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Geomicrobiological investigation of two different mine waste tailings generating acid mine drainage

Dagmar Kock, Axel Schippers *

Section Geomicrobiology, Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2, 30655 Hannover, Germany

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Abstract

The impact of microbiological metal sulfide oxidation on acid mine drainage generation was studied for two different mine tailings. Microorganisms were quantified using different methods: (1) SYBR Green II direct counting, (2) TaqMan quantitative, real-time PCR (Q-PCR), (3) catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) and (4) most probable number (MPN) cultivation of acidophilic Fe(II) oxidizers. Potential pyrite or pyrrhotite oxidation rates were measured by microcalorimetry.

In the uncovered, pyrrhotite-containing tailings near Selebi-Phikwe, Botswana, acidophilic Fe(II)-oxidizing microorganisms were present in high numbers (MPN) of up to 10^7 cells g^{-1} dw (mean value 3×10^6 cells g^{-1} dw) throughout the entire water unsaturated, oxidized zone of about 25 m (at the tailings dam periphery) with a paste pH in the range of 3–4. Mean numbers of living Bacteria (CARD-FISH) and total microorganisms (SYBR Green II) were 1×10^7 cells g^{-1} dw and 8×10^7 cells g^{-1} dw, respectively. Cell numbers obtained by Q-PCR analysis were in the same range. The average potential pyrrhotite oxidation rate measured by microcalorimetry was 3.4×10^{-4} mol pyrrhotite m^{-3} tailings s^{-1} at 25 °C. About half of the pyrrhotite oxidation activity was biologically catalyzed.

By contrast, in the covered pyrite-containing tailings in Impoundment 1 in Kristineberg, northern Sweden, acidophilic Fe(II)-oxidizing microorganisms (mean value 5×10^5 cells g^{-1} dw) were only detected in a distinct zone of oxidized tailings between the cover and the unoxidized tailings where low pH values down to 3 prevailed. Bacterial numbers obtained by Q-PCR analysis were much higher (mean value 3×10^8 cells g^{-1} dw). The proportion of biological pyrite oxidation was up to 100% for the oxidized zone. The average potential pyrite oxidation rate was 1.6×10^{-5} mol pyrite m^{-3} tailings s^{-1} at 10 °C, an order of magnitude lower than that for the pyrrhotite-containing tailings.

Keywords: Acid mine drainage; Mine tailings; *Acidithiobacillus ferrooxidans*; Quantitative real-time PCR; CARD-FISH; SYBR Green II

1. Introduction

Acid mine drainage (AMD) is a strongly acidic solution containing high amounts of heavy metals and sulfate threatening groundwater quality. AMD is generated by chemical and biological oxidation of pyrite, pyrrhotite and other metal sulfides in mine waste heaps or in tailings from sulfidic ore processing [1–3]. The pyrite oxidation rate depends on temperature, pH, humidity and the availability of oxygen in the tailings, which is mainly controlled by diffusion. In addition, the oxidation rate strongly depends on the abundance of acidophilic Fe(II)- and metal sulfide-oxidizing microorganisms, which accelerate the kinetics of pyrite

* Corresponding author.
E-mail address: a.schippers@bgr.de (A. Schippers).

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oxidation 30–300-fold [4,5]. Geomicrobiological and geochemical studies of pyrite and pyrrhotite-containing mine tailings pointed out the important role of acidophilic Fe(II)-oxidizing microorganisms, such as *Acidithiobacillus ferrooxidans* for the generation of AMD [6–11]. Only a few studies included the measurement of pyrite or pyrrhotite oxidation rates [9,12,13].

