APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 2930–2935 Vol. 61, No. 8
0099-2240/95/$04.00+0

Microbial Diversity in Uranium Mine Waste Heaps

AXEL SCHIPPERS, REGINE HALLMANN, SUSANNE WENTZIEN, AND WOLFGANG SAND*

Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg, D-22609 Hamburg, Germany

Received 6 October 1994/Accepted 17 May 1995

Two different uranium mine waste heaps near Ronneburg, Thuringia, Germany, which contain the remains of the activity of the former uranium-mining Soviet-East German company Wismut AG, were analyzed for the occurrence of lithotrophic and chemoorganotropic leach bacteria. A total of 162 ore samples were taken up to a depth of 5 m. Cell counts of ferrous iron-, sulfur-, sulfur compound-, ammonia-, and nitrite-oxidizing bacteria were determined quantitatively by the most-probable-number technique. Sulfate-, nitrate-, ferric iron-, and manganese-reducing bacteria were also detected. In addition, the metabolic activity of sulfur- and iron-oxidizing bacteria was measured by microcalorimetry. Generally, all microorganisms mentioned above were detectable in the heaps. Aerobic and anaerobic microorganisms thrived up to a depth of 1.5 to 2 m. Up to 99% of *Thiobacillus ferrooxidans* cells, the dominant leaching bacteria, occurred to this depth. Their numbers correlated with the microbial activity measurements. Samples below 1.5 to 2 m exhibited reduced oxygen concentrations and reduced cell counts for all microorganisms.

Mining of sulfide ores may cause severe environmental problems. Pollutants like heavy metals are mobilized by bacterial leaching and are introduced into soil and groundwater, if the ground beneath mine waste heaps is permeable (38, 53). Uranium ores are often associated with metal sulfides like pyrite. Pyrite oxidation results in a release of uranium ions and radioactive disintegration products of uranium. Exhalation of gaseous radon, one of the most environmentally significant disintegration products, threatens the health of creatures living in the neighborhood of the heaps.

A large uranium mining area with severe environmental problems is located near Ronneburg, Thuringia, Germany. During a period of more than 40 years, 13 mine waste heaps of low-grade black schist ore have been accumulated, with a volume of 179×10^6 m^3 covering an area of 5.5 km^2. The mining was stopped in 1990 after the reunification with West Germany. Historical and geological characteristics of the mining area have been described previously (28, 29). To minimize the deleterious effects of acid mine drainage and radon emission, a rehabilitation program has been developed in the past few years. A shortfall of this program is the inadequate knowledge about the contributing microbiological processes. Thus, the present work was undertaken to thoroughly investigate the microbial diversity inside the heaps.

Only a few investigations on microbial ecology in sulfidic mine waste have been carried out (4, 16, 18, 22, 52, 54). The lithotrophic bacterium *Thiobacillus ferrooxidans* is assumed to be most important for leaching of mine waste. However, little information is available about the role of the lithotrophic leaching bacteria *T. thiooxidans* and *Leptospirillum ferrooxidans* in such biotopes. Moderately acidophilic thiobacilli like *T. neapolitanus*, *T. intermedius*, and *T. novellus* were found predominantly in ore samples with neutral pH values. However, up to now, their role in the leaching process has not been fully understood.

Several methods are used for determination of microbial leach activity. Most of them suffer from limitations caused by the extremely acidic environment. In this work, the metabolic activity of lithotrophic sulfur- and ferrous iron-oxidizing bacteria was determined by microcalorimetry (46, 49, 51).

Nitrifying bacteria were detected in leach biotopes containing carbonaceous ores (34, 63). Leach experiments in the laboratory with sulfidic ore containing carbonates revealed a low but measurable release of metal ions (26). For this reason, the occurrence of these microorganisms was also analyzed.

There are no reports on the occurrence of anaerobic bacteria in mine waste heaps. However, oxygen concentrations are known to decrease inside heaps (2, 10, 17, 20, 21). Since organic substrates like wood, oil, and sewage sludge are often dumped during the formation of a heap, growth of anaerobic bacteria like sulfate-, nitrate-, ferric iron-, and manganese-reducing bacteria seems possible. The detrimental effect of anaerobes results from their ability to reduce, for example, insoluble ferric iron or manganese compounds to the soluble ferrous or manganous ions (6, 31, 32, 43). These may migrate and pollute other sites. In addition, iron and/or manganese precipitates often contain coprecipitates of other metals; these could also be mobilized, causing increased heavy metal pollution.

(This paper is based in part on the results of studies by A. Schippers for a Ph.D. from the Faculty of Biology, University of Hamburg, Hamburg, Germany.)

MATERIALS AND METHODS

Sites. Two uranium mine waste heaps were investigated. The black schist ore from the heaps contained about 0.05% uranium, between 0.5 and 7% pyrite or marcasite, and different concentrations of carbonate. Heap A (Absetzerhalde) was 2,000 m long, 1,500 m wide, and 40 m high. About half of the heap was covered with a layer of loamy soil material. The cover was between 0.6 and 1 m thick and grown with birch trees. Heap G (Gessenhalde) was 700 m long, 500 m wide, and 40 m high. It is a heap which had been bioleached for 15 years for uranium. Sulfuric acid had been added to overcome the buffering capacity of the carbonate material. This heap was not covered.

Sampling. All 162 ore samples were taken between May and October 1993: 55 from the uncovered part of heap A, 81 from the covered part of heap A, and 23 from heap G. Of these samples, 22 were taken from the surface and 137 were taken from depths up to 5 m. The remaining three were fluid samples from acid mine drainage ditches. For the depth samples, a hollow steel cylinder was used to collect the ore material. Every 30 to 40 cm, a sample was taken from the inner

* Corresponding author. Mailing address: Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg, Ohnhorststrasse 18, D-22609 Hamburg, Germany. Phone/Fax: 040/82282-423.

part of the core inside the cylinder. The temperature of the samples was determined on site (impac Tastotherm D 200). The oxidation-reduction potential was measured on site as well (Ingold electrodes and Knick Portamess digital pH meter).

