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TECHNICAL ARTICLE

Microbial Abundance in the Schwertmannite Formed in a Mine Water Treatment Plant

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Abstract Treatment of acidic iron- and sulfate-rich mine waters in a pilot plant at the opencast lignite mining pit Nochten (Lusatia, Germany) involves microbial iron oxidation and subsequent precipitation of the iron-oxyhydroxysulfate schwertmannite. To determine if recirculation of schwertmannite can stabilize and optimize the oxidation process, cell number, viability, and diversity of the bacterial community in schwertmannite precipitated on carrier material and stored schwertmannite were analyzed. In schwertmannite on carrier material, the total cell number decreased slightly with increasing mineral depth, whereas the percentage of viable cells decreased significantly. The microbial community, investigated by fluorescence-in-situ-hybridization (FISH) and terminal restriction fragment length polymorphism (T-RFLP), revealed the presence of the iron-oxidizing bacterium "*Ferrovum*" sp. and relatives of *Gallionella*, independent of mineral depth. Analysis of the viability of microorganism in stored schwertmannite indicated an almost constant percentage of viable cells over 42 days.

Keywords Microbial iron oxidation · Live/dead-stain · Microbial community analysis · "*Ferrovum*" · Saxony · Germany

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Introduction

Mining exposes metal ores and sulfide minerals (e.g. pyrite) to oxygen and water. The resultant oxidative dissolution generates acidic iron- and sulfate-rich waters, known as acid mine drainage (AMD), which is often contaminated with other metals and metalloids (Banks et al. 1997; Nordstrom 2000). At the opencast lignite mines in Lusatia (Germany), millions of liters of iron- and sulfate-rich acidic water are formed annually and wastewater treatment is needed to avoid surface- and groundwater contamination. Biological wastewater treatment systems are an interesting alternative to conventional chemical treatment, since the immobilization of iron can be achieved by iron oxidation catalyzed by microorganisms at low pH (Glombitza et al. 2007; Hedrich et al. 2011). Accordingly, less amounts of neutralizing agents are required, which reduces operational costs and sludge volumes. Such an approach is realized in a pilot plant at the open pit Nochten (Lusatia, Germany) (Glombitza et al. 2007). Indigenous bacteria oxidize iron, which subsequently precipitates as schwertmannite (idealized formula: $Fe_8O_8(OH)_6SO_4$) on carrier material within the pilot plant (Hedrich et al. 2011). Since sulfate constitutes a part of schwertmannite, the load of iron and sulfate is lowered; in addition, the precipitated mineral has the potential for further industrial application (Glombitza et al. 2007).

Analyses of the microbial community of the pilot plant using 16S rRNA gene clone libraries revealed a dominance of representatives of *Betaproteobacteria* in the water as well as in the schwertmannite (Heinzel et al. 2009a). The majority of clones of this phylogenetic group belonged to the iron-oxidizing bacteria "*Ferrovum myxofaciens*" (formerly: "*Ferribacter polymyxa*"), while others were related to *Gallionella*. Sequences from other iron-oxidizing

bacteria like *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and *Ferrimicrobium acidiphilum* were only detected in minor percentages. Another significant phylogenetic group was the *Alphaproteobacteria,* from which relatives of *Acidocella* sp., *Acidiphilium* sp., *Sphingomonas* sp., *Caulobacter* sp., and *Caedibacter* sp. were identified (Heinzel et al. 2009a). The occurrence of *Acidocella* sp. and *Acidiphilium* sp. in association with iron-oxidizing bacteria is quite common (Hallmann et al. 1992; Hamamura et al. 2005; Harrison 1984; Johnson et al. 2001; Okabayashi et al. 2005; Schippers et al. 1995). The synergistic interactions between autotrophic iron oxidizers and heterotrophic bacteria are not completely understood, but the iron oxidation rate is increased when both partners are present (Fournier et al. 1998; Marchand and Silverstein 2003).