In mine waste, pyrite is oxidized according to Eq. (1):

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (1)$$

Pyrrhotite oxidation occurs according to Eq. (2) [14]:

$$Fe_{1-x}S + (2-x/2)O_2 + xH_2O \rightarrow (1-x)Fe^{2+} + SO_4^{2-} + 2xH^+ \quad (2)$$

Based on the mineralogical composition of pyrrhotite, x ranging between 0 and 0.125 Fe(II) may be further oxidized to Fe(III) hydroxide according to Eq. (3):

$$Fe^{2+} + 0.25O_2 + 2.5H_2O \rightarrow Fe(OH)_3 + 2H^+ \quad (3)$$

In the present study, the uncovered pyrrhotite-containing tailings dam near Selebi-Phikwe, Botswana [15], and the covered pyrite-containing tailings in Impoundment 1 in Kristineberg, northern Sweden [16–21], were geomicrobiologically analyzed including the microcalorimetric measurement of potential pyrite oxidation rates [9,10,22,23]. The abundance of bacteria in the tailings has been quantified using four different techniques: (a) SYBR Green II staining, (b) TaqMan quantitative, real-time PCR (Q-PCR), (c) catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) and (d) most probable number (MPN) cultivation of acidophilic Fe(II)-oxidizers.

2. Materials and methods

2.1. Site description and sampling

2.1.1. Covered pyrite-containing tailings in Impoundment 1 in Kristineberg, northern Sweden

The annual precipitation in the humid Kristineberg area, northern Sweden, varies between 400 and 800 mm $year^{-1}$ and the annual mean temperature is 0.7 °C. The design, the mineralogy and the chemistry of the pyrite-containing tailings in Impoundment 1 have been described in detail [16–21]. Briefly, the tailings in Impoundment 1 cover an area of 0.1 km^2 and have a thickness of up to approximately 11 m, with an average thickness of 6–8 m. They were covered in 1996 with a soil cover consisting of 0.3-m compacted till and 1.5-m unspecified till. From the 1940s until 1996, the impoundment was unremediated and sulfide oxidation occurred in distinct depth layers (oxidized tailings). Based on the chemical composition, the sulfide mineral content of the unoxidized tailings ranges from 10% to 30%, totally dominated by pyrite. In the oxidized tailings, the sulfide content is generally lower [16]. Three boreholes at different locations (cores K, O and Q) were drilled using a drill-rig. The drill cores were split into 30 subsamples from the oxidized and the unoxidized tailings for laboratory analysis. Brown precipitates of iron (hydrox)oxides due to pyrite oxidation were found in the oxidized tailings at all three locations.

2.1.2. Uncovered pyrrhotite-containing tailings dam near Selebi-Phikwe, Botswana

The climate in Selebi-Phikwe, Botswana, is semiarid with an average annual temperature of 21 °C. The mine waste tailings dam near Selebi-Phikwe generates high amounts of AMD, which are collected in a drainage ditch surrounding the dam. To prevent contamination of surface water, AMD is purified in a plant by addition of limestone to increase the pH and to precipitate metals. The geochemistry of the tailings dam has been previously described [15]. Briefly, the tailings dam consists of waste from about 32 years of Ni-, Cu-, Zn- and Co-sulfidic ore processing. The original material from the flotation plant consists of about 35% solid material with an average grain size diameter of about 0.1 mm. The solid material contains about 11% pyrrhotite, about 1.5% other metal sulfides and hornblende and feldspar as major gangue minerals. The approximately 40-m high dam (2003) covers an area of ca. 1 km^2. The final height shall be 50 m in the year 2014. Currently, the surface of the central part is water covered, whereas the periphery of the dam surface is dry. Here, three holes (B-H1 = core 1, B-H2 = core 2, B-H3 = core 3) were drilled through the water unsaturated down to the saturated zone at about 25 m depth. Brown precipitates of iron (hydrox)oxides due to pyrrhotite oxidation were found throughout the entire unsaturated zone. A high proportion of the originally deposited pyrrhotite has been already oxidized within the first years of tailings deposition. Altogether, 65 solid samples were taken in 1-m intervals.

2.2. Geochemical analysis

The paste pH was measured with an electrode after shaking of 5-g sample in 12.5 mL 1 M KCl for 1 h. Humidity was determined as weight difference after

drying. The elemental composition of the solid material was determined by XRF analysis. The total amount of sulfur (S_{total}) was measured with the instrument LECO CS 200. Total reduced inorganic sulfur (TRIS) was determined as chromium reducible sulfur following a previously described procedure [15,24].