Gas samples. Sixteen gas samples from different depths of four holes in heap A were taken with a gas-sampling device (Meta BLPS-204; Messtechnische Systeme GmbH). A hollow steel pipe with a lateral opening at the bottom was used. Three inflatable rubber seals at different heights ensured sampling at the correct depth. About 10 liters of gas was pumped through a glass vessel (100 ml) before it was closed by two gas taps. Gas analysis of O_2, CO_2, CH_4, and H_2 was done by gas chromatography.

Determination of microbial activity. For determination of microbial activity, the heat output of ore samples was measured with a thermal activity monitor (type 2277, Thermometric, Stockholm, Sweden, or C3-Analysentechnik, Baldham, Germany) equipped with an ampoule cylinder (type 2277-201). The size of the ore particles was restricted to less than 3 mm because of the diameter of the ampoule mouth; additionally, care was taken to have a sample mixture of fine and coarse particles. A 3-ml glass ampoule containing 2 g of ore in air was inserted in the measuring cylinder, and, after thermal equilibration, the heat output resulting from microbial degradation of metal sulfides was recorded, as previously described (46, 59). Heat output caused by chemical oxidation was measured in control experiments. For the differentiation, 1 ml of chloroform was added to the ore sample. After 24 h, the chloroform was removed by vacuum evaporation and the heat output was measured again.

Enumeration of aerobic microorganisms. After the microbial activity had been measured, the ore sample (2 g) was removed from the ampoule and suspended in 10 ml of a 0.9% NaCl solution. The suspension was incubated for 2 h on a rotary shaker (Infors TR type) at 200 rpm to detach cells from the substratum. The turbid suspension was diluted in 10-fold steps to 10^{-6}. For estimating the numbers of aerobic lithotrophic bacteria, a three-tube most-probable-number technique was applied. Chemoorganotrophic microorganisms (COT) were counted by plating on agar. Cultures were incubated in the dark at 28°C.

For enrichment of *T. ferrooxidans* and *L. ferrooxidans*, a medium with ferrous iron as the substrate was used (35). Tubes were incubated on a rotary shaker (200 rpm) for 3 weeks. Tubes in which ferric iron was formed (indicated by a reddish brown color) were counted for bacterial enumeration, and microscopy revealed the presence of bacteria. Differentiation between the two iron oxidizers was done by light microscopy. The highest positive dilutions were analyzed for rods (indicating *T. ferrooxidans*) or curved and vibrioid-shaped cells (indicating *L. ferrooxidans* [47]).

In addition, pyrite was used as a substrate to detect pyrite-oxidizing bacteria, which did not grow with soluble ferrous iron. These samples were incubated for 8 weeks.

T. thiooxidans was enriched with a medium containing sulfur as substrate (25). Moderately acidophilic thiobacilli were cultivated with thiosulfate as the substrate (37). Tubes were incubated for 3 weeks on a rotary shaker. The tubes were counted as positive if the pH value had dropped below 2 for *T. thiooxidans* and below 5 for moderately acidophilic thiobacilli.

Ammonia-oxidizing bacteria were enriched with a medium as described by Krümmel and Harms (27). However, $CaCO_3$ was added as the buffer instead of $CaCl_2$. Nitrite-oxidizing bacteria were enriched with a medium for lithotrophic growth (5). Tubes were incubated for 6 weeks. In the case of ammonia-oxidizing bacteria, tubes were evaluated positively if nitrite and/or nitrate was detectable. In the case of nitrite-oxidizing bacteria, tubes in which nitrate was detected or nitrite had disappeared were counted.

Neutrophilic chemoorganotrophic microorganisms were counted by plating on DEV agar (Merck 10685), and acidophilic chemoorganotrophic microorganisms were counted by plating on *Acidiphilium* agar (23).

Thermophilic sulfur- or pyrite-oxidizing bacteria were enriched with media for *T. ferrooxidans*. The substrate was sulfur or pyrite instead of ferrous iron. The pH values ranged between 2 and 5. The tubes were incubated for 6 weeks at 60°C.

Quantification of anaerobic bacteria. Samples of 1 g were suspended in 5 ml of a 0.9% NaCl solution under anaerobic conditions (Whitley anaerobic cabinet, type du-Scientific, with an atmosphere of 2% CO_2, 10% H_2, and 88% N_2). Tubes containing media for enrichment of the different anaerobic bacteria were incubated for 3 weeks at 30°C after inoculation. Sulfate-reducing bacteria were enriched on a medium described by Postgate (42) and modified by Lapage et al. (30). Nitrate-reducing bacteria that used organic compounds as electron donors were enriched with a medium for nitrite-oxidizing bacteria without nitrite. Instead, 2 g of sodium nitrate, 1.5 g of yeast extract, 1.5 g of peptone, and 0.55 g of sodium pyruvate were added per liter. Tubes were evaluated as positive if at least 25% of the nitrate was missing. Nitrate-reducing bacteria that used thiosulfate as the electron donor were enriched with a medium for *T. denitrificans* (56). A positive evaluation resulted if at least 25% of the nitrate and 25% of the thiosulfate were missing. Ferric iron- or manganese-reducing bacteria that used organic compounds (31, 32) or sulfur as electron donors (57) were detected by production of ferrous or manganous ions.

Chemical measurements. The ore suspension used for the enumeration of aerobic microorganisms was also used for chemical measurements. pH values were measured with a WTW-pH-mV-2000 meter and Ingold electrodes. Ferrous and ferric ions were measured spectrophotometrically and thiosulfate was measured by titration (40). The metals Ca, Mg, Mn, Ni, Cu, Zn, and Co were measured by atomic absorption spectrophotometry with a 1100 B type model (Perkin-Elmer, Überlingen, Germany). Sulfate was determined indirectly by using atomic absorption spectrophotometry for measurement of the barium remaining in solution after precipitation of barium sulfate with excess barium chloride (12). Ammonium, nitrite, and nitrate were determined by high-pressure liquid chromatography (HPLC) (3, 9) (Kontron HPLC; autosampler 460, pump 420, detector SFM 25, UV detector 430; column/Hypersil-ODS-5μ).

