitate was filtered onto an ash-free filter, ignited at 650°C, and weighted after cooling (Kauritschev, 1986). Total cadmium was determined in a 2 mol.L^{-1} HNO_3 (1:5 w:v) extract after 2 hours shaking. Soluble cadmium was measured in the 0.01 mol.L^{-1} $CaCl_2$ (1:10 w:v) (Houba et al., 1986). The yield of biomass was determined after drying of shoots at 105°C. Cadmium content in the biomass was determined by dry combustion at 450°C in an oven and dissolution of the resulting ash in 3M HCl. Cadmium in solutions was measured using a Philips PU 9485 atomic adsorption spectrophotometer.

6.3. RESULTS AND DISCUSSION

6.3.1. Oxidation of elemental sulfur

The course of pH-changes in the soil pots is presented in Figure 6.1. Initial pH in all treatments was 5.2-5.35. Both biological sulfur treatments showed immediate acidification from the beginning of experiments. Within 40 days of incubation, soil pH in the high biological sulfur treatment reached a plateau of 3.4. Afterwards, pH did not change significantly for 20 days. Between 60-90 days, a further pH decrease was observed, reaching a final value of 2.8, when the experiment was terminated No such plateau level was observed in the variant with medium biological sulfur treatment; instead, a steady decrease in pH was observed throughout the entire experimentation period, reaching the final pH of 3.25.

Acidification in the soil amended with sulfur flower proceeded initially at a much slower rate. A steady and slow decline in pH, from 5.2-5.25 to pH 4.9-5.1, was observed during first 50 days for both levels of sulfur flower addition. Afterwards, a rapid decrease in pH was observed in the high sulfur flower treatment; the rate showing again after 70 days to reach a final level 3.8. Similarly, the medium sulfur flower treatment started to acidify faster after 60 days of incubation, although at much lower rate than the high level treatment.

Although the soil pH is an important variable controlling the behaviour of heavy metals, it may be a misleading parameter in the evaluation of the process of sulfur oxidation due to the presence of pH-buffering systems which may consume newly produced acidity in the soil (Alloway and Jackson, 1991; Berthelsen et al.,

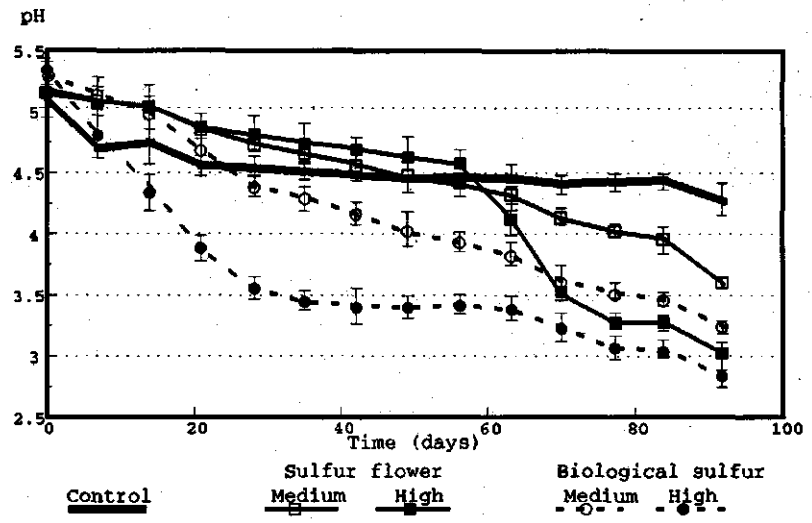

Figure 6.1 Changes of the soil pH with time in pots treated with biological sulfur, sulfur flower and control.

1994; Tuin and Tels, 1990a). Therefore, the anionic component of the sulfuric acid, i.e. sulfate, which adsorbs only negligibly in the soil (Ervio, 1991; Germida and Janzen, 1993), was analyzed. As expected from the stoichiometry of S^0 oxidation, sulfate concentration was highly correlated with the concentration of acidity (calculated from the soil pH). Relationships were identical for all treatments (data not shown).

The rate of sulfate production ($d(SO_4)/dt$) was calculated as:

$$dSO_4 / dT = \frac{C_2 - C_1}{T_2 - T_1} \qquad 6.1$$

Here, C_1 and C_2 are two successive concentrations of sulfate in the soil measured at times T_1, T_2, respectively. This rate was used as an indicator of the elemental sulfur oxidation (Figure 6.2). Only treatments with high level of sulfur application are presented. The oxidation rate of biological sulfur was high sulfur from the very beginning of the experiment. Between 30 and 50 days the sulfur oxidation rate decreased. Afterwards, sulfur oxidation accelerated, reaching maximum at 70 days after application (0.91 mmol SO_4 $kg^{-1}.day^{-1}$). In contrast, sulfate production from the sulfur flower was negligible within the first 50 days of incubation. Afterwards, the sulfur flower oxidation rate increased to a maximum of about 0.4 mmol SO_4 $kg^{-1}.day^{-1}$ for the remainder of the experiment.

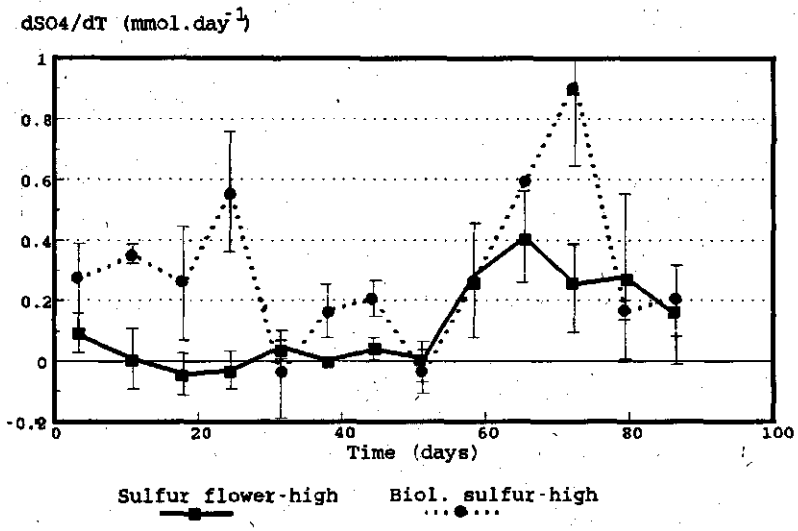

Figure 6.2 Changes of the sulfate production rate (mmol SO_4 $kg^{-1}.day^{-1}$) in the soil with high biological sulfur and sulfur flower treatments.

The changes in sulfur oxidation rate described above correspond well with the observation of local pH-plateaus during the experiments, indicating that declining pH was directly associated with the increasing sulfate concentration in the soil. The variations observed in sulfur oxidation rate can not be clearly interpreted. Two factors may have influenced the process:

A) Altered activity of soil microbial communities responsible for sulfur oxidation.

B) Altered bio-availability of elemental sulfur, due to dynamic changes of specific surface or hydrophobicity of the sulfur particles.

A) Altered activity of soil microbes. Changes in activity of soil microbes can affect the rate of oxidative processes in the soil (Witter et al., 1994). Although the water content of the soil pots was maintained relatively constant, conditions of open-air exposure may have affected the microbial activity. Moreover, different succession of microbes, as a result of changing soil pH, may have contributed. Blais et al. (1993b) reported a two-stage successive change in a mixed population of thiobacilli predominantly oxidizing reduced sulfur compounds in a sewage sludge. Less-acidophillic thiobacilli were initially observed until pH dropped below 4, then a second group of thiobacilli appeared, with a pH-optimum for growth below 3.5. In our pot experiments with biological sulfur, the initial high oxidation rate (first 25 days) corresponded with a pH decrease to 3.5. At that point oxidation was suspended, and recommenced only after 60 days of incubation. We speculate that

this behaviour may be attributed to the successive shift of two different microbial populations.

B) Altered bio-availability of sulfur to microbes. Availability of the elemental sulfur to microbial oxidation is affected by the surface area of the S^0-crystals (Germida and Janzen, 1993; McCaskill and Blair, 1986; Watkinson and Blair, 1993) and by hydrophillicity of this surface (Chapter 3; Janssen et al., 1996). The effects of surface area clearly demonstrated in our experiments with biological sulfur with a specific surface area of 2.5 $m^2.g^{-1}$ oxidizes at a considerably higher rate than with sulfur flower (0.01 $m^2.g^{-1}$ *on average*) (Janssen et al., 1996). Furthermore, we recorded previously (Chapters 3 and 4) that, during oxidation of biological with a suspension of thiobacilli, the finest particle-size fractions were attacked first. When the fine particles were depleted, oxidation proceeded at considerably lower rate (Chapters 3 and 4).

The second factor influencing the sulfur bioavailability is the presence of wetting agents (Schaeffer et al., 1963; Solari et al., 1992). The considerable lag in sulfur flower oxidation was likely due to the hydrophobicity of sulfur surface (Solari et al., 1992). Bacterial wetting agents are, however, already present at the surface of biological sulfur (Janssen et al., 1996; Schaeffer et al., 1963) and account probably for the fact that no lag in oxidation was observed in our experiments.

6.3.2. Cadmium solubilization

As was expected, cadmium became increasingly solubilized with decreasing soil pH. To describe this process, we applied partition coefficient, K_d, expressed as:

$$K_d = \frac{Q}{10 \cdot C} \qquad 6.II$$

where C is the equilibrium cadmium concentration in the soil solution ($mg.L^{-1}$), Q the concentration of cadmium remaining in the soil ($mg.kg^{-1}$) and the value of 10 represents the liquid/soil ratio used in extractive determination ($L.kg^{-1}$). Dissolved cadmium (C), was assumed to correspond to the cadmium determined in the 0.01 $mol.L^{-1}$ $CaCl_2$ extract (Houba et al., 1986). Q was calculated by subtracting the amount in solution from the total cadmium levels (HNO_3-extractable). The values of K_d can be interpreted as a strength of sorption: with increasing value of K_d, more cadmium is adsorbed by the soil, i.e. less cadmium is solubilized.

Values of K_d are given as a function of soil pH in Figure 6.3. Both biological sulfur and sulfur flower treatments produced similar effects on Cd solubility. Possible differences between the two sulfur types might occur due to

different velocities of acidification which might interfere with the process of cadmium solubilization and reduce dissolution rates. However, this was not observed in our experiments and we may conclude that the pH was the sole controlling factor in cadmium solubilization. However, it should be noted that our experiments were performed with freshly contaminated soil, i.e. the cadmium contamination was not subjected to aging. Aging may, in certain cases, considerably slow down the solubilization rate (Eriksson, 1989; Tuin and Tels, 1990a) resulting in lower K_d values, especially during earlier stages of acidification.

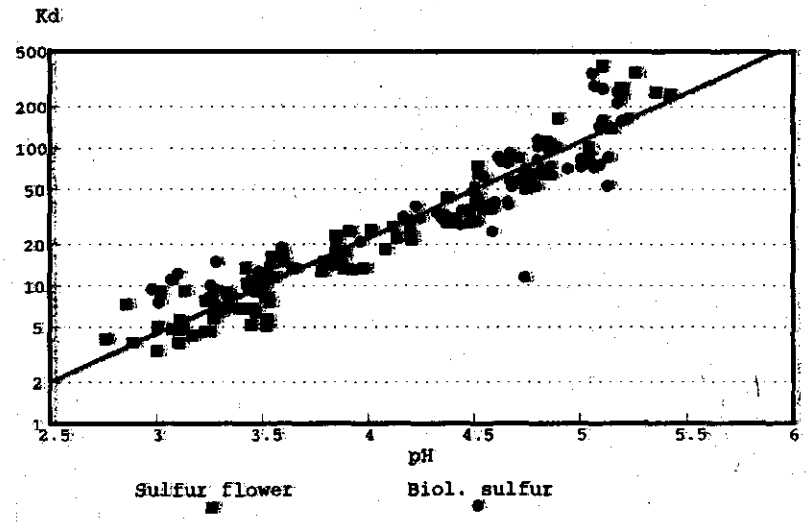

Figure 6.3 Cadmium partition coefficient (K_d, Eq. 6.II) as affected by soil pH. Data include all measurements in time and both sulfur types at both levels.