2.3. Potential pyrite or pyrrhotite oxidation rate

The potential pyrite or pyrrhotite oxidation rate at atmospheric oxygen partial pressure was determined by microcalorimetry [9,10,22] because the reaction rate correlates with the heat output. A Thermal Activity Monitor Thermostat type 2277 (Thermometric; Järfälla, Sweden) equipped with 20 mL Ampoule Micro Calorimetric Units (type 2230–000) was used to measure the heat output (μW) due to the exothermal pyrite or pyrrhotite oxidation in 10-g sample each. The heat output caused by chemical pyrite or pyrrhotite oxidation only was measured in a second run after inactivation of microorganisms at 65 °C for 6 h. The difference between the values of the two measurements per sample is the heat output due to potential biological pyrite or pyrrhotite oxidation in μW.

Measurements for the pyrite-containing tailings were done at 10 °C close to the average summer temperature in Kristineberg, northern Sweden. A complete oxidation of FeS_2 to Fe(III) and sulfate produces a reaction energy $\Delta_f H^0$ of −1546 kJ mol^{-1}. Using this value, the molecular mass of FeS_2 of 0.12 kg mol^{-1}, the measured heat output a (μW) and the sample weight w (g), the pyrite oxidation rate r was calculated according to the following equation:

$$r\ (\mu g\ kg^{-1}\ s^{-1}) = -1.546^{-1}\ (mmol\ kJ^{-1})0.12 \times (kg\ mol^{-1})a\ (\mu W)w^{-1}\ (g^{-1})$$

The rate was converted to mol m^{-3} s^{-1} using a tailings density of 2186 kg m^{-3} calculated from values given for water content, grain density and porosity for 10 tailings samples [20].

Measurements for the pyrrhotite-containing tailings dam near Selebi-Phikwe, Botswana were done at 25 °C close to the average annual temperature of 21 °C. A complete oxidation of FeS to Fe(III) and sulfate produces a reaction energy $\Delta_f H^0$ of −940 kJ mol^{-1}. The molecular mass of FeS was 0.088 kg mol^{-1} and the estimated tailings density was 1700 kg m^{-3}.

Since the microcalorimetric measurements were done at atmospheric and not at in situ oxygen partial pressure, the obtained rates are considered as potential oxidation rates.

2.4. Quantification of microorganisms

The total number of microorganisms (including living and dead cells) was determined by counting of SYBR Green II stained cells in tailings material on filters under a fluorescence microscope as described elsewhere [25]. The tailings material had been previously fixed in formaldehyde after sampling following a published procedure [26]. To quantify the abundance of Bacteria and Archaea by Q-PCR analysis [27], high-molecular-weight DNA was extracted from 3 g of frozen sample following a modified FastDNA Spin Kit for Soil (Bio101) protocol [28], and the 16S rDNA copy numbers of prokaryotes, Archaea and Bacteria were determined using TaqMan assays [29,30]. 16S rDNA gene copy numbers were converted to cell numbers using a conversion factor of 3.6 [31]. The number of living Bacteria and Archaea in formaldehyde-fixed material was determined by catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) as previously described [27,32]. The number of metal sulfide-oxidizing bacteria of the type *A. ferrooxidans* was quantified by the "most probable number technique" using a medium for enrichment of acidophilic Fe (II)-oxidizers [33].

3. Results

The geomicrobiological analyses of the unsaturated, oxidized zone of the uncovered pyrrhotite-containing tailings dam at Selebi-Phikwe, Botswana, and the oxidized zone of the covered tailings in Impoundment 1 in Kristineberg, northern Sweden showed significant differences for both tailings. Mean values of all parameters from analyses of the three different cores for each tailings are given in Table 1. Depth profiles of selected parameters are shown for one core of each tailings in Figs. 1 and 2.