RESULTS

The environmentally detrimental impact of heaps A and G was confirmed by data on the ion concentrations in the effluents from the heaps and the radon emission as radioactivity in the air above the heaps (7). The highest mean values of radioactivity, more than 1000 nGy/h, were measured for heap G. Between 200 and 1,000 nGy/h was detected at the uncovered part of heap A. The values ranged below 200 nGy/h for the covered part of heap A. The effluent samples had an average pH of 2.8 and an average oxidation-reduction potential of +400 mV. Mean ion concentrations (in milligrams per liter) were as follows: Fe^{3+}, 5,200; Mn^{2+}, 280; Ni^{2+}, 97; Zn^{2+}, 40; Cu^{2+}, 28; Co^{2+}, 16; Mg^{2+}, 3,700; Ca^{2+}, 67; and SO_4^{2-}, 30,000. Thus, SO_4^{2-} and Fe^{3+}, resulting from pyrite oxidation, and Mg^{2+}, resulting from dissolution of the carbonaceous gangue material, were the main components. To evaluate the contribution of microorganisms, especially leaching bacteria like *T. ferrooxidans*, to this release of toxic compounds, the following data were collected.

Physical and chemical data. (i) Ore samples. At six sampling sites in heaps A and G, the temperatures ranged between 7 and 45°C. At one covered sampling site in heap A at a depth of 2 m, a temperature of 100°C was measured. The pH values were around 7 at the samples from the covered part of heap A. This finding was probably a result of the carbonaceous gangue material of the ore. In the uncovered part of heap A, the pH values fluctuated between 5 and 8. In heap G, pH values around 4 prevailed. The latter samples contained about 250 mg of Fe^{2+} and Fe^{3+} per kg, 75 mg of Mn^{2+} per kg, 20 mg of Cu^{2+} per kg, and 18 mg of Zn^{2+} per kg. The values for the oxidation-reduction potential in the samples from heap G were quite uniformly around +600 mV, whereas those in the samples from heap A fluctuated around +400 mV at the uncovered part and varied between +200 and −100 mV at the covered part.

(ii) Gas samples. A drop in the oxygen concentration from 19 to 10% and an increase in the carbon dioxide concentration to 3% were detected at a depth of 2 m in the uncovered part of heap A. At a depth of 1 m in the covered sites, the oxygen concentration was even further reduced to 3%, while the carbon dioxide concentration amounted to 13%. Obviously, the cover functions as a gas barrier. Hydrogen was not detectable. Methane could be detected only in samples from the covered part of heap A. Concentrations of up to 7% methane were measured.

Microbiological data. (i) Sampling site with high temperatures. Mesophilic lithotrophic bacteria were not detectable at the sampling site with high temperatures. Furthermore, attempts to enrich moderately thermophilic bacteria at 45°C remained unsuccessful. Instead, aerobic thermoacidophilic sulfur- and pyrite-oxidizing bacteria were found. The COT exhibited cell counts of up to 10^3 cells per g of sample. A strain of a thermoacidophilic bacterium was isolated from enrichments. The organism resembled members of the *Sulfolobus*/*Acidianus* group in physiology and morphology (13). The pure culture was able to grow autotrophically with sulfur and pyrite in a pH range of 1 to 5. The cells can be described as lobed cocci (data not shown).

(ii) Sampling sites with moderate temperatures. Mesophilic ferrous iron-, sulfur-, sulfur compound-, ammonia-, and nitrite-oxidizing bacteria, as well as acidophilic and neutrophilic COT, were detected. At these sites nitrate-, sulfate-, manganese-, and ferric iron-reducing bacteria were also detectable. Attempts to enrich moderately thermophilic bacteria remained unsuccessful. *T. ferrooxidans* dominated among ferrous iron-oxidizing bacteria. *L. ferrooxidans* was detectable only in assays with pyrite as the substrate.

The importance of lithotrophic bacteria was evaluated by comparing their cell counts with the cell counts of COT. The data for the samples from the covered part of heap A and from heap G were classed in four depth steps, whereas the data for the samples from the uncovered part of heap A were classed in six depth steps. The mean cell counts of aerobic bacteria as a function of depth are shown in Fig. 1 for the three sites. The cell counts of all groups decreased with increasing depth at the uncovered part of heap A and at heap G. At the covered part of heap A, four of five groups were still present up to a depth of 4 m, whereas at this depth organisms were not detectable in the uncovered heap G. In case of the covered part of heap A, the cell counts of all groups, except *T. ferrooxidans*, were nearly independent of depth. *T. ferrooxidans* counts were highest immediately below the loamy cover and decreased with depth. Approximately 99% of all *T. ferrooxidans* cells were found up to a depth of 1.5 to 2 m. In the samples from the uncovered heap G, *T. ferrooxidans* was the most frequent lithotrophic organism, whereas in heap A, moderately acidophilic thiobacilli dominated.

T. thiooxidans was generally less widespread than *T. ferrooxidans*. Usually, a difference of at least one tenth power was noted.

Among the COT, the neutrophilic COT dominated, with about 10^4 cells per g of sample in the sites on heap A because of the mostly neutral to slightly alkaline pH. In heap G, the acidophilic COT were about 10 times more common than the neutrophilic COT, irrespective of the sample depth. A positive correlation exists between the cell counts of acidophilic COT and those of *T. ferrooxidans*. Calculation of the cell counts (*T. ferrooxidans* versus acidophilic COT), irrespective of the site and the sampling depth, gave a regression coefficient of 0.81. The correlation was significant. Nitrifying bacteria were only rarely detected. They occurred in surface samples from heaps A and G at less than 10 cells per g of sample (data not shown).