Since the investigations of Heinzel et al. (2009b) revealed the stability of microbial ferrous iron oxidation against changing parameters like seasonal impacts and different chemical mine water compositions, commercial use of the pilot plant could be considered. However, in contrast to the chemical parameters, the retention time of the water in the plant was a more pivotal process parameter. A decrease from average values of 7–10 h to approximately 4 h resulted in a dramatic decline in the oxidation rate, reportedly due to a washout of the biomass suspended in the oxidation basin (Heinzel et al. 2009b). Therefore, procedural optimization of the pilot plant was necessary. Hedrich and Johnson (2012) described a promising approach using a modular bioremediation system to remove iron selectively from mine waters containing other metals like aluminum, copper, manganese, or zinc by the precipitation of ferric minerals, especially schwertmannite. The designed system contained three different reactors operating successively: (1) catalysis of ferrous iron oxidation at pH around 2 by "*F. myxofaciens*", (2) precipitation of ferric iron at pH 3.5 after the addition of sodium hydroxide and a flocculation agent, and (3) reducing the residual soluble iron concentration in a packed-bed bioreactor inoculated with "*F. myxofaciens*" (Hedrich and Johnson 2012). Another approach to stabilize the oxidation process of a mine water treatment plant is to increase the biomass in the system (Janneck et al. 2010). However, the iron oxidation mechanism of the bacteria detected in the schwertmannite seems not to be influenced by the surrounding ferric mineral, because electron-microscopic investigations showed no direct interactions between the bacterial cell and the mineral-like incrustation of the cell surface or deposition of minerals inside the cell (Hedrich et al. 2011).

Therefore, schwertmannite itself is a reservoir for iron-oxidizing bacteria and recirculation of the mineral phase may potentially improve the process. Since it was unknown how many cells were immobilized in the mineral, we investigated the cell number, the viability of microorganisms, and the composition of the bacterial community in schwertmannite precipitated on a carrier material. Various storage conditions were simulated in the laboratory to investigate how various factors affected the viability of the bacterial cells in stored schwertmannite, which could also be recirculated.

Materials and Methods

Sampling Site

The AMD pilot-scale treatment plant located at the open-cast lignite mining pit Nochten (Lusatia, Germany) was constructed to facilitate microbial ferrous iron oxidation and the precipitation of ferric minerals, and was described in detail by Glombitza et al. (2007) and Heinzel et al. (2009a). The retention time of the water was 10.5 h and the water chemistry varied slightly (Table 1; Fig. 1). In previous investigations, the precipitated minerals had been identified as schwertmannite with traces of jarosite and goethite (Hedrich et al. 2011). Images of the treatment plant as well as of the mineral precipitate can be seen in supplemental Fig. A1. Supplemental figures and tables accompanies the online version of this paper, which can be accessed and downloaded for free by all journal subscribers.

Sampling of Schwertmannite

Schwertmannite was collected in triplicate samples from the carrier material using a sterile hollow drill (diameter: 2.1 cm) for depth profile analysis and using a sterile flask for storage analysis. Sessil® is used as the carrier material in the mine-water treatment plant, which has been shown to be a good material for bacterial attachment (Hedrich et al. 2009). Samples were cooled on ice and immediately

Table 1 Chemical characteristics of the water in the oxidation basin of the pilot plant during precipitation of the ferric mineral; average values for July 2010 are shown

	pH	E_h (mV)	Temp. (°C)	Fe(II) (mg/l)	Fe(III) (mg/l)	SO_4 (mg/l)	PO_4 (mg/l)	NH_4 (mg/l)
July 2010	2.8	295	18	110	90	2,300	0.4	0.2