6.3.3. Cadmium uptake by biomass

The dependence of cadmium concentration in mustard shoots on pH (Figure 6.4) is described by the linear regression:

$$Cd_{shoots}(mg.kg^{-1}) = 362.3 - 52.9 \cdot pH \qquad 6.III$$

However, the correlation coefficient for the regression was low (r^2=0.39 for 32 data pairs). Cadmium concentration in shoots decrease with increasing. Accumulation coefficients, i.e. Cd in plants ($mg.kg^{-1}$)/total Cd in soil ($mg.kg^{-1}$),

reached maximum values of 12-17 at lowest pH, with average of 3.2. Numerous authors have reported a close relationship between plant-available cadmium and Cd-concentration in $CaCl_2$ soil extracts (Houba et al., 1986; Smilde et al., 1992; Tichý et al., 1997). However, no significant correlation was observed in our experiments.

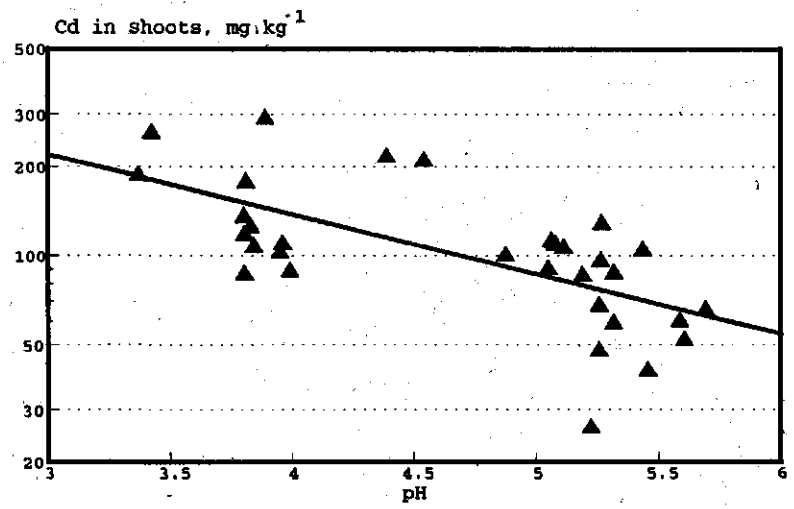

Figure 6.4 Cadmium concentration (mg Cd .kg^{-1} d.w.) in shoots of common mustard as affected by soil pH.

The yield of dry shoots decreased considerably with decreasing pH (Figure 6.5). The linear regression was:

$$\textit{Yield (g dry biomass per pot)} = 17.56 \cdot pH - 61.82 \qquad \textbf{6.IV}$$

with $r^2=0.64$ for 32 data pairs. The two parameters affecting the cadmium removal by plant biomass, i.e. the cadmium concentration in shoots and the shoots dry yield, were combined to show the resulting cadmium uptake effect (Figure 6.6). The curve in Figure 6.6 was produced by combining the regressions (3) and (4). Although the correlation coefficients of the original regressions were low, the existence of pH-optimum for maximum removal of cadmium can be identified in the range of pH 5-5.5. At higher pH, the uptake of cadmium declines, regardless higher biomass production, since the concentration of Cd in shoots is low. At lower pH values, increasing cadmium accumulation was offset by lower biomass production, resulting in low vegetative removal rate.

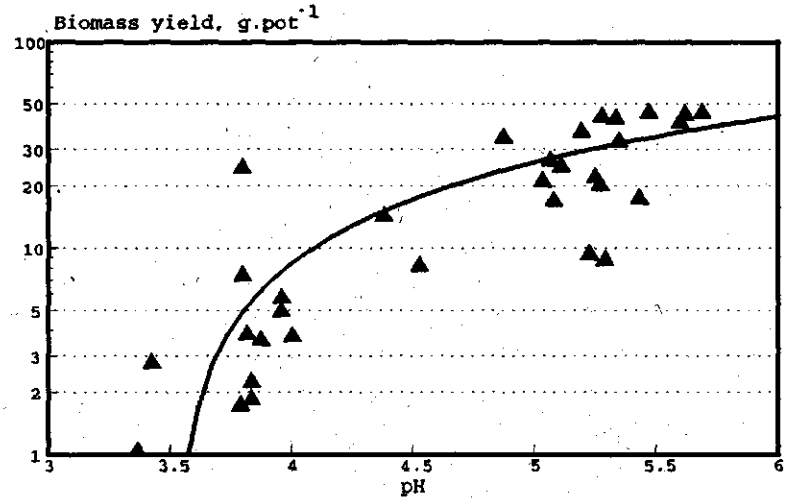

Figure 6.5 Shoots yield of common mustard as affected by soil pH.

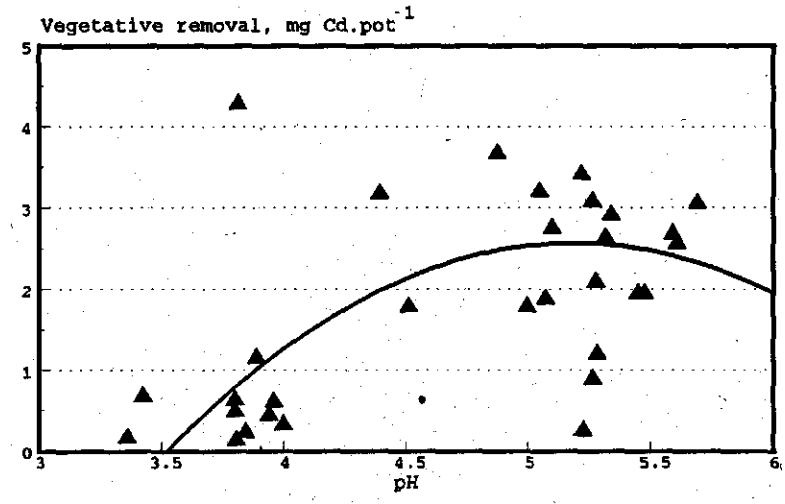

Figure 6.6 The removal of cadmium by biomass, as expressed in mg Cd per 1 soil pot. The curve is produced by combining Eqs. 6.III and 6.IV.

6.4. CONCLUSIONS

The addition of elemental sulfur resulted in considerable decreases in soil pH. Biologically-produced elemental sulfur was oxidized without a lag period after the addition to the soil. The commercially available orthorhombic sulfur flower showed a considerable delay prior to the oxidation, and the process of oxidation proceeded at lower rate. These results agree with the previous findings on the kinetics of microbial oxidation of both sulfur types in a suspension (Chapter 3). This indicates high potential for application of biologically produced hydrophillic sulfur for soil treatment. Such treatment would be possible either as an in-situ technique or in a heaped soil (Rulkens et al., 1995). Moreover, since this sulfur is a waste-product, its price is marginal exclusive the transport costs.

Acidification of soil resulted in the solubilization of cadmium. Soil pH was found to be the primary controlling factor in cadmium solubilization. This acidification can be used as a pre-treatment method for decontamination of metal-polluted soils if an efficient technique for metal removal from the soil solution is provided. Such a technique may involve enforced soil solution percolation followed by proper treatment of the percolate, electroreclamation, or vegetative uptake.

The experiments on vegetative uptake of cadmium by common mustard (*Sinapis alba*, cultivar JARA) showed a strong dependence of cadmium accumulation in plants on soil pH. However, the biomass yields decreased with decreasing pH and adversely affected total cadmium vegetative uptake and removal. It is possible that uptake may be increased when other crop species more tolerant to the low soil pH are used. Maximum Cd concentration reached in our experiments was 300 mg of Cd per kg of dry biomass. At the pH-optimum for maximum uptake, cadmium concentrations were 50-150 $mg.kg^{-1}$. Such high concentrations exceed most legal normatives for standard processing of the biomass, e.g. composting or fodder. Therefore, a finding of proper treatment method for such biomass would be required.

CHAPTER 7

BIOLEACHING OF METALS FROM A WETLAND SEDIMENT LOADED PREVIOUSLY WITH MINE-DRAINAGE

Abstract: *Bioleaching can be one of few techniques applicable for the removal of toxic metals from polluted soils or sediments. Its principle is a microbial production of sulfuric acid and subsequent leaching of metals. Bioleaching can benefit from the use of low-cost substrates and from a possible coupling to other processes of microbial sulfur cycle, like sulfate reduction to treat spent bioleaching liquor, or partial sulfide oxidation to recycle sulfur. For the evaluation of bioleaching, different leaching strategies are considered, i.e. intensive or extensive extraction. The intensive extraction uses high concentrations of acid at short extraction times, whereas low acid additions and long treatment times are used in extensive processes. On a reference study with the wetland sediment receiving mine drainage we demonstrated that the bioleaching is a typical extensive process. The bioleaching experiments involved the use of the different sulfur substrates, i.e. orthorhombic sulfur flower and microbially produced, recycled sulfur from partial sulfide oxidation process. The latter type of sulfur substrate performed considerably better.*

Partly published in *Water Science and Technology* **37/8** (1998), 119-128, authors: R. Tichý, W. H. Rulkens, J. T. C. Grotenhuis, V. Nýdl, C. Cuypers, J. Fajtl.

7.1. INTRODUCTION

Mine drainage water presents considerable environmental risks worldwide. It is characterized by high concentrations of sulfate, cationic metals like Fe, Zn, Cu, Cd, and sometimes extremely low pH (Pascoe et al., 1993; Richards et al., 1993). It is usually produced in high volumes and only few techniques are feasible for its treatment. One of the broadly considered alternatives is the use of wetlands (Wildeman and Laudon, 1988; Eger 1994; Tichý and Mejstřík, 1996). The treatment of the mine drainage water in a wetland involves several mechanisms like sedimentation and filtration of particulate pollution like ferric hydroxides, iron oxyhydroxysulfates or jarosites (Smith et al., 1988; Karathanasis and Thompson 1995), cation exchange and sorption on mineral and organic matrices (Tarutis and Unz, 1990; Bolt and van Riemsdijk, 1987), microbial sulfate reduction leading to alkalization and precipitation of metal sulfides (Machemer and Wildeman, 1992; Eger, 1994; Tarutis and Unz, 1994; Farmer et al., 1995), or ferric iron reduction and subsequent alkalization of the sediment (Vile and Wieder, 1993).

The retention of heavy metals by the sediment is sustained under anaerobic conditions. An introduction of air into the system can happen e.g. during a period of draught, as a result of dikes break, or during dredging and further handling of the sediment when the basin fills up with solid material and has to be regenerated (Gambrell, 1994). Once the air is introduced, a rapid decrease of pH is encountered, followed by increased solubilization of toxic metals. This was repeatedly demonstrated for the polluted river sediment (Maass and Miehlich, 1988; Calmano, 1992; Förstner, 1995) and wetland sediment (Gambrell et al., 1991; Evangelou and Zhang, 1995). The processes involved during sediment oxidation comply oxidation of reduced sulfide-containing compounds (Marnette et al., 1992), oxidation of ferrous iron and precipitation of ferric hydroxides (Förstner, 1995). Acidification of the environment results in further dissolution of non-sulfidic metal precipitates and in desorption of cationic metals (Förstner, 1995). By the abovementioned processes, the polluted sediment can posses hazardous properties and its treatment is required.

However, only limited experience exists with the treatment of sediments loaded with the mine-drainage water (Rulkens et al., 1995; Tichý and Mejstřík, 1996). Apart from immobilization and solidification techniques, only few alternatives are available for the removal of the contaminants and clean-up of the sediment bulk. These include particle-size separation and chemical extraction with acids. Recently, the use of bioleaching was proposed to decontaminate soils and sediments (van der Steen et al., 1992; Tichý et al., 1993a). This method uses specific microorganisms which are able to oxidize reduced sulfur and ferrous-iron containing compounds, leading to acidification of the medium and solubilization of metals. Large-scale bioleaching has been applied in practise only in microbial mining of metals

(Bruynesteyn, 1989; Rawlings and Silver, 1996). Technological trials to use it for decontamination of sewage-sludge (Couillard and Mercier, 1990, 1992; Sreekrishnan and Tyagi, 1995) and to desulfurize coal (Bos and Kuenen, 1990; Rossi, 1993; Loi et al., 1994) were reported as well.