The occurrence of anaerobes as a function of depth is shown in Fig. 2 for the sites on heap A. The occurrence of anaerobic bacteria decreased with depth in the samples from the uncovered part of heap A. The cell counts of anaerobic bacteria in samples from the covered part of heap A show wide fluctuations. The most abundant anaerobic bacteria were nitrate-reducing bacteria. Lithotrophic nitrate-reducing bacteria were detected in all surface samples. In more than 50% of these samples, sulfate-reducing bacteria were detected as well. Below a depth of 3 m, they were not detectable any more. Ferric iron- and manganese-reducing bacteria occurred in only a few samples from heap A and were not detectable at all in samples from heap G. Only nitrate- and sulfate-reducing bacteria were found in the samples from heap G (in less than 20% of the samples [data not shown]).

Microbial leach activity was significantly correlated with cell counts of *T. ferrooxidans* in all samples from the surface to a depth up to 1.5 m. The values of the heat output were plotted against the cell counts (10^2 to 10^3, 10^3 to 10^4, . . . , $>10^6$) to calculate a possible correlation. A positive correlation resulted (Fig. 3). Below a depth of 1.5 m, the heat output comprised both biological and chemical reactions (probably autoxidation

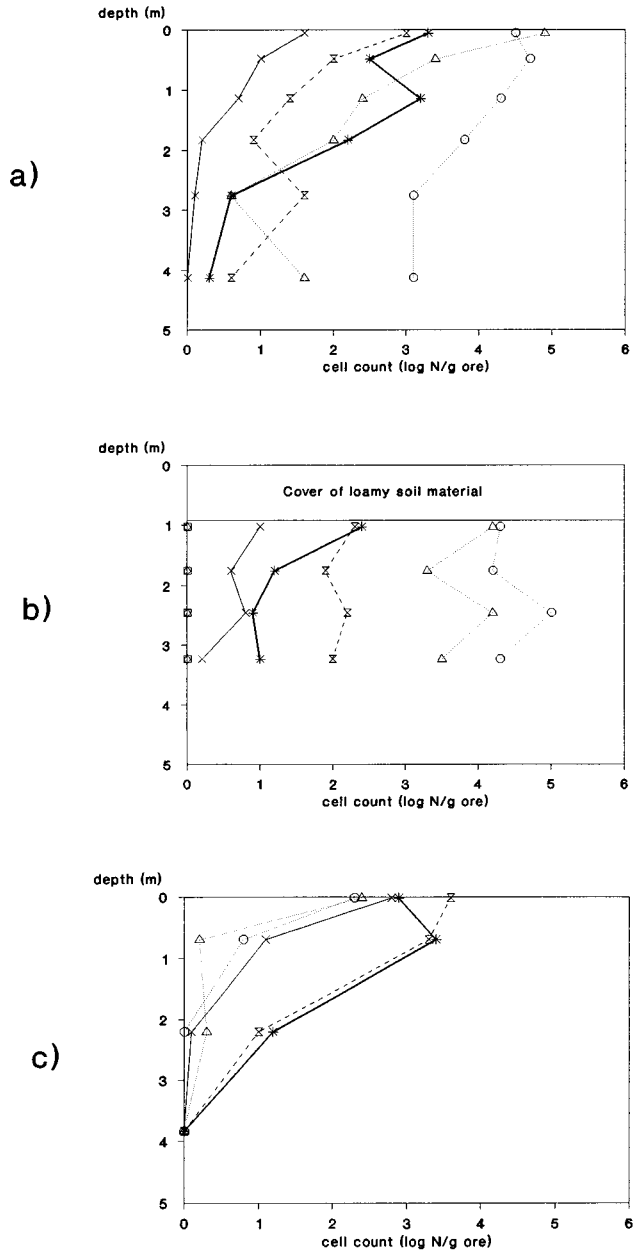

FIG. 1. Cell counts of aerobes as a function of depth. (a) Uncovered part of heap A; (b) covered part of heap A; (c) uncovered heap G. Symbols: ∗, *T. ferrooxidans*; ×, *T. thiooxidans*; △, moderately acidophilic thiobacilli; ⧖, acidophilic COT; ○, neutrophilic COT. Standard deviations of cell counts were around 10^{-1}.

of reduced metal ions or sulfur compounds with oxygen) and was not used for further calculations.

To compare the leach activity of *T. ferrooxidans* in the samples from Ronneburg with values from other biotopes, three additional linear correlations were included in Fig. 3. These biotopes were the Mina Ilba in Romania (in situ leach experiment [44, 46]), laboratory experiments in percolators (47, 50), and waste heaps from Cu, Zn, and Pb mining near Cartagena, Spain (44). Leach activity and cell counts of *T. ferrooxidans* in the samples from these biotopes were determined in the same manner as described for samples from Ronneburg. The highest microbial leach activity was measured in the laboratory sam-

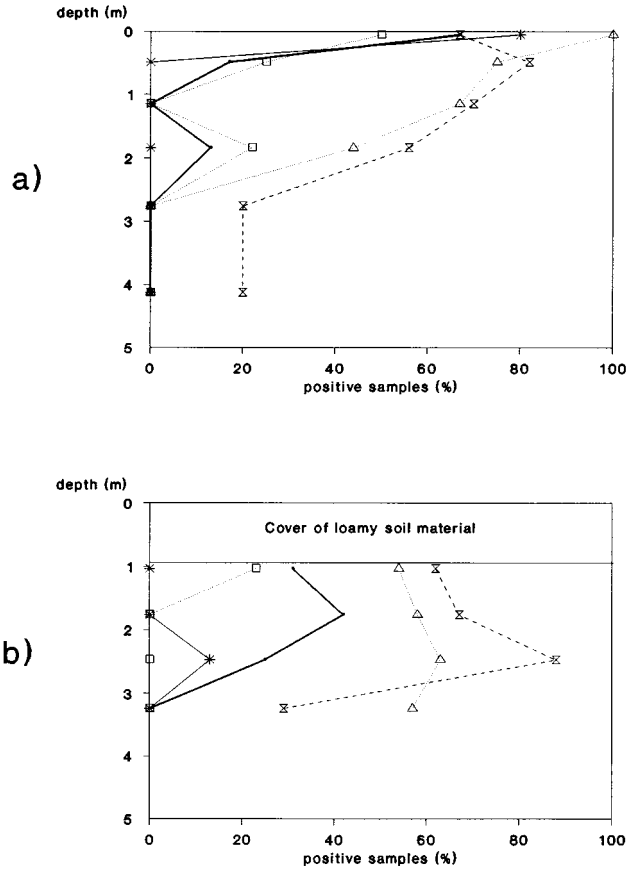

FIG. 2. Occurrence of anaerobes as a function of depth. (a) Uncovered part of heap A; (b) covered part of heap A. Symbols: △, lithotrophic nitrate-reducing bacteria; ⧖, organotrophic nitrate-reducing bacteria; ●, sulfate-reducing bacteria; ∗, Fe^{3+}-reducing bacteria; □, Mn^{4+}-reducing bacteria.

