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Microbial iron reduction during passive in situ remediation of an acidic mine pit lake mesocosm

Katrin Wendt-Potthoff*, Elke Bozau, René Frömmichen [1], Jutta Meier, Matthias Koschorreck

UFZ Helmholtz Centre for Environmental Research, Brückstraße 3a, D-39114 Magdeburg, Germany

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ABSTRACT

Ferric iron reduction was studied in a pilot-scale enclosure experiment for passive biological remediation of an acidic mine pit lake in Lusatia, Germany. The metabolic properties of prokaryotes involved in Fe(III) reduction may be important for the outcome of biological remediation, as chemolithotrophic Fe(III) reduction can counteract the desired pH increase, but heterotrophic Fe(III) reduction will provide the necessary Fe(II) for precipitation of sulfide minerals following sulfate reduction. Therefore, vertical profiles of sediment parameters related to iron and sulfur cycling were determined in conjunction with viable counts of different ferric iron-reducing micro-organisms using selective media. Findings were compared to an untreated reference site. The addition of organic matter stimulated ferric iron reduction and sulfate reduction in the enclosure and led to elevated pH and accumulations of ferrous iron and reduced sulfur compounds. Numbers of neutrophilic heterotrophic Fe(III) reducers increased during treatment, those of acidophilic heterotrophic Fe(III) reducers remained similar, and those of acidophilic chemolithotrophic Fe(III) reducers decreased. Zones of ferric iron-reducing activity corresponded well with microbial depth profiles; however, viable counts of neutrophilic or acid-tolerant Fe(III) reducers must have been underestimated based on the corresponding observed activity levels. Ferric iron reduction by chemolithotrophic acidophiles seemed to be of minor importance, so a lowering of pH values due to Fe(III) reducing activity is unlikely.

Introduction

In many parts of the world, acidic pit lakes have formed and continue to develop as a result of opencast lignite mining, or existing lakes are acidified by inflowing acid mine drainage, due to pyrite oxidation in the dumps and tailings (Evangelou, 1995). This results in water bodies with pH values below 4 and high sulfate and iron concentrations, often accompanied by increased contents of other metals. Due to extreme chemistry, biological productivity in these lakes is low (Lessmann and Nixdorf, 2002). In cases where acidification of adjacent rivers or aquifers becomes an environmental problem or where the use of the lake for fishery and recreation is planned, the lake has to be treated in a way that acidity is neutralized and iron and sulfate concentrations are lowered to appropriate levels. Since chemical neutralization is costly and has to be repeated frequently due to continuing acidity supply, remediation strategies based on stimulation of microbial sulfate and iron reduction by organic carbon addition are being tested. The process can be summarized using the equation of Frömmichen et al. (2004):

$$15\langle CH_2O\rangle + 6FeOOH + 7SO_4^{2-} + 14H^+ \rightarrow 15CO_2 + 6FeS + S^0 + 25H_2O$$

In order to revert pyrite oxidation, sulfide minerals have to be regenerated, making sulfate reduction and Fe(III) reduction key processes for biological neutralization. Alkalinity generation by Fe(III) reduction has been demonstrated in wetlands for acid mine drainage treatment (Vile and Wieder, 1993). Ferric iron reduction is also important with respect to establishment of reducing conditions and inhibition of further pyrite oxidation by exhaustion of Fe(III). When ferric solids are reduced, acidity is consumed (Bilgin et al., 2005), and by precipitation of non-sulfidic Fe(II) minerals (e.g. carbonates, phosphates) this effect is stabilized and re-oxidation of Fe(II) in the oxic watercolumn is prevented. Whereas sulfate reduction may be inhibited by low pH, ferric iron reduction is well known to take place under acidic conditions, and rates may exceed those measured in more neutral freshwater habitats or paddy soils (Frenzel et al., 1999; Küsel, 2003a; Roden and Wetzel, 1996). Ferric iron reduction may create conditions feasible for sulfate reduction, but at the same time both processes compete for electron donors.