The treatment of metal-polluted sediment can be achieved via various techniques, amongst which is also a bioleaching, i.e. the process using microbial production of mineral acids to solubilized heavy metals. It can be achieved in various technological configurations like sediment slurry or a heap leaching. In this study, we focus on the sediment slurry. The key parameter in bioleaching is the pH of a sediment: the lower are pH values, the higher are metal extraction yields. To achieve sufficiently low pH, proper amount of reduced sulfur or ferrous iron must be present in the treated material. In some cases, the indigenous concentration of reduced sulfur or ferrous-iron containing compounds in the treated material is not high enough to reach sufficiently low pH. Therefore, additional substrate has to be introduced, like ferrous iron (Couillard and Mercier, 1990) or elemental sulfur (Tichý et al., 1993a; Tyagi et al., 1994; Sreekrishnan and Tyagi, 1995). We demonstrated previously (Chapters 3, 4 and 6) a novel bioleaching substrate - microbially produced elemental sulfur. This material appears as a waste product during the microbial treatment of sulfide-containing waste water (Buisman et al., 1990; Janssen et al., 1995). This process is a part of microbial sulfur cycle (Chapter 1, Figure 1.1: sulfide partial oxidation). The microbially produced sulfur provides considerably higher specific surface area, and higher hydrophillicity, compared to the orthorhombic sulfur flower. This leads to much higher oxidation rates of microbially produced elemental sulfur, compared to sulfur flower (Chapter 3; Janssen et al., 1996).

To investigate the possible use of bioleaching for treatment of mine-drainage loaded wetland sediment, firstly the bioleaching tests were carried out using a sediment without amendment of sulfur or acid to demonstrate the auto-acidification potential of the material. Thereafter, experiments with adding sulfuric acid were performed as reference tests, and these results were compared to bioleaching tests amended with sulfur flower or microbially produced elemental sulfur.

7.2. MATERIALS AND METHODS

Sediment: The sediment used in our study originates from a wetland close to settlement Lukavice (district Chrudim, 110 km East from Prague, Czech Republic). The system evolved naturally inside of a pool with broken dike closure, and its age is estimated for 20-30 years. The wetland receives water from a quarry of granite, which contains elevated content of pyrite and some toxic metals. We used the sediment from anoxic layer, which was characterized by dark-grey colour. The sediment was taken after the screening of the upper, aerobic (orange) layer and ca. top 10 cm of the anaerobic layer, and filled in closed 10-L plastic buckets together with the wetland water, which kept the sediment from the exposition to air during the transport. In the laboratory, the sediment was manually homogenized and sieved wet at a 1 mm mesh.

Assessment of the auto-acidification potential: A sediment slurry was prepared by weighing the wet sediment and adding it into distilled water at a ratio of 1g fresh sediment : 2.5 mL water. The dry solid:liquid ratio was 0.0752:1 (g:mL). The slurry was agitated by a magnetic stirrer (90 rpm), and air was pumped to the bottom at a flow rate of 3 L per minute. The aeration proceeded in the dark at 25°C. At chosen times, pH, oxidation-reduction potential (ORP), and metal content was monitored (see below). The experiment was carried out in three independent runs.

Leaching tests with sulfuric acid: The slurry for the leaching tests was prepared by mixing the fresh anoxic sediment with distilled water at ratio 1 g fresh weight : 2.5 mL water. The dry solid:liquid ratio was 0.0603:1 (g:mL). The dry solid:liquir ratio slightly differs from that used in assesssment of autoacidification potential. This is due to varying water content in the sediment sampled prior to the tests. 500 mL shaking bottles were used with 400 mL of the suspension in each. The bottles were closed with seals penetrated by four 5 mm glass tubes to ensure the exchange with atmosphere and proper oxidative status of the slurry. The suspension was acidified by 1 mol.L^{-1} H_2SO_4 to achieve acid concentrations of 5; 10; 20; 30; 60; 100; 150; 200; 250 mM. The bottles were agitated in a rotatory horizontal shaker (100 rpm) in the dark at 25°C.

Bioleaching tests: The experimental setup for bioleaching tests was identical to that applied for leaching tests with acid. Instead of sulfuric acid, additions of 0, 0.1, 0.5, 1, and 5 g of elemental sulfur per 1 litre of sediment slurry were applied. This corresponds to the addition of 0, 3.115, 15.58, 31.15, and 155.8 mmol.L^{-1} of S^0. Orthorhombic sulfur flower (Sulfur praecipitatum) was obtained from Lachema Brno, Czech Republic. Microbially-produced elemental sulfur was produced by a pilot plant treating sulfide-rich wastewater in Eerbeek, The Netherlands. The sulfur suspension was decanted, twice washed with aliquot volumes of distilled water, and

dried at 60°C. Its content of elemental sulfur was 97.5 % by weight, the rest being microbial biomass debris and other impurities originating from the sulfide-containing wastewater. Experiments were carried out in two independent runs.

Analyses: The total Cd, Cu, Fe, Pb, and Zn content in the sediment was determined after HNO_3/H_2O_2 wet digestion using the U.S. EPA method 3050 (U.S. EPA, 1987). pH of the sediment slurry was measured with a Hanna Instruments combined pH-electrode HI 1332 B, connected to a Radelkis (Budapest, Hungary) precision digital pH-meter (OP-208/1). Oxidation-reduction potential (ORP) was measured with an Ingold PT4805-S7/120 combined redox electrode, connected to the pH-meter described above. Both pH and ORP measurements were done directly in the sediment slurry, kept in suspension by slow swirling. To determine the sulfate and metals dissolved in the aqueous phase, slurry was centrifugated (15 minutes, 3000 rpm) and filtrated over a Synpor 5 μm membrane filter (Pragochema, Prague, Czech Republic). Sulfate was determined by isotachophoresis and contact conductivity detector after Vacík and Muselasová (1985). ICP/AES (PU 7450, Leemans Laboratories, U.S.A.) method was used to determine metals in the liquor.

Numeric data processing: For all data processing, a standard statistical software package Statgrafics 2.6 was applied. Non-linear regression was performed by least-square iteration using Marquart method. The end-criterium for iterations was set as change of least square $< 10^{-14}$.

7.3. RESULTS AND DISCUSSION

7.3.1. Assessment of the auto-acidification potential

The aerated sediment slurry showed instantenous decrease of pH and increase of ORP after the introduction of air (Figure 7.1). Nearly linear decrease of pH from 6.8 down to 5.15 was observed within the first 30 hours of aeration. This was accompanied by a steep increase of ORP from -145 meV to +150 meV. Afterwards, the acidification rate was retarded. Finally, the sediment slurry stabilized at pH 4.2 after 120 hours of aeration. Accordingly, the increase of ORP was less steep in the second half of the experiment, reaching the final values of +300 meV.

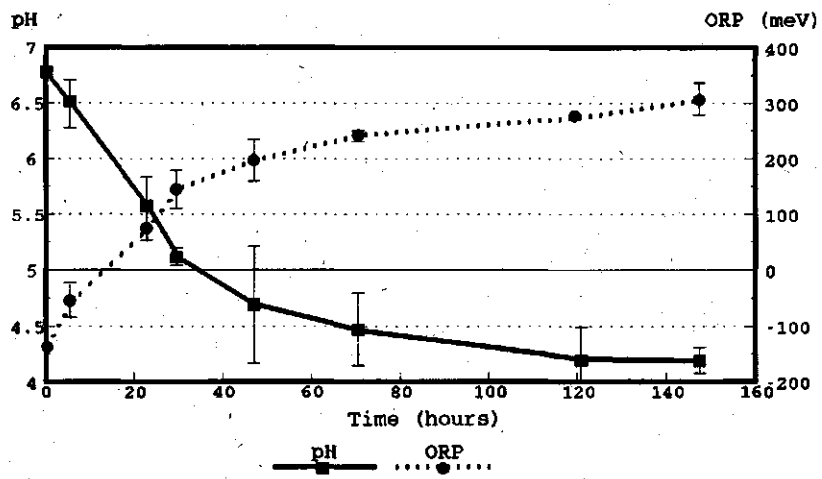

Figure 7.1 Changes of pH and oxidation reduction potential (ORP) in a sediment slurry with no sulfur amendment.

The intensive changes in pH and ORP within the first 30 hours of aeration were accompanied by a fast increase of sulfate and iron in the liquor (Figure 7.2). The sulfate increased from ca. 1 mmol.L^{-1} to 6.35 mmol.L^{-1} after 30 hours. Afterwards, sulfate concentrations did not change. The increasing concentration of sulfate in the liquor is a result of chemical and biological oxidation of reduced sulfur compounds, particularly, sulfides present in the sediment. The concentrations of iron demonstrated also the initial increase with maximum at 40 hours of aeration. Afterwards, a decrease in soluble iron was observed. We speculate that this phenomenon was caused by two different mechanisms: initially, the iron was predominantly present in its reduced Fe^{2+} form, since the sample was originally anoxic. The decrease of pH resulted in desorption of Fe^{2+} from the adsorbed phase and in dissolution of pyritic crystals (Eger, 1994; Evangelou and Zhang, 1995). This was manifested by its increasing concentration in the aqueous phase. Afterwards, however, the Fe^{2+} iron was oxidized into the Fe^{3+} form, which is poorly soluble in water (Smith et al., 1988; Karathanasis and Thompson, 1995). Its precipitation caused a steep decrease of iron concentration in the liquor. However, we can not prove this statement by experimental observations, since we did not performed the analysis of Fe^{2+}/Fe^{3+} species.

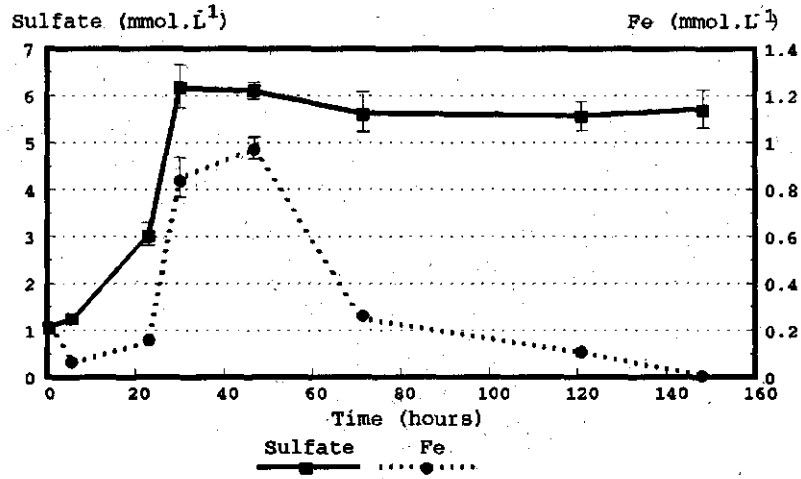

Figure 7.2 Concentrations of sulfate and total iron in a sediment slurry with no sulfur amendment.

The aeration and decrease of pH of the slurry evoked the solubilization of Cd, Cu, and Zn (Figure 7.3). Lead was not detected in the aqueous phase during the assesssment of auto-acidification. Concentrations of Cu and Zn were stable and low within the first 30-40 hours of aeration. Afterwards, the increase was recorded to a maximum of ca 0.12 $mg.L^{-1}$ of Cu and ca. 0.9 $mg.L^{-1}$ of Zn. This maximum was reached after 70 hours of aeration. After 70 hours, the concentrations of Cu and Zn were stable. Solubilization of cadmium was less affected by time. Its maximum concentration in the liquor reached 0.078 $mg.L^{-1}$. However, the initial concentration of cadmium in the liquor was 0.055 $mg.L^{-1}$, i.e. 70% of the terminal soluble cadmium.

Although the pH of the slurry reached values of 4.2, i.e. rather low, the solubilization of metals was not high enough to record a substantial removal effect. When compared to the total concentrations of metals in the sediment (data not shown), only maximum 16% of Cd, 0.4% for Cu, and 3.7% of Zn was solubilized. Therefore, we conclude that the auto-acidification potential of the studied wetland sediment is not satisfactory to promote efficient removal of metals.

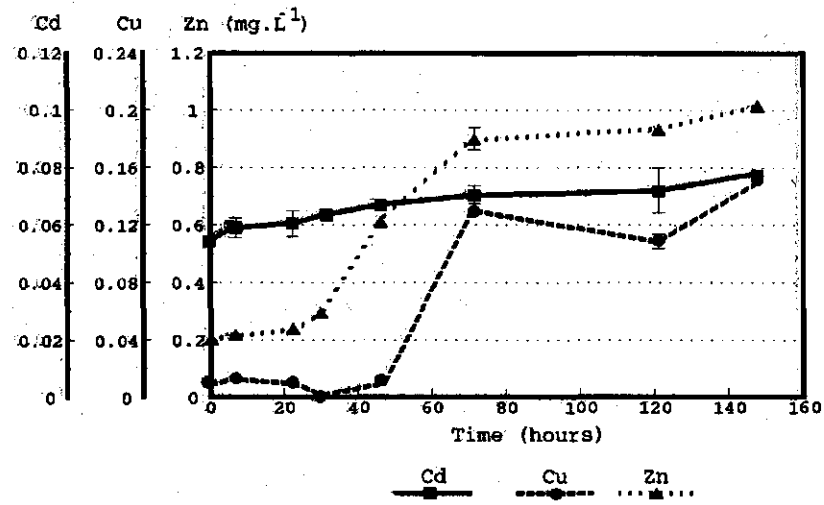

Figure 7.3 Solubilization of Cd, Cu, and Zn in a sediment slurry with no sulfur amendment.