In the covered, pyrite-containing tailings iron (hydro) oxides were visible only in a distinct zone of oxidized tailings between the cover and the unoxidized tailings. Significant differences between the about 0.5-m-thick zone of oxidized tailings and the unoxidized tailings in core O are shown in Fig. 1. In the oxidized tailings, the amounts of S_{total} and the pH are lower, and the number of Bacteria determined by Q-PCR is much higher than in the unoxidized tailings. The total cell number (SYBR Green II) in the oxidized tailings is identical to the number of Bacteria (Q-PCR). Acidophilic Fe(II)-oxidizing microorganisms of the type *A. ferrooxidans* (MPN) were only detected in the oxidized tailings, which correlates with a high proportion of up to 100% of

Table 1
Mean values of geochemical and geomicrobiological parameters for the unsaturated, oxidized zone of the uncovered pyrrhotite-containing tailings at Selebi-Phikwe, Botswana, and the oxidized zone of the covered tailings in Impoundment 1 in Kristineberg, northern Sweden

	Oxidized zone of tailings at Selebi-Phikwe, Botswana	Oxidized zone of tailings in Kristineberg, Sweden
Main metal sulfide	Pyrrhotite	Pyrite
Soil cover	No	Yes
In operation (2003)	Yes	No
Annual mean temperature	21 °C	0.7 °C
S_{total}	4.4%	5.3%
Total reduced inorganic sulfur (TRIS)	3.8%	nm
Fe_{total}	14%	9%
Humidity	12%	24%
pH	3.8	4.8
Total number of microorganisms (SYBR Green II)	8×10^7 cells g^{-1} dw	3×10^8 cells g^{-1} dw
Number of prokaryotes (Q-PCR)	6×10^7 cells g^{-1} dw	nm
Number of bacteria (Q-PCR)	3×10^7 cells g^{-1} dw	3×10^8 cells g^{-1} dw
Number of living bacteria (CARD-FISH)	1×10^7 cells g^{-1} dw	nm
Number of *Acidithiobacillus ferrooxidans*-like bacteria (MPN)	3×10^6 cells g^{-1} dw	5×10^5 cells g^{-1} dw
Potential pyrite or pyrrhotite oxidation rate at atmospheric oxygen pressure (weight per weight)	17.5 μg pyrrhotite kg^{-1} tailings s^{-1} at 25 °C	0.9 μg pyrite kg^{-1} tailings s^{-1} at 10 °C
Potential pyrite or pyrrhotite oxidation rate at atmospheric oxygen pressure (moles per volume)	3.4×10^{-4} mol pyrrhotite m^{-3} tailings s^{-1} at 25 °C	1.6×10^{-5} mol pyrite m^{-3} tailings s^{-1} at 10 °C
Proportion of biological pyrite or pyrrhotite oxidation	52%	80%

nm = not measured.

biological pyrite oxidation. The average potential pyrite oxidation rate measured by microcalorimetry was 1.6×10^{-5} mol pyrite m^{-3} tailings s^{-1} at 10 °C.

In the uncovered, pyrrhotite-containing tailings brown precipitates of iron (hydro)oxides were discernible and microorganisms were present in high numbers throughout the entire water unsaturated, oxidized zone of about 25 m (at the tailings dam periphery). The paste pH shows low variation in the range of 3–4. The amounts of S_{total} and total reduced inorganic sulfur (TRIS) are almost identical, which means that pyrrhotite is the dominant sulfur-containing mineral. The quantification of microorganisms by the different methods exhibits comparable results. SYBR Green II total counts comprise living and dead cells and give the highest mean value of 8×10^7 cells g^{-1} dw. The Q-PCR values for total prokaryotes and total Bacteria are somewhat lower but in the same order of magnitude. The mean number of living Bacteria detected by CARD-FISH was 1×10^7 cells g^{-1} dw. Archaea could be quantified neither by Q-PCR nor by CARD-FISH, which exhibits the dominance of Bacteria in the tailings. A high proportion of the CARD-FISH detectable, living Bacteria (30%) could be detected via MPN cultivation of acidophilic Fe(II)-oxidizing microorganisms of the type *A. ferrooxidans*. Their numbers were significantly lower at the tailings surface than at deeper layers, but a depth trend could not be observed. These organisms are able to oxidize pyrrhotite throughout the entire unsaturated zone. The average potential pyrrhotite oxidation rate measured by microcalorimetry was 3.4×10^{-4} mol pyrrhotite m^{-3} tailings s^{-1} at 25 °C. The proportion of biological to chemical pyrrhotite oxidation was highly variable, did not show a depth trend and was found to be 52% in average.