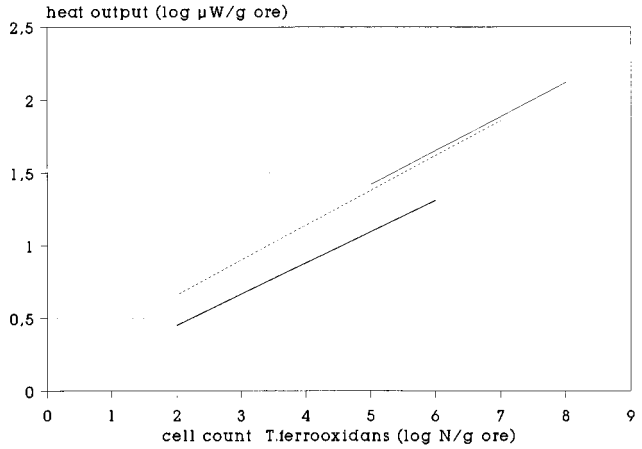

FIG. 3. Variation of microbial activity in different habitats. ▬, Ronneburg (50 samples, $r = 0.93$); – – –, Cartagena (66 samples, $r = 0.96$); ·····, Ilba (82 samples, $r = 0.97$); ——, percolators (22 samples, $r = 0.96$). *r*, correlation coefficient. The habitats are uranium mine waste heaps near Ronneburg (this work); waste heaps from Cu, Zn, and Pb mining near Cartagena, Spain (44); the Mina Ilba in Romania (in situ leach experiment [44, 46]); and laboratory experiments in percolators (47, 50).

ples (optimum conditions for temperature, pH, oxygen content, and sufficient substrate), while the heaps near Ronneburg exhibited the lowest activity. Even the values in samples from the heaps near Cartagena were considerably higher than those in the samples from the heaps at Ronneburg. A heat output of 10 µW/g of ore required 10^3 cells per g ore in samples from Cartagena and in the laboratory samples, whereas in those from Ronneburg, 10^4 cells per g ore were necessary. Thus, *T. ferrooxidans* in the latter case had only one-tenth of its maximum activity.

DISCUSSION

Thiobacilli dominate the microflora in the samples of the Ronneburg heaps, although the cell counts of COT seemed to be higher in many samples. Nevertheless, because lithotrophic bacteria including thiobacilli were measured by the most-probable-number technique, the real cell count was considerably underestimated (8, 48, 62). This results from the growth mode of lithotrophs. Organisms growing in a biofilm cannot be separated into single cells. This holds for leaching bacteria as well. Hallmann et al. (19) demonstrated attached cells of *L. ferrooxidans* on ore particles.

T. ferrooxidans was regularly detected in the samples, whereas *L. ferrooxidans* only rarely occurred. Similar results were described by Groudev and Groudeva (18), Goodman et al. (16), and Sand et al. (46) for Bulgarian, Australian, and Romanian leach biotopes, respectively. Obviously, the growth requirements of *L. ferrooxidans* (temperature above 20°C and availability of water [19, 47]) are not met. However, with the substrate pyrite, *L. ferrooxidans* could be detected in several samples. This raises the possibility that there is a population of *L. ferrooxidans* which is not culturable with soluble ferrous iron. Further experiments are planned to address this question.

The use of microcalorimetry enabled us to determine that at the Ronneburg site the leach activity of *T. ferrooxidans* was 10 times lower than elsewhere. Obviously, *T. ferrooxidans* is considerably inhibited at this site. The reason for this inhibition remains to be elucidated. The loam layer covering heap A seems not to be decisive. The alkaline pH value, due to the carbonaceous gangue material, and/or the considerable degradation of the ore, resulting in a muddy consistency of the top layers, may be responsible.

Moderately acidophilic thiobacilli were detected more often than *T. ferrooxidans* at the heap A sites, where the pH values were mainly around 7. The question of their substrate arises, because small numbers of *T. ferrooxidans* and *T. thiooxidans* were found at these sites. According to the equation of the indirect leaching mechanism (33), sulfur would be available. However, a few sulfur producers cannot produce enough sulfur to feed many moderately acidophilic sulfur-consuming thiobacilli. Furthermore, the leach mechanism requires acidic pH values. However, preliminary data indicate that the leach equations must be revised (45). Thiosulfate and ferrous iron are proposed to be intermediates of the oxidation of pyrite by ferric iron (39). There is evidence that thiosulfate instead of sulfur is the primary product of microbial pyrite leaching under acidic conditions (48). This finding suggests the existence of an altered sulfur chemistry based on sulfane monosulfonic acids (55, 60). Thiosulfate undergoes a sequence of chemical reactions which finally may give rise to the easily detectable and slowly biodegradable sulfur. Furthermore, thiosulfate, trithionate, and tetrathionate have previously been detected in the course of the oxidation of pyritic mine spoil material (41) and during chemical oxidation of pyrite at pH values between 6 and 9 (15). Thiosulfate is known to be a good substrate for the moderately acidophilic thiobacilli. These bacteria are able to

grow if pyrite is chemically oxidized at neutral pH values (1). This may explain, why moderately acidophilic thiobacilli occur in large cell numbers in samples from the covered part of heap A, where neutral pH values are found. They were detected in small cell numbers in heap G, where pH values around 4 were measured.