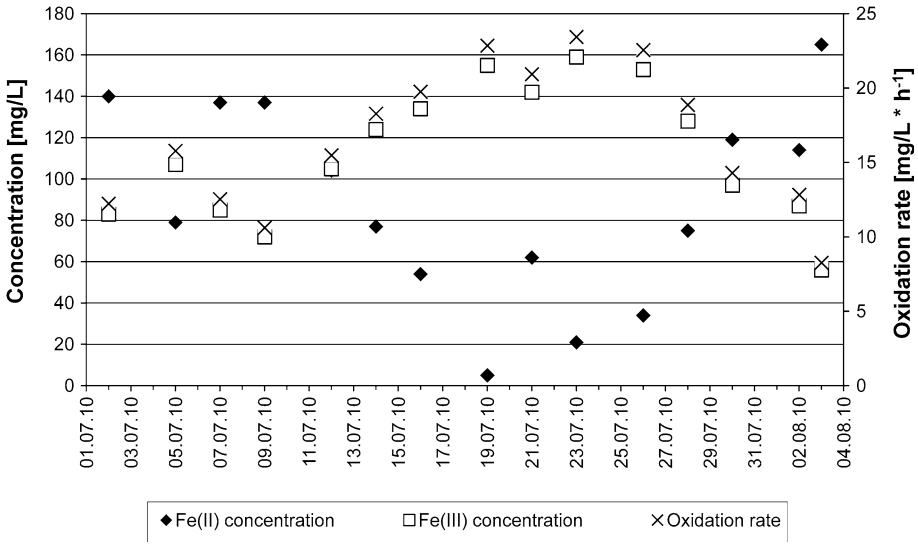

Fig. 1 Development of the ferrous and ferric concentration of the mine water and of the ferrous-iron oxidation rate in July 2010. Schwertmannite precipitated on the carrier material during this period and was sampled on August 3, 2010

transported to the laboratory. There, the core was cut into four layers of 0.5 cm depth each; the 0–0.5 cm layer was the one exposed to the water and the 1.5–2.0 cm layer was next to the carrier material. The bacterial community of each layer was analyzed after dissolution of the mineral phase.

Preparation of Storage Experiment

The schwertmannite was stored in 40 g (wet weight) aliquots under the following conditions: (i) normal atmospheric conditions in the presence of water, (ii) reduced oxygen concentration in the presence of water, and (iii) reduced water content under atmospheric conditions. To reduce the oxygen concentration, the bottles with schwertmannite were flushed with nitrogen for 5 min and subsequently incubated in tightly closed bottles. The minerals in approach (i) and (ii) were covered with 10 mL of sterile filtered pilot plant water. To reduce the water content in approach (iii), the mineral samples were centrifuged at 4,500×*g* for 5 min and the supernatant was removed. All samples were incubated at 20 °C for 42 days. The mineral sample was mixed daily to avoid the formation of nutrient gradients. Samples were taken at the beginning of the experiment and after 5, 8, 15, 21, 32, and 42 days.

Dissolution of the Mineral Phase

The mineral phase of samples was dissolved in an aqueous solution of 0.2 M oxalic acid prior to microbial analyses. The cells were harvested after 2 h incubation at 4 °C by centrifugation at 15,500×*g* for 15 min and washed with phosphate-buffered-saline (PBS) water. The resuspended cells were used for microbiological analysis.

Microbiological Analyses

Cell Count and Fluorescence-in-situ-Hybridization (FISH)

The number of viable and non-viable cells were determined using the LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, L13152), as described by Boulos et al. (1999) and by Invitrogen (LIVE/DEAD® BacLight™ instructions manual). The total cell number was calculated as the sum of viable and non-viable cells.

In PBS resuspended cells were fixed for FISH analyses by mixing the resuspended cells with 4 % paraformaldehyde (1:3, v/v). After incubation at 4 °C overnight, the fixed cells were washed with PBS, resuspended in equal volumes of PBS and absolute ethanol, and stored at −20 °C (Hallberg et al. 2006). The FISH analyses were performed according to Hallberg et al. (2006) with oligonucleotide probes (Eurofins MWG Operon) targeting "*Ferrovum*" sp. (BSC0459_deg) and *Gallionella* relatives (GALTS0048) (Table 2). The cells were counterstained with DAPI. To ensure a correctly working protocol, cells of a "*Ferrovum*"-containing culture or *Escherichia coli* cells containing a cloned 16S rRNA gene of *Gallionella* relatives, respectively, were used as positive controls. Hybridization with both probes revealed a signal for BSC0459_deg in the *Ferrovum*-containing culture and a signal for GALTS0048 for *E. coli* containing the 16S rRNA gene of the *Gallionella* relatives.