4. Discussion

For the first time, the microbial community in sulfidic mine tailings has been quantified using four different methods. The fluorochrome SYBR Green II (as well as acridine orange or DAPI) binds unspecifically to nucleic acids and thus does not provide information on the viability of the cells. Potentially, a major part of the counted cells could be dormant or even dead and yet retain stainable DNA [34,35]. Consequently, for the pyrrhotite-containing tailings, the highest mean cell number was obtained with SYBR Green II direct counting. A slightly lower mean number produced the Q-PCR method, which targeted high molecular weight DNA. The different mean numbers for these two methods may be explained by the fact that SYBR Green II also binds to degenerated DNA which is not detectable by Q-PCR. However, both methods gave identical results for the pyrite-containing tailings.

RNA, in contrast, is much more labile and is readily degraded in cells that become inactive due to starvation [35]. Cell death in pure cultures accelerates when less than half of the RNA remains. Starved cells may still maintain an intact cell membrane and nucleic acids such

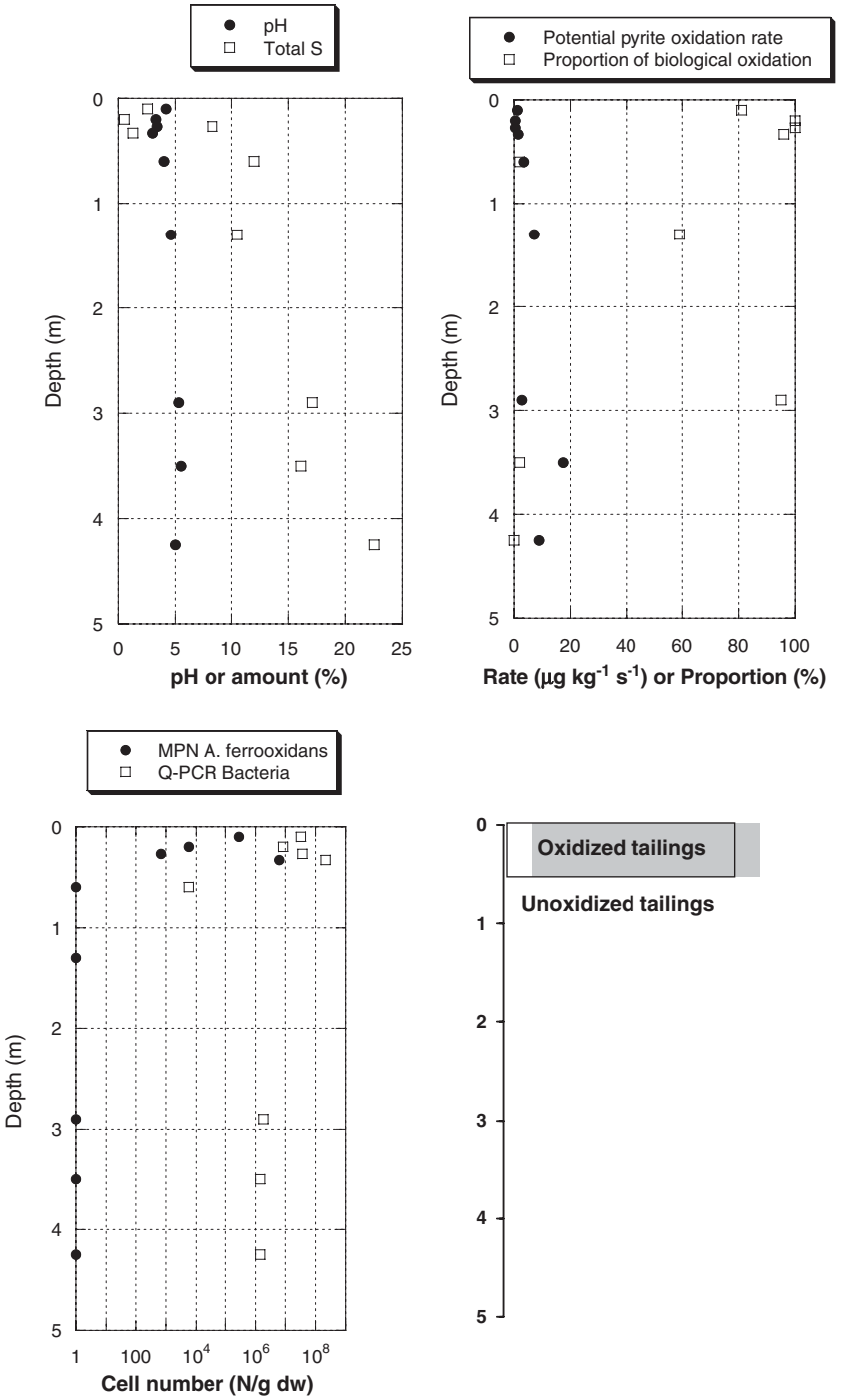

Fig. 1. Geomicrobial and geochemical data for core O of the covered, pyrite-containing tailings in Impoundment 1 in Kristineberg, northern Sweden.

as DNA or tRNA, but they rapidly loose their ribosomes [36]. The experience from pure culture studies is that cells with a significant ribosome content are living and metabolically active. Therefore, ribosomal RNA is an indicator of living cells, which could be quantified by CARD-FISH, as has recently done for deeply buried marine sediments [27]. The CARD-FISH analysis of the pyrrhotite-containing tailings gave a mean number of living Bacteria, which is eight times lower than the number of the SYBR Green II direct counts. However,