However, a significant correlation between the occurrence of bacteria and the pH could not be demonstrated. This probably resulted because the ore material contains more than 90% shale. Suspending the samples during processing resulted in an increased availability of carbonaceous compounds. This caused a change of the original pH in microniches of the ore to an overall (mixed) value.

The physical parameters temperature and gas composition are important for the diversity of the microflora. As a consequence of the high temperature, a thermoacidophilic bacterium resembling the leach bacteria *Sulfolobus/Acidianus* spp. could be detected at a hot sampling site. The gas composition inside the heaps changed with depth. The oxygen concentration decreased, and the carbon dioxide concentration increased. Corresponding to the decrease in oxygen concentration, the number of *T. ferrooxidans* cells decreased. Obviously, at a depth of 1.5 to 2 m, the oxygen concentration became limiting for the growth of *T. ferrooxidans*. Concomitantly, the carbon dioxide concentration increased (mainly as a result of the dissolution of the carbonaceous gangue material). Similar data were demonstrated for Australian mine waste heaps (21) and for other leach biotopes (4, 18, 52, 54). Consequently, the cell counts of *T. ferrooxidans* remained constant up to a depth of 5 m in Australian mine waste heaps under microaerophilic conditions (16).

Generally, it is accepted that lithotrophic bacteria feed acidophilic COT by the excretion of organic compounds (19, 24, 36, 58, 61). The cell counts of the latter correlated with the cell counts of *T. ferrooxidans*, obviously in agreement with this hypothesis. The decrease in the cell counts of the acidophilic COT at the uncovered sites may be explained by the depth-dependent decrease in the *T. ferrooxidans* cell counts. For all other microorganisms, a similar decrease with depth was noted irrespective of aerobic or anaerobic metabolism. The main nutrient source for the microorganisms is the pyrite oxidation by *T. ferrooxidans*. If this process does not function, nutrients for the other types of microorganisms soon become exhausted. Atmospheric deposit by rain or dust seems to be negligible.

Anaerobically growing bacteria may be important members of the biocoenosis in leach dumps because they are able to reduce insoluble metal compounds to a soluble and, hence, mobile species. The reductive solubilization of ferric iron and manganese oxides and hydroxides may result in a release of coprecipitated heavy metal ions (14). Among the anaerobic bacteria, the nitrate-reducing ones are the most important. Nitrate was regularly detectable in the samples, ensuring a constant supply for these bacteria. The nitrate might result from the activity of nitrifiers present in the heaps, although the general importance of nitrifying bacteria is obviously low. They occurred only at the surface of the heaps and were present in small numbers. Sulfate-reducing bacteria were detected in samples from the surface, although aerobic conditions exist. This might be explained by the finding that some species are able to use oxygen as an electron acceptor (11).

A large microbial diversity exists in mine waste heaps. Rehabilitation measures must be scrutinized for their effect on the microbial biocoenosis and the leach activity, if they are to be successfully realized. The microcalorimetric leach activity measurements constitute a notable advancement for this task. The effect of measures becomes detectable within a reasonable time span. To take the right action, the leach process must be thoroughly understood and further research is needed to elucidate the sulfur chemistry in leach heaps.

ACKNOWLEDGMENTS

This work was supported by grant AZ02042 from the Deutsche Bundesstiftung Umwelt to W.S.

We thank the Wismut GmbH for cooperation in sampling and for supplying ore material. We also thank the Institut für Bodenkunde, Universität Hamburg, and the Bundesanstalt für Geowissenschaften und Rohstofforschung, Hannover, Germany, for gas analysis. In addition, we thank J. M. Shively for critical reading of the manuscript.