Bacteria able to reduce ferric iron are phylogenetically and physiologically highly diverse, and a variety of mesophilic micro-organisms may contribute to iron reduction in pit lake sediments.

* Corresponding author: Tel.: +49 391 8109810; fax: +49 341 459810.
E-mail address: katrin.wendt-potthoff@ufz.de (K. Wendt-Potthoff).
[1] Present address: Trinkwasserversorgung Magdeburg GmbH, Herrenkrugstraße 140, D-39114 Magdeburg, Germany.

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The most well-known iron reducers belong to the *Geobacteraceae*. These bacteria are generally neutrophilic, obligate anaerobes and utilize acetate and other organic acids, alcohols, some aromatic compounds or molecular hydrogen as electron donors (Lovley, 2000). Their presently known lower pH limit for Fe(III) reduction is pH 5.5 where activity is already low (Cummings et al., 2000). They have been detected in DNA extracted from mine pit lake sediments by polymerase chain reaction (PCR) with specific primers, especially in zones of elevated pH (Blöthe et al., 2008), but their overall relevance in these habitats is still unclear. A second group of well-studied neutrophilic ferric iron reducers comprises the facultative anaerobes of the genus *Shewanella*. They have a more restricted spectrum of electron donors, utilizing hydrogen, some sugars and organic acids (Lovley, 2000). To date no findings about these organisms in mining environments have been reported, and attempts to detect them either by PCR with a specific primer set (Blöthe et al., 2008) or by plating sediment suspensions on triple sugar iron agar (Wendt-Potthoff, unpublished) were unsuccessful. Considering the fact that many *Shewanella* sp. were isolated from marine environments and require seawater salinity (Bowman et al., 1997), it is unlikely that they play an important role here. Another group of Fe(III)-reducing bacteria that might be important in mining-impacted freshwater habitats is the genus *Ferribacterium*, which comprises strict anaerobes that utilize organic acids (Cummings et al., 1999).

There are also two groups of obligately acidophilic bacteria that reduce ferric iron: Firstly, facultatively anaerobic heterotrophs of the genus *Acidiphilium* have been found to be highly relevant Fe(III) reducers in mining environments (Küsel, 2003a; Rowe et al., 2007). Their pH range for iron reduction is 3–5, and they utilize glucose and other sugars, ethanol and hydrogen as electron donors, but are inhibited by acetate and other organic acids. Interestingly, they can carry out ferric iron reduction under microaerobic conditions (Johnson and McGinness, 1991; Küsel, 2003a). Secondly, *Acidithiobacillus ferrooxidans*, an autotrophic organism well-known for ferrous iron and pyrite oxidation, is also capable of ferric iron reduction under anoxic conditions, provided elemental sulfur is available as electron donor. The pH range is pH 1.3–4.5 for this organism. Ferric iron reduction by *A. ferrooxidans* is an unwanted reaction with regard to biological neutralization, since the pH decreases during this process (Pronk et al., 1992):

$$S^0 + 6Fe^{3+} + 4H_2O \rightarrow H_2SO_4 + 6Fe^{2+} + 6H^+$$

Few studies exist on the passive treatment of acidic mine pit lakes using microbial processes (e.g. Brugam et al., 1995; Brugam and Stahl, 2000; Koschorreck et al., 2002; Martin et al., 2003), and not much is known about the microbiology of ferric iron reduction under such conditions (Wendt-Potthoff et al., 2002; Meier et al., 2005). It is an open question, which are the physiological properties (such as pH-preferences or electron donors) of iron reducers in a sedimentary habitat with shifting pH values and electron donor composition like an acidic pit lake sediment under organic carbon amendment. In order to find out which physiological types are competitive under certain conditions and what the consequences for biological alkalinity generation might be, we studied ferric iron reduction in treated and untreated pit lake sediments using a combination of process-oriented parameters and selective cultivation. The media composition reflected the pH and electron donor requirements of the above-mentioned bacterial groups. The importance of these three groups was further investigated by means of microcosms amended with the main electron donors in a separate study (Porsch et al., 2009).