Microscopic analyses were performed on diagnostic slides (8 wells, Roth) and visualized by a Nikon Eclipse E1000 epifluorescence microscope. 400–500 cells per well were counted and divided by the number of counted grids to get a mean value of cells per grid.

Table 2 Oligonucleotide probes used for FISH analysis

Probe	Target	Probe sequence (5′ → 3′)	References
BSC0459_deg[a]	*Ferrovum* sp.	TCCAGRTTATTCGCCTGA	Hallberg et al. (2006) modified by S. Hedrich (pers. communication)
GALTS0048[b]	Uncultured *Gallionella* sp. clone TrefC4	CCACTAACCTGGGAGCAA	Hallberg et al. (2006)
GALTS h1[c]	Upstream of GALTS0048	GATATATTACTCACCCGTTCG	Hallberg et al. (2006)
GALTS h2[c]	Downstream of GALTS0048	GCCCCCAGGCCCGTTCGA	Hallberg et al. (2006)

[a] Cy3-labeled; [b] FITC-labeled; [c] Unlabeled helper probes

DNA Preparation and Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

DNA was extracted using the UltraClean® microbial DNA isolation kit (MO BIO, Laboratories, Inc.). The bacterial 16S rRNA gene was amplified in three separate PCR mixtures using the PCR Master Mix (Fermentas), 5 % (v/v) DMSO (Sigma), 0.2 µg/µl bovine serum albumin (Fermentas), 0.24 µM Cy5-labeled 27f (Lane 1991), and 0.24 µM unlabeled 1387r-primer (Marchesi et al. 1998) (Eurofins MWG Operon). The following PCR protocol was applied for T-RFLP analysis: 2 min initial denaturation, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C, and a final incubation step at 72 °C for 5 min. To exclude possible contamination of the PCR reagents, negative controls containing the PCR mixture but no DNA template were run in parallel to each amplification process. PCR products and negative controls were checked using an agarose-TAE (1 %) gel with subsequent DNA-staining by ethidium bromide. The pooled PCR products were purified with Sure-Clean Plus (Bioline) and then 120 ng of purified PCR product was digested with 1 U of the restriction enzymes *Alu*I and *Hae*III (Fermentas) at 37 °C overnight. The digested DNA was separated and analyzed with the Beckman Coulter CEQ8000 genetic analysis system, according to Heinzel et al. (2009b). The TRFs detected were assigned based on a TRF database obtained in former studies of the pilot plant (Heinzel et al. 2009b). TRFs resulting from the digestion with *Alu*I and *Hae*III were averaged. The relative abundance of a TRF signal was calculated according to Hallberg et al. (2006), where the peak area of the respective TRF is divided by the total peak area of the T-RFLP pattern.

Results and Discussion

Availability and Diversity of Cells in Schwertmannite on the Carrier Material

To assess the availability of microbial cells in a depth profile, the core obtained from schwertmannite precipitated on carrier material was cut into four sections and analyzed with the LIVE/DEAD® kit. The total cell number decreased very slightly inside the mineral precipitate, from approximately 1.9×10^5 cells/g (dry weight schwertmannite) in the mineral layer exposed to the water to 1.5×10^5 cells/g in the layer next to the carrier material. This indicates a permanent ferric mineral precipitation with a simultaneous immobilization of the microorganisms and no significant cell loss, for example, by cell lysis (Fig. 2).

However, the number of viable cells decreased significantly inside the mineral precipitate. While 1.4×10^5 cells/g (corresponding to 72 % viable cells) were detected in the 0–0.5 cm layer next to the water, just 7.2×10^4 cells/g (corresponding to 46 % viable cells) were counted in the layer next to the carrier material (Fig. 2). This represents a viability reduction of 26 %. Microscopic images can be seen in supplemental Fig. A2.