REFERENCES

1. **Arkesteyn, G. J. M. W.** 1980. Pyrite oxidation in acid sulfate soils: the role of microorganisms. Plant Soil **54:**119–134.
2. **Bennett, J. W., J. R. Harries, G. Pantelis, and A. I. M. Ritchie.** 1989. Limitations on pyrite oxidation rates in dumps set by air transport mechanisms, p. 551–562. *In* J. Sally, R. G. L. McGready, and P. L. Wichlacz (ed.), Biohydrometallurgy. Canmet, Ottawa, Canada.
3. **Bergmann, R., and M. Meinke.** Unpublished data.
4. **Bhappu, R., P. Johnson, J. Brierley, and D. Reynolds.** 1969. Theoretical and practical studies on dump leaching. AIME Trans. **244:**307–320.
5. **Bock, E., H. Sundermeyer-Klinger, and E. Stackebrandt.** 1983. New facultative lithoautotrophic nitrite-oxidizing bacteria. Arch. Microbiol. **136:**281–284.
6. **Brock, T. D., and J. Gustafson.** 1976. Ferric iron reduction by sulfur- and iron-oxidizing bacteria. Appl. Environ. Microbiol. **32:**567–571.
7. **Bundesminister für Wirtschaft.** 1993. Wismut, Stand der Stillegung und Sanierung. Dokumentation no. 335. Kölnische Verlagsdruckerei, Cologne, Germany.
8. **Cooper, A. B.** 1983. Population ecology of nitrifiers in a stream receiving geothermal inputs in ammonium. Appl. Environ. Microbiol. **45:**1170–1177.
9. **Corbin, J. L.** 1984. Liquid chromatographic-fluorescence determination of ammonia from nitrogenase reactions: a 2-min assay. Appl. Environ. Microbiol. **47:**1027–1030.
10. **Daniel, J. A., J. R. Harries, and A. I. M. Ritchie.** 1980. Temperature distribution in an overburden dump undergoing pyritic oxidation, p. 630–636. *In* P. A. Trudinger, M. R. Walter, and B. J. Ralph (ed.), Biogeochemistry of ancient and modern environments. Springer-Verlag KG, Berlin.
11. **Dilling, W., and H. Cypionka.** 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiol. Lett. **71:**123–128.
12. **Dunk, R., R. A. Mostyn, and H. C. Hoare.** 1969. The determination of sulfate by indirect atomic absorption spectroscopy. Atomic Absorption Newsl. **8**(4)**:** 79–81.
13. **Emmel, T., W. Sand, W. A. König, and E. Bock.** 1986. Evidence for the existence of a sulphur oxygenase in *Sulfolobus brierleyi*. J. Gen. Microbiol. **132:**3415–3420.
14. **Francis, A. J., C. J. Dodge, A. W. Rose, and A. J. Ramirez.** 1989. Aerobic and anaerobic microbial dissolution of toxic metals from coal wastes: mechanism of action. Environ. Sci. Technol. **23:**435–441.
15. **Goldhaber, M. B.** 1983. Experimental study of metastable sulfur oxyanion formation during pyrite oxidation at pH 6-9 and 30°C. Am. J. Sci. **283:**193–217.
16. **Goodman, A. E., A. M. Khalid, and B. J. Ralph.** 1981. Microbial ecology of Rum Jungle I: environmental study of sulphidic overburden dumps, experimental heap leach piles and tailings dam area. Report AAEC/E531. Australian Atomic Energy Commission, Sutherland, New South Wales, Australia.
17. **Goodman, A. E., T. Babij, and A. I. M. Ritchie.** 1983. Leaching of a sulphide ore by *Thiobacillus ferrooxidans* under anaerobic conditions, p. 361–376. *In* G. Rossi and A. E. Torma (ed.), Recent progress in biohydrometallurgy. Associazione Mineraria Sarda, Iglesias, Italy.
18. **Groudev, S. N., and V. I. Groudeva.** 1993. Microbial communities in four industrial copper dump leaching operations in Bulgaria. FEMS Microbiol. Rev. **11:**261–268.
19. **Hallmann, R., A. Friedrich, H. P. Koops, A. Pommerening-Röser, K. Rohde, C. Zenneck, and W. Sand.** 1993. Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and physicochemical factors influence microbial metal leaching. Geomicrobiol. J. **10:**193–206.
20. **Harries, J. R., N. Hendy, and A. I. M. Ritchie.** 1988. Rate controls on leaching in pyritic mine wastes, p. 233–241. *In* P. R. Norris and D. P. Kelly (ed.), Biohydrometallurgy. Science and Technology Letters, Kew, Great Britain.
21. **Harries, J. R., and A. I. M. Ritchie.** 1983. The microenvironment within waste rock dump undergoing pyritic oxidation, p. 377–392. *In* G. Rossi and A. E. Torma (ed.), Recent progress in biohydrometallurgy. Associazione Mineraria Sarda, Iglesias, Italy.
22. **Harrison, A. P., Jr.** 1978. Microbial succession and mineral leaching in an