7.3.2. Leaching tests with sulfuric acid

The pH of the sediment slurry during the aeration and at varying additions of sulfuric acid was obviously affected by two major trends: a) the decrease of pH due to chemical and microbial oxidative changes (at low acid additions), and b) the delayed increase of pH due to the pH-buffering, protonation of solid components, proton-adsorption and diffusion into the particles (at high acid additions). These two trends worked adversely and resulted in a rather complex behaviour of pH in the sediment slurry, as shown in Figure 7.4, measured points. After the addition of H_2SO_4 at concentrations ≤ 100 mmol.L^{-1} H_2SO_4, the pH immediately started to increase. After 5 hours, however, the pH in these treatment begun to decrease. In treatments with acid addition higher than 150 mmol.L^{-1} H_2SO_4, the subsequent pH-decrease was not recorded.

To predict the pH of a sediment slurry as a function of time and concentration of added acid, we developed an empirical model as follows:

$$pH = P1 + P2 \cdot \log(Y) + P3 \cdot e^{(P4 \cdot Y + P5 \cdot t)} + \frac{P6}{1 + P7 \cdot t} \quad .I \qquad 7$$

Here, Y denotes a concentration of added sulfuric acid (mmol.L^{-1}), t represents the

time of extraction (hours), and P1, P2, P3, P4, P5, P6, P7 are model parameters. The log symbol stands for a natural logarithm and e is the base of natural logarithm. Non-linear fitting of this model yielded the correlation coefficient of $r^2=0.982$ for 121 data points. The curves predicted by the model are plotted in Figure 7.4. The values of model parameters are summarized in Table 7.1.

Table 7.1 Fitted parameters in the Equation 7.1. The correlation coefficient r^2 for 121 data points yielded **0.982**.

P1	5.308 ± 0.270
P2	-0.3719 ± 0.0195
P3	8.2404 ± 1.0180
P4	-0.00257 ± 0.00047
P5	-0.04063 ± 0.00200
P6	-6.7078 ± 1.3060
P7	0.06363 ± 0.0095

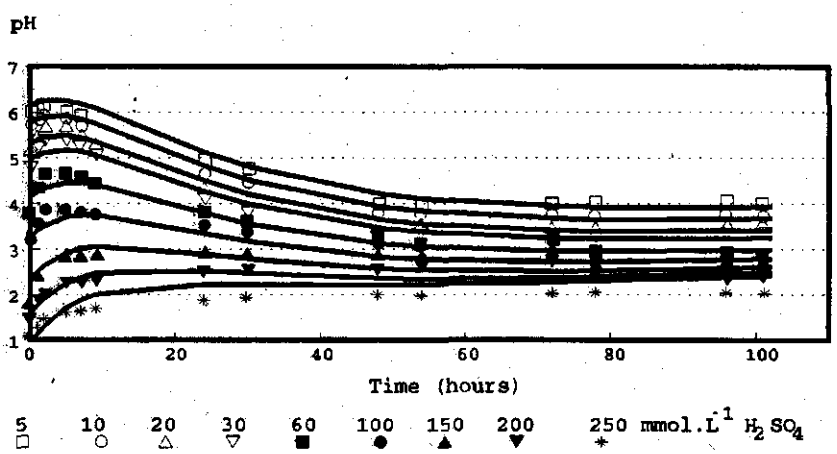

Figure 7.4 Changes of pH of a sediment slurry after the addition of sulfuric acid.

The concentrations of solubilized metals in the liquor followed changes of pH and time. To combine both parameters, we applied the exposure variable, i.e. time multiplied by protons activity. The exposure was successfully demonstrated as a control variable e.g. in solubilization of pyrite by acid (van der Zee and de Wit, 1993). The concentrations of Cd, Cu, Pb, and Zn followed a clear sigmoidal dependence on exposure (data not shown). Therefore, we applied a regression using modified Gompertz function:

$$Me(mg/L) = e^{Q1 + Q2 \cdot e^{Q3 \cdot (t \cdot (H^+))^{Q4}}} \qquad 7.II$$

Here, Me denotes a concentration of particular metal in the solution ($mg.L^{-1}$), t is time (hours), (H^+) is the activity of protons ($mmol.L^{-1}$) calculated from the slurry pH, and Q1, Q2, Q3, Q4 are model parameters. Resulting values of parameters for the four studied metals are summarized in Table 7.2.

Table 7.2 Fitted parameters in the Equation 7.II. N stands for the number of data points.

	Cd	Cu	Pb	Zn
Q1	-4.690±0.821	-2.151±2.418	-1.301±1.122	-1.226±0.690
Q2	3.405±0.847	5.694±2.478	4.749±1.119	3.928±0.690
Q3	-0.557±0.189	-1.976±1.530	-2.663±1.100	-0.545±0.134
Q4	-0.382±0.075	-0.494±0.095	-0.530±0.069	-0.487±0.049
r^2	**0.892**	**0.942**	**0.912**	**0.964**
N=	110	110	106	109

It should be noted that the developed empirical model, Equation 7.2, will work for the conditions of diluted slurry, since it does not take into account the volume of bulk liquid. This empirical model has an upper limit, to which it approaches at exposure approaching infinite. The values of these limits can be interpreted as extrapolated maximum concentrations of given metal in the solution. Numerically, they were similar to the values of theoretical maximum concentrations in the liquid, which were calculated by multiplying the sediment total concentrations by the solid:liquid ratio.

The total concentrations of metals, theoretical maxima in the solution, actual maxima in the solution and maximum achieved extraction yields are represented in

Table 7.3. The actual maximum concentrations reach 54.2-66.9% of the total metal content. The 54.2% solubilization of Pb is rather unexpected result, since $PbSO_4$ is believed to be poorly soluble in water (Evans, 1989). However, the low pH-values probably enforced Pb solubilization, so that its concentrations in the liquor were percentically comparable to other metals. More detailed discussion on this subject is given in Chapter 8.4 of this thesis.

Table 7.3 Total metals in the sediment, theoretical and actual maximum concentrations of solubilized metals, and maximum extraction yields.

	Cd	Cu	Pb	Zn
Mean ($mg.kg^{-1}$)	6.402	485.2	576.1	356.2
Standard deviation	0.453	8.1	31.9	10.3
Theor. maximum in solution ($mg.L^{-1}$)[A]	0.386	29.31	34.76	21.49
Actual maximum in solution ($mg.L^{-1}$)[B]	0.229	19.61	18.85	13.27
Maximum extraction yield (%)[C]	59.3	66.9	54.2	61.7

A: Total metal content in the sediment multiplied by the solid:liquid ratio. We assume that the given solid:liquid ratio of 0.0752:1 sufficiently represents the highly diluted suspension.
B: Concentration of metal in the solution after 100 hours extraction with 250 $mmol.L^{-1}$ H_2SO_4.
C: Percentage of actual maximum soluble metal from theoretical maximum.

Combining the two equations, i.e. 7.I and 7.II, the efficiency of extraction with sulfuric acid (added directly or produced by microbes) can be simulated. In Figure 7.5, this simulation is presented with H_2SO_4 of concentrations of 3.125, 15.625, 31.25, 156.25 and 250 $mmol.L^{-1}$. The resulting extraction curves revealed three different phases: 1. nearly horizontal line initially, 2. the increasing phase, and 3. terminal phase with the curve asymptotically approaching a horizontal line. For Cd and Zn, the first phase occurred within the first 10 hours of leaching, and the third phase took place at time >80 hours. Similar findings for Cu and Pb were found only with the highest simulated concentration of H_2SO_4. Lower concentration of H_2SO_4 showed low extraction yields, and the typical three regions were not pronounced. The maximum applied H_2SO_4 concentration (250 $mmol.L^{-1}$), indicated by thick lines in Figure 7.5, showed nearly horizontal line for Cd and Zn. It shows

an increase with time for Cu, Pb, however, the three phases noticed above were also not pronounced.

Our results indicate that at short extraction times, i.e. within 20 hours, high concentrations of sulfuric acid must be applied to gain a significant extraction yield. A prolonged extraction with high acid concentration is redundant, since it does not substantially improve the extraction yield. Comparably high extraction yield can be obtained with lower concentrations of acid, however, at prolonged extraction. These findings indicate that two different strategies are applicable for leaching, i.e. **intensive**, which uses high concentrations of acid at short extraction, and **extensive**, which uses long extraction with low acid concentrations. This corresponds to our previous findings with leaching of clay, silt, and sandy soils artificially polluted with zinc (Chapter 5), and seems to have a general validity.

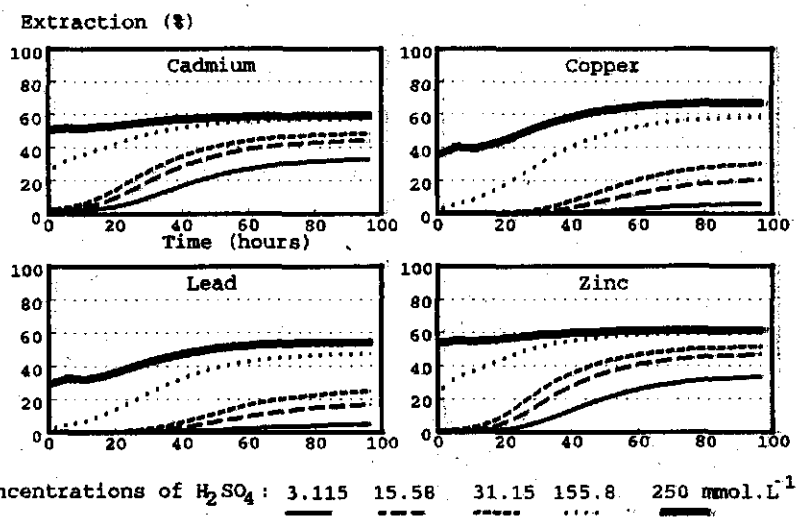

Figure 7.5 Simulated extraction yields of Cd, Cu, Pb, and Zn at varying additions of H_2SO_4.

7.3.3. Bioleaching tests

The pH recorded during bioleaching tests with microbially produced sulfur and orthorhombic sulfur flower is presented in Figure 7.6. To demonstrate a relevance of the pH-changes for the bioleaching efficiency, the 50% extraction isolines for Cd, Cu, Pb, Zn were calculated from the empirical model (Eq. 7.II) and added to the Figure 7.6. The extraction isolines should be interpreted as sets of extraction time and pH values which yield the 50% extractability of a given metal. The space above the isoline contains points with lower extraction efficiency, points

below this isoline yield higher extraction efficiency. In all treatments, the microbially produced sulfur acidified faster than the sulfur flower. This phenomenon was described earlier for a sediment-free water suspension (Chapter 3) and for conditions of undisturbed soil profile (Chapter 6). For both types of sulfur, the treatment with 31.1 and 155.8 mmol.L^{-1} sulfur, i.e. 1 and 5 g $S^0.L^{-1}$, respectively, yielded similar results. It means that the concentration of 31.15 mmol.L^{-1} already approached the maximum oxidative capacity of the indigenous microbial community, and higher addition of sulfur was redundant. The microbial sulfur oxidation is controlled by the process of microbial adhesion to the sulfur particles (Kelly, 1982). Assuming a constant area which is occupied by one microbe, the sediment population of thiobacilli needs for adhesion and oxidation only a certain surface area of elemental sulfur. If more sulfur is added than is needed for hosting the microflora, the oxidation rate will not increase. We speculate that this is an explanation for no difference in acidification of the 31.15 and 155.8 mmol.L^{-1} sulfur additions.

Generally, the differences among acidification with different concentrations of microbially produced sulfur were less pronounced, compared to the sulfur flower. This also suggests the limitation by maximum oxidizing capacity of the microflora, since the microbially produced sulfur provides considerably higher surface area and more hydrophillic surface characteristics, compared to the sulfur flower (Chapter 3). Therefore, much lower additions of microbially produced sulfur reached maximum oxidizing capacity.