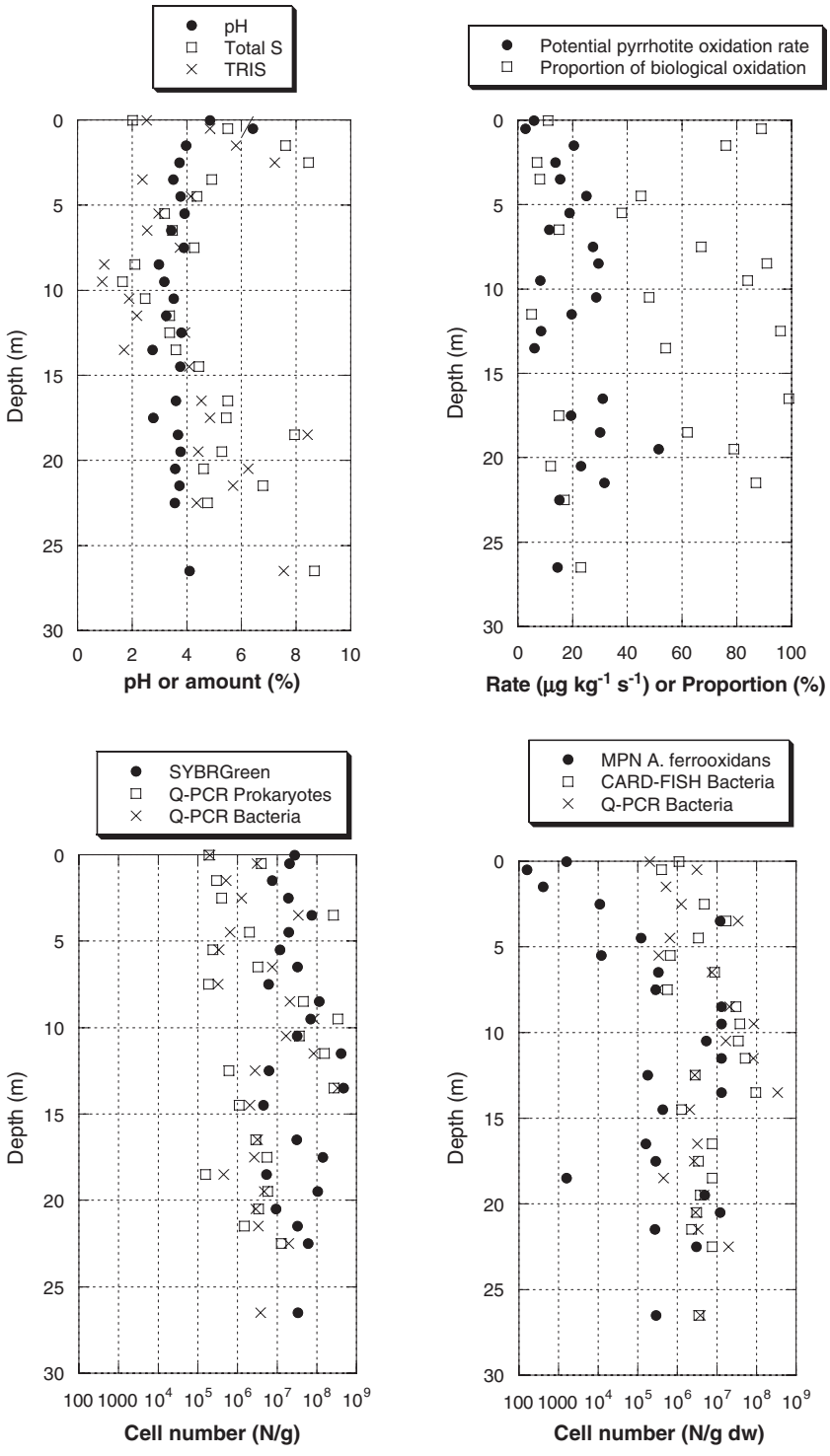

Fig. 2. Geomicrobial and geochemical data for core 1 of the uncovered, pyrrhotite-containing tailings dam near Selebi-Phikwe, Botswana.

the mean cell numbers of the SYBR Green II direct counts as well as those of the molecular methods Q-PCR and CARD-FISH were in the same order of magnitude, which shows that a high proportion of the detectable microorganisms are alive. Both molecular methods, Q-PCR and CARD-FISH, documented the dominant role

of Bacteria in the tailings since Archaea could not be quantified due to their low abundance.

A high proportion of 30% of the living Bacteria could be detected as acidophilic Fe(II)-oxidizers of the type *A. ferrooxidans* by the cultivation based MPN technique. Thus, acidophilic Fe(II)-oxidizers were a dominant group of the microbial community in the pyrrhotite-containing tailings. In contrast, only a minor proportion of less than 1% of the Bacteria was detected as acidophilic Fe(II)-oxidizers by MPN for the pyrite-containing tailings. Other not determined species of the Bacteria may also contribute to the overall metal sulfide oxidation activity. Further work is in progress to quantify *A. ferrooxidans* and other acidophilic Fe(II)-oxidizers by CARD-FISH. Numbers of *A. ferrooxidans*-like bacteria determined by MPN in this study are comparable or higher than those in other tailings [6–10].

FISH was previously used to analyze the microbial populations in an acid mine drainage outflow of the extreme environment Iron Mountain, California, USA [37–39]. In the mine tailings studied here, less cells were quantified in selected samples using conventional FISH with Cy3-monolabeled probes in contrast to the much more sensitive CARD-FISH (data not shown). Presumably, the amount of ribosomes, which determines the intensity of the fluorescence signal in conventional FISH, was lower in the cells in the mine tailings than in the cell from Iron Mountain. Since a high amount of ribosomes indicates a high activity status of a cell [35], the bioleaching activity at Iron Mountain is most likely much higher than that in the mine tailings. Unfortunately, pyrite oxidation rates for Iron Mountain had not been measured to be able to verify this assumption.