Ferris et al. (2004) and Kappler et al. (2005) reported that cells can be incrusted by iron minerals, which may cause a loss of metabolic activity and cell death. Electron-microscopic investigations by Hedrich et al. (2011) could not identify any incrustation of "*F. myxofaciens*" strain

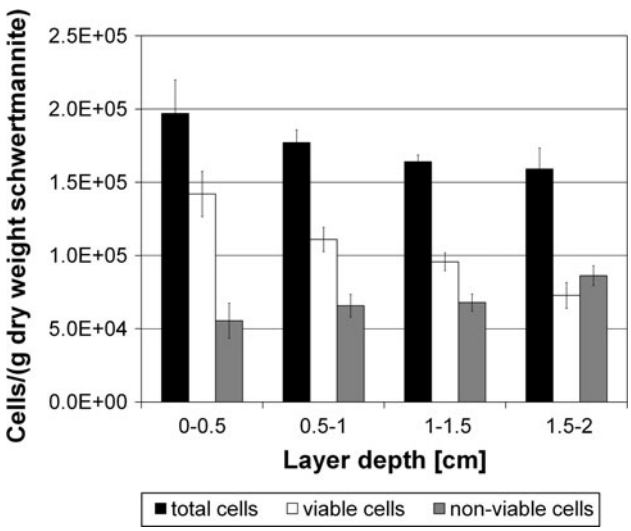

Fig. 2 Numbers of total, viable, and non-viable cells (in triplicate) in various depths of schwertmannite deposits on carrier material determined with the LIVE/DEAD® BacLight™ bacterial viability kit. The total cell number was calculated as the sum of viable and non-viable cells. The 0–0.5 cm layer was exposed to the water, while the 1.5–2 cm layer was located next to the carrier material

EHS6 and cells of the pilot plant by precipitated minerals. Since the iron-oxidizing bacteria derive their energy from the oxidation of the electron donor ferrous iron, a lack of incrustation enables the cells access to ferrous iron (Hedrich et al. 2011). Thus, the ferrous iron concentration probably decreases inside the mineral precipitate due to microbial iron oxidation. Hedrich and Johnson (2012) showed the ability of "*F. myxofaciens*" to continue ferrous iron oxidation in the presence of schwertmannite in a packed-bed reactor. Therefore, it may be assumed that the significant decrease of the viable cells was result of nutrient or oxygen limitation rather than incrustation. Besides ferrous iron, phosphate, which is an essential nutrient for all organisms, may also be a limiting factor, since only traces of phosphates (4×10^{-3} mM) were detected in the treatment plant water. Okido et al. (2008) reported that phosphate is adsorbed by schwertmannite, which further reduces its bioavailability for microorganisms.

The composition of the microbial community in schwertmannite precipitated on the carrier material was determined using FISH and T-RFLP. FISH analyses were prepared with probes targeting relatives of the iron-oxidizing bacteria "*Ferrovum*" sp. and of *Gallionella* (Hallberg et al. 2006). Inside the mineral precipitate 40–50 % of the cells were detected as "*Ferrovum*" sp. and approximately 10 % as relatives of *Gallionella* (Fig. 3; Fig. A3). Nearly 50 % of the cells showed no signals with either of the two probes.

To get more information about these cells, samples were additionally examined by T-RFLP. The results indicated that the microbial community was composed of 25–35 % "*Ferrovum*" sp. and 15–20 % relatives of *Gallionella*, independent of the mineral depth (Fig. 4). In previous studies using 16S rRNA gene clone libraries of Heinzel et al. (2009a), "*F. myxofaciens*" (67 %) dominated the microbial community in the precipitated schwertmannite as well. However, relatives of *Gallionella* could not be detected in the mineral, which could be due to the low percentage of *Gallionella* relatives (2 %) in the water (Heinzel et al. 2009a). 13 % *Gallionella* relatives could be observed in the pilot plant water, when the samples for the herein described analysis was taken (data not shown). This indicates that the microbial community found in the schwertmannite may reflect the microbial community of the pilot plant water during mineral precipitation.