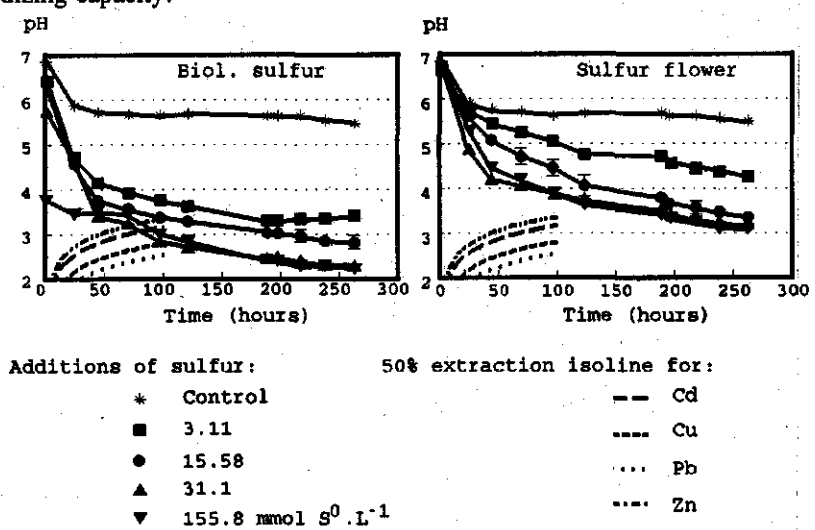

Figure 7.6 Bioleaching with microbially produced sulfur and sulfur flower with indicated 50% extraction isolines for Cd, Cu, Pb, and Zn.

7.4. CONCLUSIONS

Although the auto-acidificiation potential of the studied wetland sediment during aeration was high, and pH dropped close to 4, it is was not sufficient to remove heavy metals without further amendment of additional sulfur substrate or acid.

Two different strategies can be applied for leaching of heavy metals from the polluted wetland sediment slurry, i.e. intensive and extensive. The intensive leaching is performed at high levels of acid (above 150 mmol.L^{-1}) and extremely low pH (<2.5) at short extraction times (max. 20 hours). The extensive leaching is performed at high extraction times (>80 hours), however, at low concentrations of acid and higher pH. Eventual use of bioleaching can be denoted as the extensive strategy.

The batch leaching process, i.e. the development of pH and solubilization of Cd, Cu, Pb, Zn, was described using two empirical models. The models use two independent variables, i.e. the extraction time and the concentration of added acid. These models offer a future perspective when both independent variables are assigned their economic value. This will ultimately lead to a simple cost-benefit analysis, which is highly needed for the true evaluation of different leaching strategies, including the bioleaching.

The use of microbially produced elemental sulfur as a substrate for bioleaching yielded considerably better results than the orthorhombic sulfur flower. As this effect has been repeatedly observed, we conclude that its use for bioleaching offers an attractive possibility.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 ENVIRONMENTAL ASPECTS OF BIOLEACHING

Bioleaching is a process with two opposite environmental aspects for human beings. On one side, it leads to a spontaneous leaching of sulfuric acid and toxic heavy metals from abandoned mines, spoil banks, dredged or dewatered sediments, on the other side, it can also posses certain benefits in controlled bioleaching of low-grade ores, removal of undesired sulfur from fossil fuels or for the decontamination of metal-polluted materials and contaminated sites.

Chemical time bombs

The reduced sulfur compounds have a strong potential to act as chemical time bombs, particularly due to the strong coupling of their oxidative changes with mobility of toxic cationic heavy metals in the environment. Reduced sulfur compounds, together with heavy metals, can be safely retained in ecosystems when anoxic conditions are stable, e.g. in aqueous sediments, wetlands, anaerobic soils. An introduction of oxygen may trigger rather vast and unexpected response and adversely affect large areas by leachates containing toxic heavy metals, sulfate, and acidity which can further negatively affect both the ecosystems, geosphere, and men. This applies for the acid mine drainage, spoil bank and mine tailing leachates, but also for aqueous sediments which come into contact with the air. Although the negative environmental aspects of solid state reduced sulfur compounds were not the main topic of this thesis, they received some attention in the survey of literature (Chapter 2). They are further demonstrated by experiments with wetland sediment which was exposed to mine drainage water for a considerable time period in the past (Chapter 7). Such a wetland system is indeed rather vulnerable and may impose a stringent limitation to the landscape downstream. In case of the wetland studied in Chapter 7, periodical depressions of water level resulted not only in negative effects of outgoing pollution on the ecosystems, but even corrosion of steel and concrete parts of a construction site few kilometres downstream was recorded.

In The Netherlands, the troubles encountered during the air introduction into freshwater sediments are receiving special concern, since huge amounts of freshwater and brackish sediments have to be dredged, mainly due to nautical reasons. Other example are recent (July 1997) sever floods in the Czech Republic. Strong water streams swirled and transported sediments which may have accumulated heavy metals, other pollutants and solid state reduced sulfur compounds over the time. After the water withdrawal, much concern arouse around the impacts of the pollutants on the soil quality, agriculture and wildlife.

The use of bioleaching to remove toxic metals from the environment

The second aspect of a bioleaching process is its possible use for the removal of toxic heavy metals from the environment. This process has been demonstrated for the recovery of some metals from the low-grade ores (biohydrometallurgy), for the microbial desulfurization of coal and for the removal of toxic heavy metals from anaerobically-pretreated polluted sewage sludge. This thesis focuses at a possible use of bioleaching for the removal of toxic metals from contaminated soils or freshwater sediments. Apparently, the applicability of bioleaching depends on the availability of substrate for acid-producing bacteria and on the strength of metals binding to the soil or sediment. Standard soils are usually predominantly aerobic environments. Therefore, no or negligible amounts of reduced sulfur species can be expected there. The bioleaching would solely depend on the addition of substrate for thiobacilli. On the contrary, freshwater sediments usually contain substantial concentrations of reduced sulfur species, including pyrite and other cationic metal sulfides. Therefore, acidification and leaching is always encountered when air is introduced, and e.g. the pH of a sediment suspension may drop within few days of swirling from pH 7-8 down to 4 (see e.g. Chapter 7, Figure 7.1). However, depending on the binding strength of metals, even pH 4 may not be the level of acidity resulting in desired efficiency of metals solubilization. In this case, additional substrate for bioleaching will be required as well.

8.2 BIOLEACHING SUBSTRATES

Bioleaching processes utilize various reduced compounds containing sulfur or ferrous iron (Fe^{2+}). Substrates like metal sulfides (e.g. pyrite), reduced ferrous iron (Fe^{2+}) or elemental sulfur are applicable. Intensive bioleaching has been reported in the literature when ferrous sulfide (pyrite) or ferrous sulfate were applied. Pyrite, due to its negligible solubility in water, has to be finely milled and

properly mixed to achieve suitable bioleaching rates. Ferrous sulfate is soluble in water. Therefore, its availability to thiobacilli is very high, compared to other insoluble substrates. However, a bottleneck of the use of ferrous iron as a bioleaching substrate is in the massive precipitation of ferric hydroxides in the treated solid material. Therefore, although the bioleaching may remove toxic heavy metals, it leaves the treated material highly burdened with ferric precipitates.

When elemental sulfur is applied as a bioleaching substrate, the apparent advantage is that the treated material is not loaded by additional chemicals like ferric precipitates. It should be realized, however, that the elemental sulfur is not soluble in water. Therefore, practical applications will face certain difficulties with a pre-treatment of substrate (pulverizing, milling, wetting), its supply to the reactor and other aspects of biotechnological use of insoluble substrate. As stated in Chapters 1 and 2, a microbially produced elemental sulfur may be used as a bioleaching substrate, and the produced sulfate may be recovered again as elemental sulfur via other processes of the microbial sulfur cycle (Chapter 1, Figure 1.1). Particularly, the primary question for the research was whether the microbially produced sulfur will be a feasible substrate for acidophillic (acid-loving) thiobacilli and what will be the rate of sulfuric acid formation, compared to the use of standard orthorhombic sulfur flower. The microbially produced elemental sulfur consists of ca. 100 nm large agglomerates of elemental sulfur, bacterial biomass, polymers of bacterial origin and sulfur groups in other oxidation state than S^0 (Janssen et al., 1996). To investigate the possible use of this sulfur for bioleaching, the experiments with batch and continuous cultivation of thiobacilli were performed. They are described in detail in Chapter 3 (batch aseptic cultivation) and Chapter 4 (continuous mixed-culture experiments).

Batch experiments comparing the orthorhombic sulfur with microbially produced elemental sulfur

Results of the batch experiments proved that the microbially produced elemental sulfur is an excellent substrate, compared to the orthorhombic sulfur flower. With both substrates, thiobacilli achieved nearly equal maximum growth rate. When the final results of batch cultivations are compared, the yield of biomass on elemental sulfur was also the same for both types of sulfur. Considerable difference was found in a duration of the interval with high growth rates, which was longer with microbially produced sulfur than with sulfur flower, and in maximum specific oxidation rate (i.e. amount of sulfur oxidized per hour and per unit of biomass in a given time of batch cultivation), which was nearly two-times higher with microbially produced sulfur than with sulfur flower. The better availability of

microbially produced sulfur is attributed to its higher hydrophillicity, higher specific surface and its presence in fine particle size fractions, and possibly also to the presence of other than elemental sulfur compounds or other than matured crystalline forms of S^0, which may be better accessible for thiobacilli. Table 8.1 summarizes the basic differences between the two sulfur types.

Table 8.1 General summary of the differences between the orthorhombic sulfur flower and microbially produced sulfur, with respect to their use as a substrate for acidophillic thiobacilli.

	Sulfur flower	Microbially produced sulfur	Reference
Specific surface area ($m^2.g^{-1}$)	0.01	2.5	Chapter 3
Surface properties:	Hydrophobic	Hydrophillic	Chapter 3
Size of particles (nm)	Variable*	100	Janssen et al., 1996
Maximum specific growth rate of thiobacilli D_2 culture (h^{-1})	0.08	0.082	Chapter 3
Maximum specific sulfate production rate by thiobacilli D_2 culture per concentration of biomass nitrogen ($mmol.mg^{-1}.h^{-1}$)	0.274	0.498	Chapter 3

* The size of sulfur flower particles depends on mechanical crushing. Generally, it is much higher than that of microbially produced sulfur particles.

Indeed, the individual contribution of the above mentioned attributes to the better availability of microbially produced sulfur is questionable. For example, the process of elemental sulfur oxidation may be predominantly ruled by the presence of very fine particle-size sulfur. During the oxidation in a suspension, the sheer and agitation forces lead to a breakdown of microbially produced sulfur particles into fine fractions, as shown in Chapter 3, Figures 3.4, 3.5 and 3.6, and are rapidly consumed by the bacteria. With microbially produced sulfur, a rapid increase in the non-filterable sulfur concentration, up to 4 $mmol.L^{-1}$, was observed within the first 24 hours of agitation. Afterwards, the non-filterable elemental sulfur concentration

decreased, until it reached a nearly stable level below 0.2 mmol.L^{-1} at 72 hours of agitation. This was not recorded for the suspension of sulfur flower, where the non-filterable elemental sulfur concentrations persisted at levels below 0.2 mmol.L^{-1}.

It may be speculated whether similar effects as with microbially produced sulfur can be achieved when the sulfur flower is pulverized to very fine particles. However, the role of hydrophillic surface of microbially produced sulfur may be substantial as well. Microbially produced elemental sulfur makes homogenous milk-like suspension in water, on the contrary from the sulfur flower, which sticks to the walls of reactor and is flotated by water bubbles in the reactor (data not shown).

Continuous microbial oxidation of the microbially produced elemental sulfur

Continuous oxidation of microbially produced elemental sulfur was studied using a series of bio-reactors with mixed populations of thiobacilli. The first vessel was the reactor producing elemental sulfur from sulfide under oxygen-limiting conditions. This process is carried out by neutrophillic thiobacilli (requiring circumneutral pH) at pH around 8.

The second reactor was supplied with excessive aeration and used in two different modes of operation (see Chapter 4, Figure 4.1): in the first run of experiments, the pH of the second reactor was maintained at constant value of 8, i.e. the same as in the reactor 1. This was done to study the capacity of autochthonous bacteria from reactor 1 to further oxidize sulfur. This "stability" of sulfur is an important factor since it determines how fast should be the process of elemental sulfur separation from the liquor or whether the neutrophillic thiobacilli should be inhibited by adding chemicals. The results show that even at high dilution rates (>0.1 $hour^{-1}$), at least 40% of the elemental sulfur is oxidized (see Chapter 4, Figure 4.5). We have shown that the microbial oxidation always removed the fine sulfur particles. This means that the recovery of elemental sulfur from the original suspension will have to be speeded up, e.g. by coagulation, centrifugation, etc.