artifical coal spoil. Appl. Environ. Microbiol. **36:**861–869.
23. **Harrison, A. P., Jr.** 1981. *Acidiphilium cryptum* gen. nov., sp. nov., heterotrophic bacterium from acidic mineral environments. Int. J. Syst. Bacteriol. **31:**327–332.
24. **Harrison, A. P., Jr.** 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. Annu. Rev. Microbiol. **38:**265–292.
25. **Hutchinson, M., K. J. Johnstone, and D. White.** 1965. The taxonomy of certain thiobacilli. J. Gen. Microbiol. **41:**357–366.
26. **Kirstein, K., und W. Sand.** 1991. Nitrifikanten aus Laugungsbiotopen, p. 89–103. *In* A. Noske (ed.), Deutsch-rumänische Zusammenarbeit. Berichte des Forschungszentrums Jülich, Jülich, Germany.
27. **Krümmel, A., and H. Harms.** 1982. Effect of organic matter on growth and cell yield of ammonia-oxidizing bacteria. Arch. Microbiol. **133:**50–54.
28. **Lange, G., and G. Freyhoff.** 1991. Geologie und Bergbau in der Uranlagerstätte Ronneburg/Thüringen. Erzmetall **44:**264–269.
29. **Lange, G., P. Mühlstedt, G. Freyhoff, and B. Schröder.** 1991. Der Uranerzbergbau in Thüringen und Sachsen—ein geologisch-bergmännischer Überblick. Erzmetall **44:**162–171.
30. **Lapage, S. P., J. E. Shelton, and T. G. Mitchell.** 1970. Medium for the maintenance and preservation of bacteria. Methods Microbiol. **3A:**120.
31. **Lovley, D. R., and E. J. P. Phillips.** 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. **51:**683–689.
32. **Lovley, D. R., and E. J. P. Phillips.** 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron and manganese. Appl. Environ. Microbiol. **54:**1472–1480.
33. **Lundgren, D. G., and M. Silver.** 1980. Ore leaching by bacteria. Annu. Rev. Microbiol. **34:**263–283.
34. **Lyalikova, N. N., and D. G. Lebedeva.** 1983. Role of nitrifying bacteria in rock weathering, p. 727–734. *In* G. Rossi and A. E. Torma (ed.), Recent progress in biohydrometallurgy. Associazione Mineraria Sarda, Iglesias, Italy.
35. **Mackintosh, M. E.** 1978. Nitrogen fixation by *Thiobacillus ferrooxidans*. J. Gen. Microbiol. **105:**215–218.
36. **Matin, A.** 1978. Organic nutrition of chemolithotrophic bacteria. Annu. Rev. Microbiol. **32:**433–468.
37. **Matin, A., and S. C. Rittenberg.** 1971. Enzymes of carbohydrate metabolism in *Thiobacillus* species. J. Bacteriol. **107:**179–186.
38. **Morin, K. A., J. A. Cherry, T. P. Lim, and A. J. Vivyurka.** 1982. Contaminent migration in a sand aquifer near an inactive uranium tailings impoundment, Elliot Lake, Ontario. Can. Geotech. J. **19:**49–62.
39. **Moses, C. O., D. K. Nordstrom, J. S. Herman, and A. L. Mills.** 1987. Aqueous pyrite oxidation by dissolved oxygen and by ferric iron. Geochim. Cosmochim. Acta **51:**1561–1571.
40. **N. N.** 1984. Bestimmung von Eisen, E_1, p. 1–10; Bestimmung des Thiosulfat-Ions, D15, p. 1–2. *In* Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung. Verlag Chemie, Weinheim, Germany.
41. **Pichtel, J. R., and W. A. Dick.** 1991. Sulfur, iron and solid phase transformations during the biological oxidation of pyritic mine spoil. Soil. Biol. Biochem. **23:**101–107.
42. **Postgate, J. R.** 1963. Versatile medium for the enumeration of sulfate-reducing bacteria. Appl. Microbiol. **11:**265–267.
43. **Sand, W.** 1989. Ferric iron reduction by *Thiobacillus ferrooxidans* at extremely low pH-values. Biogeochemistry **7:**195–201.
44. **Sand, W., T. Gehrke, R. Hallmann, K. Rohde, B. Sobotke, and S. Wentzien.** 1993. In-situ bioleaching of metal sulfides: the importance of *Leptospirillum ferrooxidans*, p. 15–27. *In* A. E. Torma, J. E. Wey, and V. I. Lakshmanan (ed.), Biohydrometallurgical technologies. TMS, Warrendale, Pa.
45. **Sand, W., T. Gehrke, R. Hallmann, and A. Schippers.** Sulfur chemistry, biofilm, and the (in)direct attack mechanism—a critical evaluation of bacterial leaching. Appl. Microbiol. Biotechnol., in press.
46. **Sand, W., R. Hallmann, K. Rohde, B. Sobotke, and S. Wentzien.** 1993. Controlled microbiological in-situ stope leaching of a sulphidic ore. Appl. Microbiol. Biotechnol. **40:**421–426.
47. **Sand, W., K. Rohde, B. Sobotke, and C. Zenneck.** 1992. Evaluation of *Leptospirillum ferrooxidans* for leaching. Appl. Environ. Microbiol. **58:**85–92.
48. **Schippers, A., and W. Sand.** Unpublished data.
49. **Schröter, A. W., and W. Sand.** 1989. Investigations on leaching bacteria by microcalorimetry, p. 427–438. *In* J. Sally, R. G. L. McGready, and P. L. Wichlacz (ed.), Biohydrometallurgy. Canmet, Ottawa, Canada.
50. **Schröter, A. W., and W. Sand.** 1992. Enhanced leaching of a sulphide ore by biological acidification. Biorecovery **2:**69–81.
51. **Schröter, A. W., and W. Sand.** 1993. Estimations on the degradability of ores and bacterial leaching activity using short-time microcalorimetric tests. FEMS Microbiol. Rev. **11:**79–86.
52. **Silver, M.** 1987. Distribution of iron-oxidizing bacteria in the nordic uranium tailings deposit, Elliot Lake, Ontario, Canada. Appl. Environ. Microbiol. **53:**846–852.
53. **Snyder, T. D., and F. T. Caruccio.** 1988. The partitioning of flow components of acidic seeps from surface coal mines and the icentification of acid-producing horizons within the backfill, p. 59–66. *In* D. S. Brown and D. P. Hodel (ed.), Mine drainage and surface mine reclamation. Report 1C 9183. Bureau of Mines, U.S. Department of the Interior, Washington, D.C.
54. **Southam, G., and T. J. Beveridge.** 1992. Enumeration of thiobacilli within pH-neutral and acidic mine tailings and their role in the development of secondary mineral soil. Appl. Environ. Microbiol. **58:**1904–1912.
55. **Steudel, R.** 1989. On the nature of the "elemental sulfur" (S^0) produced by sulfur-oxidizing bacteria—a model for S^0 globules, p. 289–302. *In* H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag KG, Berlin.
56. **Taylor, B. F., and D. S. Hoare.** 1971. *Thiobacillus denitrificans* as an obligate chemolithotroph. Isolation and growth studies. Arch. Mikrobiol. **80:**262–276.
57. **Thamdrup, B., K. Finster, J. Würgler Hansen, and F. Bak.** 1993. Bacterial disproportionation of elemental sulfur coupled to chemical reduction of iron or manganese. Appl. Environ. Microbiol. **59:**101–108.
58. **Tuttle, J. H., and P. R. Dugan.** 1976. Inhibition of growth, iron and sulfur oxidation in *Thiobacillus ferrooxidans* by simple organic compounds. Can. J. Microbiol. **22:**719–730.
59. **Wentzien, S., R. Hallmann, and W. Sand.** 1994. Mikrokalorimetrische Zellzahlbestimmungen von acidophilen lithoautotrophen Bakterien, p. 297–304. *In* W. Dubbert and J. M. L. Pila (ed.), Fachtagung Überwachungsmethoden Gentechnik-Texte 29/94. Umweltbundesamt, Berlin, Germany.
60. **Wentzien, S., W. Sand, A. Albertsen, and R. Steudel.** 1994. Thiosulfate and tetrathionate degradation as well as biofilm generation by *Thiobacillus intermedius* and *Thiobacillus versutus* studied by microcalorimetry, HPLC, and ion-pair chromatography. Arch. Microbiol. **161:**116–125.
61. **Wichlacz, P. L., and T. L. Thomson.** 1988. The effect of acidophilic heterotrophic bacteria on the leaching of cobalt by Thiobacillus ferrooxidans, p. 77–86. *In* P. R. Norris and D. P. Kelly (ed.), Biohydrometallurgy. Science and Technology Letters, Kew, Great Britain.
62. **Wolters, B.** 1986. Untersuchungen zur Nitrifikantenflora am Kölner Dom. Diplom thesis, University of Hamburg, Hamburg, Germany.
63. **Zenneck, C.** 1988. Mikrobiologische Bestandsaufnahme in zwei rumänischen Erzbergwerken und Charakterisierung der lithoautotrophen Flora. Diplom thesis, University of Hamburg, Hamburg, Germany.