The remaining 50 % consisted of various bacteria, most of them belonging to the *Alphaproteobacteria* (*Acidiphilium* sp., *Acidocella* sp., *Caedibacter* sp., *Sphingomonas* sp., *Caulobacter* sp.) or the *Betaproteobacteria* (*Oxalobacter* sp., *Delftia* sp.), which seem to be irregularly distributed through the mineral layers (Fig. A4). The species detected were previously observed in the schwertmannite precipitated in the treatment plant (Heinzel et al. 2009a). The bacteria identified are characteristic for such acid mine waters and were detected by other researcher as well (Gonzalez-Toril et al. 2011; Hallberg et al. 2006; Hamamura et al. 2005; Okabayashi et al. 2005).

Although the distribution of the relative abundance of "*Ferrovum*" sp. and relatives of *Gallionella* was determined with two comparable methods, there were differences due to methodical discrepancies. In contrast to FISH, T-RFLP is a PCR-based technique. Thus, PCR biases, 16S

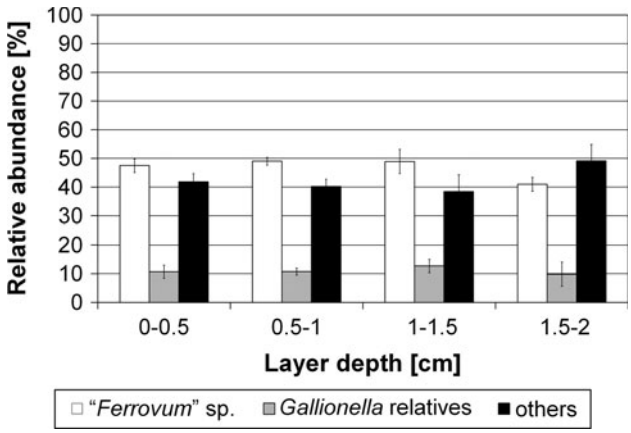

Fig. 3 The relative abundance of *Ferrovum* sp. and relative of *Gallionella* was determined by FISH in triplicate at various depths of a schwertmannite deposit on carrier material. Samples were analyzed with rRNA-probes targeting *Ferrovum* sp. (BSC0459_deg) and the uncultured *Gallionella* sp. clone TrefC4 (GALTS0048). The cells were counter-stained with DAPI. The 0–0.5 cm layer was exposed to the water, while the 1.5–2 cm layer was located next to the carrier material

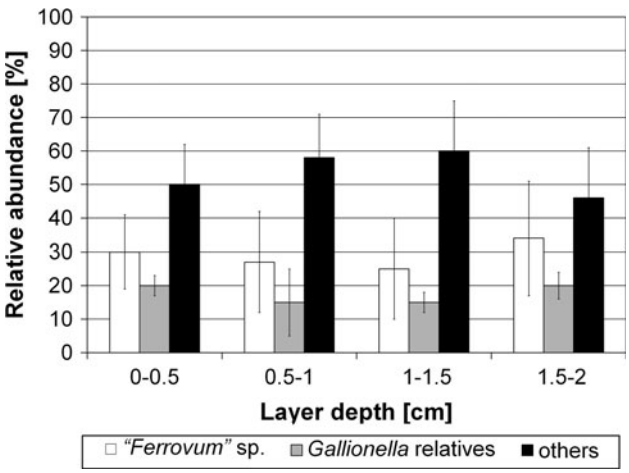

Fig. 4 The relative abundance of relatives of *Ferrovum* sp. and of *Gallionella* was determined by T-RFLP in various depths of schwertmannite deposit on carrier material, using the restriction enzymes *Alu*I and *Hae*III. Besides *Ferrovum* sp. and relatives of *Gallionella Caulobacter* sp., *Oxalobacter* sp., *Shingomonas* sp., *Acidiphilium* sp., and *Acidocella* sp. were present in low percentages (pooled in others). The 0–0.5 cm layer was exposed to the water, while the 1.5–2 cm layer was located next to the carrier material. The mean value of two mineral samples and the standard derivation are shown