In the second run of experiments, no pH maintenance was performed in the excessively aerated reactor 2. Therefore, the reactor liquor rapidly acidified due to the oxidation of elemental sulfur to sulfuric acid. Due to the non-aseptic conditions we let the population of acidophillic thiobacilli to evolve spontaneously. The continuous culture achieved highly acidic conditions (at dilution rate lower than 0.05 $hour^{-1}$, the pH dropped below 1.65, see Chapter 4, Figure 4.7). Maximum production rate of sulfuric acid was observed at a dilution rate of 0.09 $hour^{-1}$ and yielded nearly 1 mmol H_2SO_4 .L^{-1}.$hour^{-1}$ (Chapter 4, Figure 4.8).

8.3 THE LEACHING BEHAVIOUR OF HEAVY METALS

Apart from the sulfuric production capacity and potential to acidification, the second crucial aspect of bioleaching efficiency is the leaching behaviour of toxic metals from soils or sediments. The leaching behaviour is generally affected by three different factors:

Technological configuration of the leaching process. The process of bioleaching may be carried out in rather different technological configurations, including the strongly agitated soil slurry reactors (soil suspension), heap leaching or in-situ extraction. Slurry reactors are usually very efficient but require high investment and operation costs. Heap leaching or in-situ processes proceed usually at lower rate, however, their costs may be considerably lower than those of soil slurry systems.

Dependence of the metals extraction efficiency on the concentration of sulfuric acid and possible unwanted consequences of bioleaching. Throughout the literature, many case studies report on leaching behaviour of cationic heavy metals. The results are, however, often strongly case-specific and only scarce fundamental or technical information can be generalized from them. Extraction efficiency and its dependence on sulfuric acid concentration are ruled by the binding strength of metals to the soil. It involves numerous factors like the quantity and quality of soil organic matter, clay minerals, total cation exchange capacity, saturation with other cations, the way how contamination has penetrated the soil or the time of the contaminated soil "aging". The leaching of cationic metals follows numerous physical-chemical processes like desorption, complexation, ion-exchange or dissolution, which are generally enhanced by the addition of acidity. The first question is, whether the amounts of sulfuric acid produced during bioleaching are sufficient to extract metals or not. The second question is whether the levels of acid achieved during bioleaching can have some adverse effects like dissolution of soil mineral matrix, e.g. aluminium.

The difference between behaviour of soils and freshwater sediments. Commonly, the soils are prevalentnly aerobic and therefore not expected to contain significant amounts of reduced compounds. Therefore, when extra air is introduced, no acidification will occur, unless an additional bioleaching substrate is supplied. On the contrary, the freshwater sediments, when their originally anoxic status is converted into the fully aerated conditions, may reveal fast changes of pH, metal solubilization and some other parameters. However, it is not known whether the pH decrease in these sediments will be sufficient to remove metals or whether an additional substrate will be required.

To study the above mentioned aspects, three experimental studies were accomplished to demonstrate the bioleaching of metals from soils and sediments (Chapters 5, 6, 7).

Leaching of zinc in an aerobic soil slurry

The first leaching study (Chapter 5) involved an artificially zinc-contaminated clay, silt, and sandy soil. The leaching behaviour was studied at varying additions of sulfuric acid. Parallel, aluminium solubilization was quantified to study the extent of soil matrix damage by extreme acidity. Whereas the zinc extractability increased with lowering pH rather monotonously, being 17-43% at pH 7 and 72-95% at pH 1.5, the solubilization of aluminium was negligible at pH > 4. At pH < 4, aluminium concentrations in the solution started to increase (see Chapter 5, Figure 5.4). When expressed in terms of sulfuric acid concentration, levels higher than 0.001-0.002 mol H_2SO_4 $.L^{-1}$ already damaged the soil structure and solubilized aluminium (Chapter 5, Figure 5.6). From the leaching tests, two different strategies can be generalized. The first one, assigned here as intensive, uses concentrations of sulfuric acid higher than the 0.01-0.02 $mol.L^{-1}$ at a soil/solution ratio higher than 0.1-0.4 $kg.L^{-1}$. The second strategy, assigned here as extensive, uses lower concentrations of acidity, however, at a considerably lower soil/solution ratio in the leaching process. The difference between the two stategies is schematized in Table 8.2.

Table 8.2 Schematic representation of the different leaching strategies.

Leaching strategy	Soil/solution ratio	Required concentration of acid	Soil damage
Intensive	high	high	high
Extensive	low	low	low

In-situ bioleaching of cadmium and its vegetative uptake

The second study (Chapter 6) aimed at the possible use of sulfur oxidation in-situ, i.e. within the unmixed soil profile. In this study, cadmium was added to the soil and the soil was cultivated in pots under the open sky. Prior to its placement into the pots, the soil was amended with sulfur flower or microbially produced sulfur and mechanically mixed to ensure its maximum homogeneity throughout the soil profile. During cultivation, the velocity of soil acidification and cadmium solubilization were monitored. The microbially produced sulfur proved faster oxidation and acidification than the orthorhombic sulfur flower (Chapter 6, Figure 6.1). The main difference between the two types of sulfur was in the initial phase, where the microbially produced sulfur revealed a higher oxidation rate, compared to the sulfur flower (Figure 6.2). This is likely attributed to the initial presence of microbially produced sulfur as much finer particles, compared to the sulfur flower.

The solubilization of cadmium into the pore water followed directly the changes of pH (Chapter 6, Figure 6.3). This means that the elemental sulfur addition or direct amendment of sulfuric acid into the soil may be applied as a sanitative option, provided that a proper removal of metal-containing solution is accomplished. This may include e.g. the collection of soil percolate, in-situ soil washing or a removal of metals from the soil water with the use of green plants. When the removal of soil percolate is concerned, at minimum pH achieved in the experiments (pH 2.75, see Figure 6.1), up to 32% of cadmium was solubilized and appeared in the pore water. Hence, the maintenance of low pH and withdrawal of pore water may be feasible. The pH of 2.75 was achieved only after 90 days of cultivation, however, higher amendment of sulfur or direct addition of sulfuric acid may considerably speed-up the process. At highest pH levels, i.e. pH 5.5, only 0.3% of cadmium appeared in the pore water.

In Chapter 6, a vegetative uptake was further studied using a common mustard (*Sinapis alba*). A 90-days growth period was studied, assuming that the roots of plants penetrated the whole soil profile by the end of cultivation. The vegetative study was done to investigate an alternative method for the removal of solubilized cadmium, other than the direct removal of soil percolate. As expected, the cadmium taken up by harvestable plant shoots was increasing with lowering pH (Figure 6.4). However, the yields of biomass also decreased with the lowering pH (Figure 6.5). When combined the dependence of cadmium uptake and plant yield on pH, an optimum pH for the vegetative uptake was determined as 5-5.5 (Figure 6.6). However, the efficiency of the process was rather low. With biomass yields and uptake quantities achieved in the pot experiment, common mustard was able to remove maximum 2.2 ± 0.9 mg of cadmium per pot (Figure 6.6). This makes only 1.5 ± 0.6% of the total cadmium present in the soil pot. In other words, assuming

the constant uptake of cadmium by plants (which is already a rather optimistic assumption), the total cleanup of the site would require 48-115 growth cycles of common mustard to achieve zero contamination with cadmium. It should be noted that the removal efficiency is then similar to that achieved with pure withdrawal of pore water without any prior amendment of sulfur or sulfuric acid, as described above.

Bioleaching of metals from anoxic sediment

In the third leaching study (Chapter 7), the sediment from a wetland receiving mine drainage was used for slurry bioleaching tests. This type of sediment was chosen because the principle of a wetland treating the mine drainage is consistent with the idea of using various processes of the sulfur cycle as extensive options for voluminous wastewater streams containing heavy metals (see Chapter 2, section 2.3.2.1). The use of wetlands and other extensive techniques, e.g. anaerobic ponds, soil filters etc., may sustain for relatively long times with limited costs and maintenance. Periodically, the extensive system would be regenerated. Here, the bioleaching may benefit from expectedly high amounts of reduced sulfur compounds retained in the wetland or similar treatment system.

In the aerated slurry experiments, the sediment acidified down to pH 4.2 without amendment of any other chemical (Chapter 7, Figure 7.1). However, it was not sufficient to achieve satisfiable extraction efficiency. Therefore, addition of sulfuric acid or elemental sulfur was required. The study firstly used the addition of sole sulfuric acid. The pH of a sediment suspension with varying sulfuric acid amendments revealed surprisingly different behaviour, depending on the concentration of added acid. With low H_2SO_4 additions, aeration caused a monotonous decrease of pH after the acid addition. Directly after the addition of sulfuric acid at high concentrations, a monotonous increase was observed during the aeration. Intermediate variants revealed both increasing and decreasing trends (see Chapter 7, Figure 7.4).

The pH-decreasing trend at low acid amendments was explained by a release of protons due to the oxidation of reduced sulfur and iron. At high acid additions, the formation of protons induced by aeration was overruled by the high concentrations of externally added acid, and thus not recorded by pH-electrode. The monotonous increase of pH at high acid additions was attributed to the proton cleavage due to precipitation/dissolution and diffusion-limited transport of protons into the sediment particles. When pH behaviour was combined with the models of metals solubilization, the overall leaching strategy could have been evaluated (Chapter 7, Figure 7.5). Similar to the first leaching study (Chapter 5), intensive and

extensive leaching strategies also were observed. Intensive leaching of a sediment involves the use of high acid concentrations at short extraction times, whereas the extensive leaching operates at lower acid additions, however, at considerably longer extraction times. The bioleaching tests using the two types of elemental sulfur were launched as well. Accordingly with the previous studies, microbially produced sulfur proved faster acidification, compared to the orthorhombic sulfur flower.

8.4 BOTTLENECKS IN USING BIOLEACHING

Many of the advantages of the possible use of bioleaching have been listed above. However, certain bottlenecks of the process should be mentioned here as well. Firstly, the acidophillic thiobacilli are sensitive to the presence of some organic compounds, especially low-weight fatty acids. This is due to the low pH of the growth medium, which results in an undissociated state of carboxyllic groups. The molecules are therefore taken up by thiobacilli as electroneutral, however, they dissociate in pH-neutral cytoplasm and thus damage the cellular metabolic equilibrium. However, no adverse effects of common organic compounds in the soils or sediments have been demonstrated for thiobacilli sofar.

Furthermore, the use of sulfuric acid supplied directly or produced by microbes may interfere with the presence of calcium. Calcium, when present in its carbonate form (lime), creates higher requirements for the acid to achieve low pH values, since it strongly neutralizes pH. Furthermore, calcium can interfere with sulfate: formation of $CaSO_4$ can have a consequence in the gypsum precipitation. At ambient temperature, the solubility product of $CaSO_4$ is 0.023 $mmol.L^{-1}$. An illustration of this phenomenon is given in Figure 8.1, using a simulation with Ecosat v. 4.4 (Wageningen Agricultural University, Department of Soil Science and Plant Nutrition). The simulation involved an aqueous system containing 0.03 $mol.L^{-1}$ sulfate, i.e. 2883 $mg.L^{-1}$, and variable pH and calcium content at ambient temperature. The system had been set up to include the exchange with atmospheric CO_2, the gas phase was included using constant partial carbon dioxide pressure equal to atmosphere.

It may be seen that the concentration of precipitated gypsum depends on pH and concentration of Ca. With increasing Ca-concentrations the formation of gypsum increases. The gypsum precipitation is affected by pH due to the protonation of sulfate at lower pH. The gypsum precipitation may have positive effects on the process performance (the outgoing water contains less sulfate), however, the sulfur can not be separated from the sediment.