The microcalorimetric data of this study clearly show that pyrite and pyrrhotite are biologically and chemically oxidized in the tailings. The microcalorimetrically determined rates are potential rates, because the microcalorimetric measurements were done at atmospheric and not at the in situ oxygen content in the tailings dam, which varies with depth. The in situ pyrite or pyrrhotite oxidation rate in the tailings is limited by oxygen diffusion, which also determines the thickness of the oxidation zone in the tailings. For other tailings, depletion of molecular oxygen within the top 0.2 to 0.6 m was measured, strongly depending on the sulfide content and the humidity of the tailings material [8,9,12,40]. For the covered pyrite-containing tailings in northern Sweden, a 0.3-m-thick oxidation zone was assumed which gives an areal potential average pyrite oxidation rate of 4.7×10^{-6} mol m^{-2} dam surface s^{-1} (18 kg FeS_2 m^{-2} $year^{-1}$). For the pyrrhotite-containing tailings in Botswana, a thickness of the oxidation zone of only 0.1 m was assumed because of the high oxygen consumption due to the high pyrrhotite oxidation rate. In this case, the areal potential average pyrrhotite oxidation rate would be 3.4×10^{-5} mol m^{-2} dam surface s^{-1} (94 kg FeS m^{-2} $year^{-1}$) [15]. This rate is about 10 times higher than the pyrite oxidation rate for the tailings in Sweden, 10 times higher than the maximum pyrrhotite oxidation rate (determined as in situ O_2 consumption rate) for a pyrrhotite-rich tailings pond in the Sudbury area, Ontario, Canada [12], and about 30 times higher than a previously measured pyrite oxidation rate in an Arctic tailings impoundment in northern Canada [9]. The latter study has shown that in situ O_2 consumption rates and microcalorimetrically determined rates are almost identical.

The different rates determined for different tailings may be explained by differences in molecular oxygen diffusion, temperature, metal sulfide reactivity and abundance of metal sulfide-oxidizing bacteria. The diffusion of molecular oxygen is mainly controlled by the water content. Consequently, variations in pyrite oxidation rates by a factor of 100 depending on the water content at different sampling sites of an Arctic tailings impoundment were found [9]. Temperature differences explained variations in pyrite oxidation rates by a factor of 2 in case of a temperature shift from 10 °C to 20 °C [9]. The reactivity of different metal sulfides varies over a wide range. The chemical pyrrhotite oxidation rate was found to be on the order of 20–100 times those measured for pyrite [41]. Metal sulfide-oxidizing bacteria such as *A. ferrooxidans* have shown to increase the chemical pyrite oxidation rate by a factor of 30 to 300 [4,5].

However, according to the microcalorimetrically determined potential average pyrrhotite oxidation rate of 94 kg FeS m^{-2} dump surface $year^{-1}$, an annual sulfate production of approximately 100 kg m^{-2} dump surface $year^{-1}$ occurs, which means an annual sulfate production of 100,000 t for the whole tailings dam in Botswana. Based on the mean TRIS value, a total sulfate formation potential of 8×10^{6} t could be calculated. Assuming a constant pyrrhotite oxidation rate over time, all pyrrhotite would be oxidized within 80 years [15].

Somewhat lower annual sulfate production rates of 56–66 kg m^{-2} dump surface $year^{-1}$ were determined for the tailings dam in Botswana based on modelling, mineralogical analysis and a humidity cell test [42]. According to these lower rates, all pyrrhotite would be oxidized within 120–140 years (constant rates assumed). The time period of AMD production is critical for the evaluation of remediation measures to be applied

after mine closure. The time period found in this study coincidences with that given in the literature for tailings. For tailings impoundments with low to moderate sulfide contents and shallow water-table positions, the duration of sulfide oxidation can be relatively short, with peak oxidation occurring over the first 20 to 30 years. In sulfide-rich tailings with a deep water table, sulfide oxidation is predicted to continue for centuries in the absence of remedial actions [43].

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