rRNA gene heterogeneities, and the GC content may have influenced the PCR (v. Wintzingerode et al. 1997). For example, during the formation of the PCR artifacts, chimeric molecules and mutations could have caused incomplete or incorrect enzyme digestion. Consequently, the relative abundance determined by T-RFLP is prone to errors. At the same time, the permeability of the bacterial cell walls and the accessibility of the target region are important for FISH analysis (Fuchs et al. 2000). Using unlabeled helper probes according to Hallberg et al. (2006), an effort was made to minimize the difficult accessibility of the 16S rRNA gene of *Gallionella* sp.

Storage of Schwertmannite

Conditions appearing in stored or transported schwertmannite were simulated in the laboratory to investigate their influence on the viability of the bacterial cells in the schwertmannite using the LIVE/DEAD® kit. Expected conditions were: i) normal atmospheric conditions in the presence of water, ii) reduced oxygen concentration in the presence of water, and iii) reduced water content under atmospheric conditions. The analyses revealed a variation of the total cell number between 2×10^5 and 6×10^5 cells/g over 42 days, indicating no significant cell loss by cell lysis (Fig. 5). However, the great standard deviations indicated an inhomogeneous distribution of the cells within the ferric mineral. Thus, it seems to be difficult to get a representative sample. The percentage of viable cells under the various conditions did not differ significantly. Over the incubation time, the percentage of viable cells varied in all three approaches between 30 and 55 % (Fig. 5). The detection of viable cells indicated the presence of sufficient nutrients for the microbial survival.

The distribution indicates that the bacteria present can sustain themselves under various conditions. Presumably, the residual ferrous iron is being oxidized by the "*Ferrovum*" sp. and *Gallionella* relatives. The ferric iron formed could be reduced by *A. ferrooxidans* and *Acidiphilium* sp. under aerobic and anaerobic conditions if an electron donor was present (Brock and Gustafson 1976; Johnson and McGinness 1991; Pronk and Johnson 1992). All mentioned species were detected in the schwertmannite of the pilot plant (Heinzel et al. 2009a). The iron-oxidizing bacteria can also deal with the reduced oxygen concentration. While *G. ferruginea* is known as a microaerophilic iron-oxidizing bacterium, *A. ferrooxidans* also grows under anaerobic conditions by gaining energy by ferric iron reduction (Hanert 1968; Pronk and Johnson 1992). Thus, a closed iron cycle may be present inside the mineral precipitate. Due to the known production of extracellular polymeric substances (EPS) by "*F. myxofaciens*" (Hallberg et al. 2006; Kimura et al. 2011), it is possible that the EPS produced serves as an electron donor for the heterotrophic and possibly the ferric iron reducing bacteria and oxygen as an electron acceptor for the ferrous iron oxidizers.

Impact on the Procedure of the Mine Water Treatment Plant

In previous studies concerning the recirculation of schwertmannite in the mine water treatment plant, the oxidation