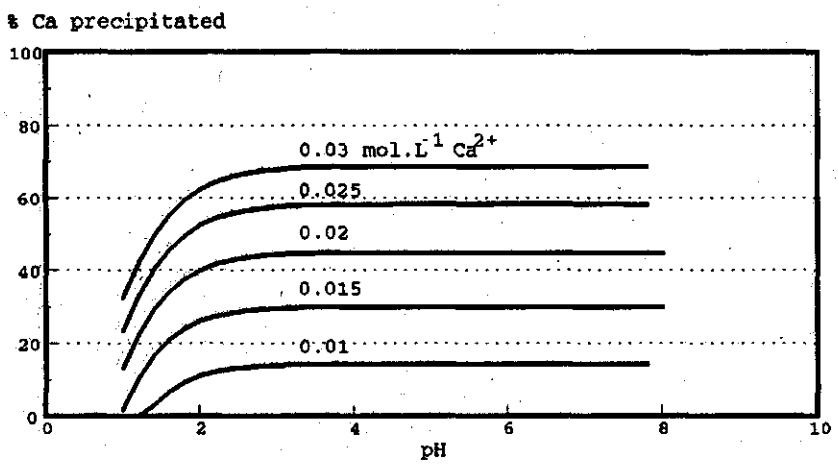

Figure 8.1 Simulated precipitation of $CaSO_4$ at ambient temperature in 0.03 mmol.L^{-1} aqueous solution of sulfate and at varying pH and Ca^{2+}.

Next environmentally important cation, which forms less soluble compounds with sulfate, is lead. Appropriate simulation using Ecosat program is shown in Figure 8.2. The total sulfate concentration is equal to 0.03 mmol.L^{-1} and aqueous system at ambient temperature was assumed. The % of precipitated lead (prevalent equilibrium mineral was $PbSO_4.PbO$) is plotted as a function of total lead concentration and pH in Figure 8.2. It can be seen that lead precipitates at pH values higher than 4, depending on the total lead concentration. Since most bioleaching operations work at pH lower than 4, no lead precipitation can be expected. It should be also noted that the concentration of lead used in our simulation were rather high: 0.005 mol.L^{-1} of Pb means approx. 1 g of Pb per litre, which is only rarely encountered during remediations. Good solubility of lead in an auto-acidifying sediment slurry was also documented experimentally in this thesis (Chapter 7). Therefore, the conclusion from the two simulations above is, that a formation of lead does not possess any bottleneck in bioleaching, whereas a strong interference with calcium can be expected.

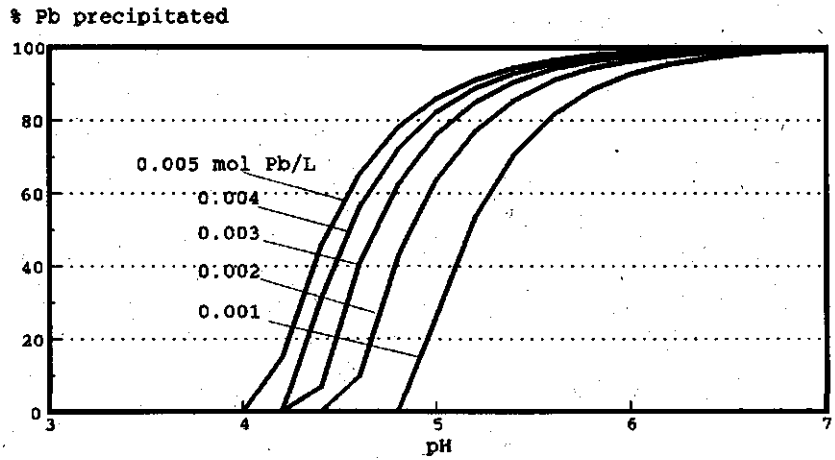

Figure 8.2 Simulated precipitation of lead at ambient temperature in 0.03 mmol.L^{-1} aqueous solution of sulfate and at varying pH and Pb^{2+}.

8.5 MAIN CONCLUSIONS

The use of microbial sulfur oxidation for the enhancement of toxic heavy metals removal from contaminated soils or sediments is technically feasible. When integrated with some other processes of the microbial sulfur cycle, i.e. the sulfate reduction or partial sulfide oxidation, it may strongly benefit from easier spent liquor processing and sulfur regeneration and re-use. Compared to these processes, the bioleaching is rate-limiting (see Chapter 4, section 4.4).

It is possible to perform bioleaching both in fully agitated soil slurries, as well as in heap-leaching or in-situ configurations, using an intensive or extensive leaching strategies. A possible use of processes with different intensity may be the main advantage of possible use of the microbial sulfur cycle to control the toxic heavy metals in the environment. For example, the use of rather extensive removal of metals from aqueous waste streams via wetlands (sulfate reduction) can be followed by a more intensive wetland sediment regeneration using bioleaching. In some cases, bioleaching may be applied to mobilize heavy metals in soils followed by a soil washing. The vegetative uptake, although supposed to be another alternative to remove solubilized metals from the soil solution, turned out to be inefficient.

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SUMMARY

Reduced inorganic sulfur species like elemental sulfur or sulfide are sensitive to changes in oxidative environments. Generally, inorganic reduced sulfur exists in natural environments in a solid phase, whereas its oxidation leads to sulfur solubilization and a production of acidity. This oxidation occurs spontaneously, due to chemical mechanisms, however, its rate can be enhanced by microbes by several orders of magnitude. The acidification which accompanies the sulfur oxidation brings about rather extreme conditions for microbial growth, the pH can drop below 2. The most common organisms capable of sulfur oxidation at extremely low pH are acidophillic thiobacilli. The microbial oxidation of reduced inorganic sulfur causes several phenomena of environmental concern. Oxidation of sulfur and subsequent production of acid is usually accompanied with a release of cationic metals from bedrock, from mineral and organic surfaces or from metal precipitates. This is happening e.g. in acid mine drainage, acidification and toxic metals release from sediments dredged for nautical reasons, contamination due to flood events, appearance of acid sulfate soils at sites rich in sulfide minerals and at metals-polluted sites which receive acidic leachates.

On the other hand, the microbial sulfur oxidation can be also applied in specific technologies. Its use for biohydrometallurgy, i.e. microbial mining of metals from low-grade ores is well known and applied in practise. Another application is microbial desulfurization of coal containing inorganic sulfur inclusions. Recently, its use for decontamination of solid wastes containing toxic metals has been proposed. The bioleaching of heavy metals from anaerobically digested sewage sludge was successfully demonstrated at technological scale.

This thesis deals with the controlled microbial leaching of toxic cationic heavy metals from contaminated soils or sediments. The bioleaching of metals from these materials provides a direct advantage when a sufficient amount of reduced sulfur is available. This applies for freshwater sediments which can accumulate substantial amounts of reduced sulfur in anoxic conditions at the bottom of freshwater bodies. The leaching affects not only the metal sulfides, but also other forms of metals encountered in anoxic sediments. Therefore, bioleaching is the most natural attempt to solubilize metals in the sediments, since the oxidative changes always occur. However, when the amount of reduced sulfur is insufficient like in most aerobic soils, additional sulfur substrate or direct addition of sulfuric acid must be considered to achieve sufficiently low pH.

Experiments were done to compare the feasibility of microbially produced elemental sulfur with sulfur flower as a substrate for bioleaching (Chapter 3). The results proved that the microbially produced elemental sulfur is a feasible substrate and it seems better available for thiobacilli, than the sulfur flower. This effect is

caused by two phenomena: (1) the surface of microbially produced sulfur is more hydrophillic than the orthorhombic sulfur flower and (2) the microbially produced sulfur consists of much smaller particles and provides thus higher specific surface area (2.5 $m^2.g^{-1}$) than the commercially available orthorhombic sulfur flower (0.1 $m^2.g^{-1}$). However, the mutual relevance of these two aspects is not fully understood and it may be speculated to which extent would be sulfur oxidation increased, when sulfur flower is pulverized to particles of similar size as the microbially produced sulfur.

The growth yields of thiobacilli on the two types of sulfur were nearly identical during batch experiments, however, because of better substrate availability, the final biomass and sulfate concentrations with microbially produced sulfur were about twice as high as with the sulfur flower. The maximum sulfur specific oxidation rate in batch cultivation was also about two-times higher with microbially produced sulfur than with the sulfur flower. During microbial sulfur oxidation, the finest particle fraction was consumed the first, which forms typically up to 40% of the microbially produced sulfur. Subsequently, the larger aggregates are oxidized, however, at a lower rate. In a continuous cultivation (Chapter 4), the lowest pH achieved by thiobacilli on microbially produced sulfur was 1.7. This was observed at a continuous reactor dilution rate of 0.04 h^{-1}. Maximum production rate of sulfuric acid was 1 $mmol.L^{-1}.h^{-1}$ at a sulfur loading of ca. 4 $mmol.L^{-1}.h^{-1}$. When compared to the maximum conversion rates of the other processes of microbial sulfur cycle, i.e. sulfate reduction and partial sulfide oxidation, the production of sulfuric acid proceeds at the lowest rate.

Three experimental studies were done to demonstrate the bioleaching of metals from soil and sediments. The first study involved artificially zinc-contaminated clay, silt, and sandy soil, and the leaching behaviour was studied by varying additions of sulfuric acid between pH 1.5-6 (Chapter 5). The measurement of aluminium solubilization was used to quantify the extent of soil matrix damage at extreme acidity. Zinc solubilization followed a monotonous increase with decreasing pH, being 17-43% at pH 7 and 72-95% at pH 1.5. However, the aluminium solubilization revealed a sharp increase at pH=4. Below this pH, Al-concentrations increased exponentially, indicating major damage to the soil mineral matrix. The study revealed two different possible strategies in leaching. The first, called here intensive, uses high concentrations of sulfuric acid to achieve satisfactory extraction efficiency and high soil/solution ratio. However, a considerable damage of the soil matrix can be expected. The second strategy, called extensive, uses lower concentrations of acid, however, the soil/solution ratio must be properly decreased.

The second leaching study aimed at the possible use of sulfur oxidation in-situ, i.e. within the soil profile after its artificial contamination with cadmium (Chapter 6). Microbially produced elemental sulfur or orthorhombic sulfur flower

were supplied to the soil prior to its placement into the soil pots and the velocity of soil acidification and cadmium solubilization were observed. The microbially produced sulfur showed faster oxidation and acidification than the orthorhombic sulfur flower: immediately after addition of microbially produced sulfur, the pH started to decrease. The pH-decrease in sulfur flower treatments was observed only after a 55 days lag phase. The solubilization of cadmium into the pore water followed directly the changes in pH. In this study, a vegetative uptake of solubilized cadmium was tested using common mustard (*Sinapis alba*). With decreasing soil pH, the concentrations of cadmium in biomass increased, however, the biomass yields decreased. When Cd concentrations and biomass yield were combined, an optimum soil pH of 5-5.5 was found for the vegetative removal. However, the overall efficiency of the vegetative removal was rather low (1.5%).

In the third leaching study, a sediment from a wetland receiving mine drainage was used, since this sediment was expected to contain high amounts of reduced sulfur (Chapter 7). 150-hours aeration of the sediment resulted in acidification down to pH 4.2, accompanied by the increase in redox-potential from -150 meV to +300 meV and an increase of sulfate concentration to ca. 6 mmol.L^{-1}. At the same time, the solubilization of Cd, Cu, Fe and Zn was recorded. Total soluble iron initially increased up to 48 mg.L^{-1} within 50 hours of aeration, followed by decrease below detection limit. This was explained by initial desorption of soluble Fe^{2+} followed by its oxidation and precipitation of the resulting Fe^{3+} ion. The minimum pH achieved by aeration of the sediment was not sufficient to achieve a satisfactory extraction efficiency of the studied metals. Therefore, addition of sulfuric acid or elemental sulfur was investigated. The study firstly involved the leaching after the treatment with varying concentrations of sulfuric acid. Two different processes were observed in the sediment slurry after acid addition: (1) the monotonous pH decrease with time, caused by oxidation of reduced sulfur or iron compounds, which was observed at low acid additions, and (2) a delayed pH increase at high acid additions due to the scavenging of acidity by various processes of pH-buffering, solubilization of minerals, diffusion etc. The solubility of Cd, Cu, Pb, and Zn was a function of exposure. Exposure is here defined as actual activity of acid (mol.L^{-1}) multiplied by the time of extraction (hours). Similar to the first leaching study, intensive and extensive leaching strategies have been distinguished, where intensive leaching involved high concentrations of H_2SO_4 and short extraction times, and the extensive leaching used no or low amendments of acid, however, at prolonged extraction times. Besides the leaching with sulfuric acid, the bioleaching tests were performed. In accordance to the previous studies, microbially produced sulfur proved faster acidification compared to the orthorhombic sulfur flower.

The use of microbial sulfur oxidation for the enhancement of toxic metals removal from contaminated soils or sediments is technically feasible. When

integrated with the other processes of the microbial sulfur cycle, bioleaching may strongly benefit from relatively easy processing of the spent extraction liquor containing heavy metals and sulfuric acid by the sulfate reduction and subsequent sulfur regeneration by partial sulfide oxidation. Compared to these processes, the bioleaching is a rate-limiting step.

It is possible to perform bioleaching both in fully agitated soil slurries, as well as in heap-leaching or in-situ configurations, using an intensive or extensive leaching strategy. The use of processes with different intensity may be the main advantage of the partial or full use of the microbial sulfur cycle to control the toxic metals in the environment. An example of the integration of processes of microbial sulfur cycle is a wetland or anaerobic pond using sulfate reduction in an extensive and sustainable way to control the pollution of voluminous aqueous streams containing metals and sulfate, followed by more intensive bioleaching. Another example is the introduction of elemental sulfur in the soil to promote slow acidification and release of metals into the pore water. The collected water can be treated further by intensive or extensive sulfate reduction.

In general, bioleaching using the microbial sulfur cycle can be applied to a broad range of polluted solid materials. It may get an increasing attention in the future, when the needs for extensive treatment methods grow.

SAMENVATTING

BIOLOGISCHE UITLOGING VAN METALEN UIT GROND OF SEDIMENTEN MET BEHULP VAN DE MICROBIOLOGISCHE ZWAVELKRINGLOOP

Gereduceerde anorganische zwavelverbindingen zoals elementair zwavel, zijn gevoelig voor veranderingen in een oxidatieve omgeving. In het algemeen is anorganisch gereduceerde zwavel in een natuurlijke omgeving een vaste stof. Wanneer het echter geoxideerd wordt gaat het in oplossing en vindt verzuring plaats. De oxidatie kan spontaan optreden door chemische mechanismen, waarbij de oxidatiesnelheid door micro-organismen in hoge mate kan worden bevorderd. De verlaging in pH die de oxidatie van zwavel met zich mee brengt veroorzaakt extreme condities voor microbiële groei. De pH kan tot minder dan 2 dalen. De meest voorkomende organismen die bij dergelijk extreem lage pH's in staat zijn tot zwaveloxidatie zijn acidofiele thiobacilli. De microbiële oxidatie veroorzaakt verschillende milieuproblemen. De oxidatieve pH-verlaging leidt tot het vrijkomen (van voorheen gebonden) kationische metalen. Het vrijkomen van metaalionen wordt waargenomen in zuur afvalwater uit (voormalige) mijnen, baggerspecie, sedimenten in uiterwaarden na overstromingen, kattekleigronden en met zware metalen verontreinigde gronden die zure regen ontvangen.

Microbiologische zwaveloxidatie wordt echter ook gebruikt in specifieke technologieën. Wel bekend is biohydrometallurgie waarbij ertsen met een laag metaalgehalte worden uitgeloogd, zodat meer rendement verkregen wordt. Een andere toepassing is de microbiële ontzwaveling van kolen door oxidatie van pyritische zwavel. Recentelijk is de gedachte ontwikkeld om deze techniek te gebruiken voor de decontaminatie van vast afval verontreinigd met giftige metalen. De bio-uitloging van zware metalen uit anaëroob vergist rioolslib is succesvol op technologische schaal uitgevoerd.

Dit proefschrift behandelt de biologische uitloging van zware metalen uit verontreinigde grond of sediment. Deze methode is vooral voordelig wanneer voldoende gereduceerd zwavel aanwezig is in het te behandelen materiaal. Dit geldt voor zoetwater sedimenten, die een aanzienlijke hoeveelheid gereduceerd zwavel kunnen accumuleren onder anoxische omstandigheden. Niet alleen metaalsulfiden worden uitgeloogd maar ook andere metaalverbindingen in anoxische omstandigheden. Bio-uitloging is de meest natuurlijke wijze om metalen uit sedimenten in oplossing te brengen, daar de biologische oxidatie spontaan optreedt. Wanneer de hoeveelheid gereduceerd zwavel onvoldoende is, zoals in veel aërobe bodems, kan zwavel als substraattoevoeging in overweging worden genomen om de pH voldoende te verlagen.

In deze studie is onderzoek verricht naar de efficiëntie van microbiologisch

elementair zwavel als substraat voor de uitloging in vergelijking met zwavelmeel (Hoofdstuk 3). Het microbiologisch gevormde zwavel of bio-zwavel is een product van de zwavelcyclus, waarbij sulfaat partieel gereduceerd wordt tot elementair zwavel. Het bleek dat bio-zwavel als substraat beter beschikbaar is voor de thiobacilli in vergelijking met de zwavelmeel. Deze beschikbaarheid van de bio-zwavel voor de micro-organismen kan worden verklaard door: 1) dat het oppervlak is meer hydrofiel en 2) de zwaveldeeltjes zijn kleiner en hebben een groter specifiek oppervlak (2,5 $m^2\ g^{-1}$) in vergelijking met commercieel othorhombisch zwavelmeel (0,1 $m^2\ g^{-1}$). De relevantie van hydrofieliteit en specifiek oppervlak is niet geheel duidelijk en het valt te bezien hoe groot het verschil in zwaveloxidatiesnelheid optreedt, wanneer het commerciële zwavelmeel vermalen wordt tot deeltjes met een vergelijkbare deeltjesgrootte als het bio-zwavel.

De groeiopbrengst was met beide substraten in batch-experimenten vrijwel identiek, maar door de betere beschikbaarheid van bio-zwavel was de eind hoeveelheid biomassa met dit substraat ongeveer twee maal zo hoog. Dit gold ook voor de maximale specifieke zwaveloxidatiesnelheid in batch-cultivatie wanneer bio-zwavel werd gebruikt. Bij de microbiële zwavel oxidatie werd de fijnste deeltjesfractie als eerste geconsumeerd. Van het microbieel geproduceerde zwavel bestond gewoonlijk 40% uit deze kleinste deeltjesfractie. De grotere deeltjes werden vervolgens met een lagere snelheid omgezet. In een continu-experiment met thiobacilli en bio-zwavel als substraat (Hoofdstuk 4) was de laagst behaalde pH 1,7, bij een reactorverdunningssnelheid van 0,04 uur^{-1}. De maximale productiesnelheid van zwavelzuur was 1 $mmol.L^{-1}.uur^{-1}$, bij een zwavel belading van 4 $mmol.L^{-1}.uur^{-1}$. In vergelijking met de maximale conversiesnelheden van de andere processtappen van de zwavelcyclus, zoals de zwavelreductie en de partiële zwaveloxidatie, was de produotie van zwavelzuur de traagste stap.

In drie experimenten werd de bio-uitloging van metalen onderzocht. Het eerste experiment betrof het uitlooggedrag van kunstmatig zinkverontreinigde klei-, zavel- en zandgrond bij pH's variërend tussen 1,5-6 door middel van toegevoegd zwavelzuur (Hoofdstuk 5). De oplosbaarheid van aluminium werd gebruikt als een maat voor de afbraak van de grondmatrix door zuur. De oplosbaarheid van zink volgde lineair de pH verlaging, bij pH 7 was deze 17-43% en bij pH 1,5 72-95%. De oplosbaarheid van aluminium gaf echter een scherpe toename te zien bij pH 4, bij lagere pH's nam de oplosbaarheid exponentieel toe, wat een verregaande afbraak van de grondmatrix deed vermoeden. Uit het onderzoek kwamen twee strategieën naar voren voor bio-uitloging. Bij de eerste strategie, hier betiteld als intensief, heeft een hoog verbruik aan zwavelzuur en een hoge grond/oplossing-ratio om een bevredigend extractierendement te halen. Dit zal echter tot een sterke beschadiging van de grond-matrix leiden. De tweede methode, hier betiteld als extensief, gebruikt minder zwavelzuur bij een lagere grond/oplossing ratio. Hierbij is meer tijd nodig

om het gewenste resultaat te behalen.

Het tweede uitlogingsexperiment had als doel het gebruik van zwaveloxidatie in situ te bestuderen. Hiervoor is aan een grondprofiel na kunstmatige verontreiniging met cadmium (Hoofdstuk 6) bio-zwavel of orthorhombisch zwavel toegevoegd. Vervolgens werd de verzuring evenals de oplosbaarheid van cadmium werden gevolgd. De grond met bio-zwavel gaf een snellere oxidatie en verzuring te zien dan het zwavelmeel, de pH-verlaging startte onmiddellijk. De pH-verlaging bij zwavelmeeltoevoeging startte pas na 55 dagen. De oplosbaarheid van cadmium in het poriewater van de grond volgde de pH-verlaging. In dit experiment werd tevens de vegetatieve verwijdering van het cadmium door mosterdzaad (*Sinapis alba*) getest. De cadmiumconcentratie in de biomassa nam toe bij pH-verlaging, maar de biomassa-opbrengst nam af. Het maximale rendement van cadmiumoplosbaarheid en vegetatieve verwijdering was bij een pH van de grond tussen 5-5,5, de vegetatieve cadmiumverwijdering was echter tamelijk laag (1.5%).

In het derde uitloogexperiment werd een moerassediment dat verontreinigd was door mijn-drainagewater gebruikt, omdat dit sediment waarschijnlijk een hoge concentratie aan gereduceerd zwavel zou bevatten (Hoofdstuk 7). Aëratie gedurende 150 uur resulteerde in een pH verlaging tot 4,2, een redoxpotentiaal verhoging van -150 meV tot +300 meV en een toename van de sulfaatconcentratie tot 6 mmol.L^{-1}. De oplosbaarheid van Cd, Cu, Fe en Zn werden gevolgd. Het totaal oplosbaar ijzer nam aanvankelijk toe tot 48 mg.L^{-1} binnen 50 uur aëratie, gevolgd door een daling tot onder de detectiegrens. Dit was te verklaren door de initiële desorptie van oplosbare Fe^{2+} door oxidatie naar Fe^{3+} ionen die precipiteerden. De laagste pH die werd bereikt door middel van aëratie was niet voldoende om een bevredigende extractie van de onderzochte metalen te realiseren. De additie van zwavelzuur of elementair zwavel werd daarom onderzocht. Als eerste werd de uitloging onderzocht na behandeling met verschillende concentraties zwavelzuur. Twee verschillende processen werden waargenomen in het sediment: (1) de continue pH-daling in de tijd, veroorzaakt door oxidatie van zwavel- en ijzercomponenten bij kleine zuuraddities en (2) een vertraagde pH toename na grote zuuraddities door de uitputting van het zuur door het buffersysteem in het sediment, het oplossen van mineralen en diffusie ed. De oplosbaarheid van Cd, Cu, Pb en Zn is een functie van de blootstelling. Hierin is de blootstelling gedefinieerd als de activiteit van het zuur (mol L^{-1}) vermenigvuldigd met de extractietijd (uur). Vergelijkbaar met het eerste uitloogexperiment (zie Hoofdstuk 5) werd onderzoek gedaan met intensieve en extensieve uitlogingsmethoden. De intensieve methode betrof een hoge dosering zwavelzuur en een korte extractietijd; de extensieve methode weinig of geen zuuradditie en een langere extractietijd. Daarnaast werd bio-uitloging met elementair zwavel bestudeerd. Overeenkomstig met eerder gevonden resultaten gaf bio-zwavel

een snellere verzuring te zien in vergelijking met zwavelmeel.

Het gebruik van microbiële zwaveloxidatie ter bevordering van de verwijdering van zware metalen uit verontreinigde gronden of sedimenten is technisch haalbaar. Wanneer het proces geïntegreerd wordt met andere stappen van de zwavelcyclus, is dat voor de bio-uitloging zeer voordelig. Dit wegens de relatief eenvoudige behandeling van het verbruikte percolaat met een hoge metaal- en zwavelzuurinhoud en verder omdat het elementaire zwavel kan worden hergebruik.

Het is mogelijk om bio-uitloging toe te passen in volledig gemengde bodems zowel als in statische in situ configuraties. Omdat de processen met een controleerde intensiviteit kunnen worden toegepast, is de oplosbaarheid van toxische metalen in het milieu te sturen. Een voorbeeld van de geïntegreerde procesvoering van de biologische zwavelcyclus is de toepassing in moerasbodems of anaërobe vijvers met een extensieve sulfaatreductie om de volumineuze stroom met zware metalen en sulfaat te reinigen, gevolgd door een intensievere bio-uitloging. De toepassing van elementair zwavel in de grond voor een langzame verzuring en extractie van metalen in het poriewater is een ander voorbeeld. Het opgevangen poriewater kan dan verder behandeld worden door middel van in- of extensieve sulfaatreductie.

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