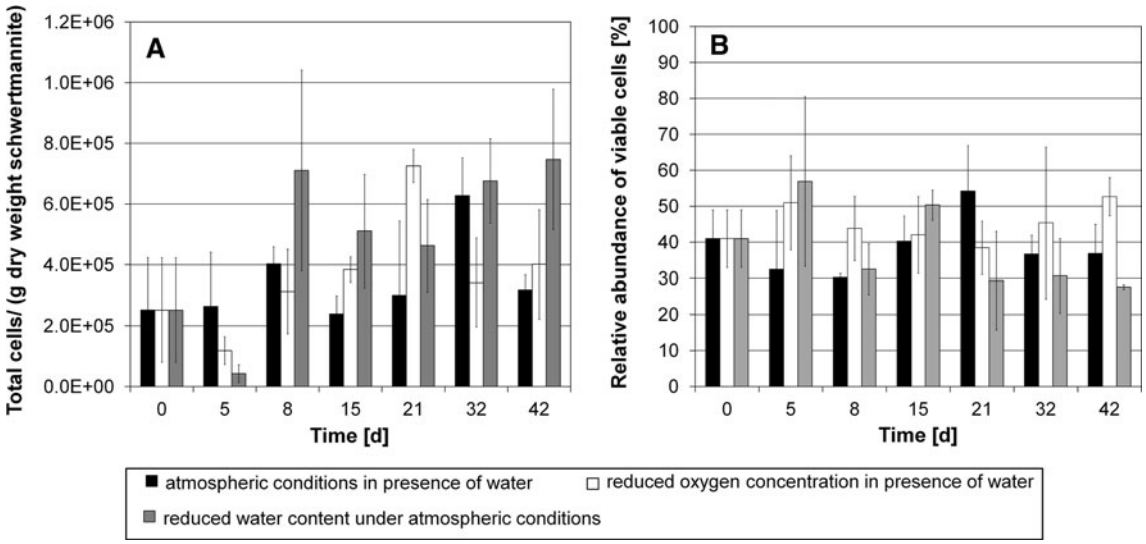

Fig. 5 Number of total cells (**a**) and the relative abundance of viable cells (**b**) in stored schwertmannite incubated under various conditions (atmospheric conditions in the presence of water, reduced oxygen concentration in presence of water, and reduced water content under atmospheric conditions) over 42 days. The determination of viable and non-viable cells was performed in triplicate with the LIVE/DEAD® BacLight™ bacterial viability kit. The total cell number was calculated as the sum of viable and non-viable cells

rate increased significantly when schwertmannite was constantly recycled and when the pilot plant was completely loaded with carrier material (Janneck et al. 2010). These investigations indicated the presence of viable iron-oxidizing bacteria in precipitated schwertmannite, which was confirmed in this study by the detection of viable cells in schwertmannite precipitated on carrier material in all mineral layers as well as in schwertmannite stored over 42 days. However, an increased oxidation rate by schwertmannite recirculation was not reproducible in the studies of Janneck et al. (2010), possibly due to recycling of material containing less viable cells. To counteract this effect, a recirculation of the outer and therefore freshest schwertmannite layer precipitated on carrier material would have the greatest potential to increase the biomass in the pilot plant, since the highest viability (72 %) was observed in this layer and, depending on the method, "*Ferrovum*" sp. (FISH: 50 %, T-RFLP: 30 %) and *Gallionella* relatives (FISH: 10 %, T-RFLP: 17 %) were detected. Furthermore, pre-investigations of the viability of the cells and the composition of the microbial community in the schwertmannite used for the recirculation enable a more specific selection of ferric mineral material preferably containing a high percentage of viable iron-oxidizing bacteria. The previous experiences of Janneck et al. (2010) regarding the recirculation of schwertmannite and the amount of carrier material combined with the results obtained in this study could lead to an efficient stabilization and optimization of the oxidation rate of the mine water treatment plant, which will have to be investigated in future tests.

Conclusion

Common fluorescent microscopic and molecular biological methods were used to detect bacteria inside the ferric minerals, allowing rapid examination of the number of viable cells and the composition of the microbial community in the mineral precipitate. In the present study, viable cells could be detected in the mineral precipitated on carrier material in all layer depths and even in schwertmannite stored for 42 days. The highest viability (72 %) was observed in the outer and therefore freshest schwertmannite layer on the carrier material, where, depending on the method, the iron-oxidizing "*Ferrovum*" sp. (FISH: 50 %, T-RFLP: 30 %) and *Gallionella* relatives (FISH: 10 %, T-RFLP: 17 %) were detected. The possibility of detecting bacteria in ferric minerals could allow selective recirculation of schwertmannite with high percentages of viable iron-oxidizing bacteria, which would potentially maintain a stable, optimized oxidation capacity, if process engineering aspects like shrinkage of available volume do not argue against that.

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