The acidic mine pit lake (ML) 111 was developed in 1963. It is a shallow lake consisting of three subbasins with a mean depth of 4.5 m and a maximum depth of 10.2 m (Büttner et al., 1998). Concentrations of sulfate and dissolved iron in the water body are 1300 and 150 mg l^{-1}, respectively, and the pH value is 2.7. It was chosen as a model lake because it is highly acidified and does not possess surface inflows or outflows. Sediment and water of ML 111 have been tested in a series of upscaling experiments to select appropriate carbon amendments to stimulate biological neutralization via ferric iron and sulfate reduction (Frömmichen et al., 2003, 2004). Carbokalk, a side product from the sugar industry, has been found to be most suitable. It is a dried carbonation mud, formed after lime clarification of raw sugarbeet juice, and it contains the non-sugar compounds of the beets and approximately 50% is lime. The Carbokalk is combined with wheat straw, which acts as long-term carbon and nutrient source and as a physical barrier to slow down mixing and oxygenation near the sediment–water interface (Koschorreck et al., 2002). The substrates were placed on the sediment surface to create a passive treatment system. In July 2001, a pilot-scale enclosure experiment was started. An enclosure with a diameter of 30 m was installed in the north basin at a water depth of 6.5 m. The enclosure holds 4500 m^3 of water, corresponding to nearly 1% of total lake volume. It was amended with 4.2 tons of Carbokalk (5.95 kg m^{-2}) and 6 tons of wheat straw (8.5 kg m^{-2}) in the form of bales, which were pinned down with iron scrap. The untreated north basin sediment near the enclosure served as reference site.

At the time of our study, conspicuous geochemical changes had taken place in the sediment as a consequence of substrate supply. Within few days after substrate addition, the pH increased to >5 at the sediment surface due to Carbokalk dissolution. The zone of elevated pH extended to deeper sediment layers in the following months, and ferrous iron and reduced sulfur species accumulated, indicating microbial Fe(III) and sulfate reduction (Koschorreck et al., 2007). However, rate measurements and porewater profiles of Fe(II) revealed that Fe(III) reduction exceeded sulfate reduction (Koschorreck et al., 2007), as found in a previous shorter and smaller field experiment with Carbokalk alone, where re-acidification occurred after one summer (Wendt-Potthoff et al., 2002). As a consequence, not all the Fe(II) produced could be fixed in the sediment in the form of minerals, and dissolved Fe^{2+} diffused out of the sediment into the oxic watercolumn where it was reoxidized, hydrolyzed and precipitated. By this process, an acidic layer of fresh sediment with high ferric iron content developed on top of the treated black sediment (Fig. 1). With regard to biological remediation, part of the added

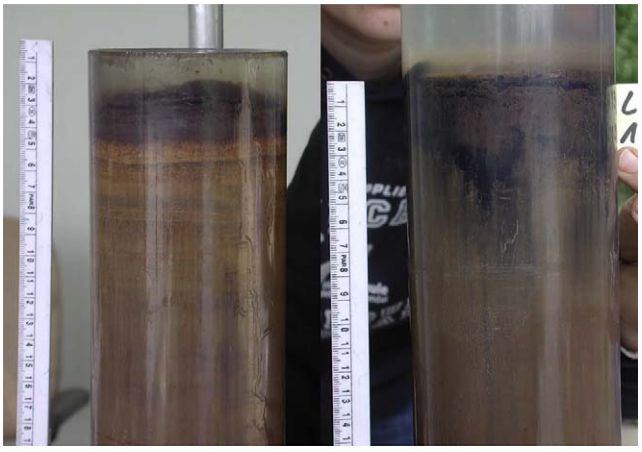

Fig. 1. Sediment cores from the untreated lake (left) and the enclosure (right) in May 2003.

electron donor was wasted without generating alkalinity. To counteract the imbalance between Fe(III) reduction and sulfate reduction, more detailed understanding that would lead to strategies allowing either more intense stimulation of sulfate reduction or a selective limitation of ferric iron reduction would be desirable. For the latter, a more detailed knowledge about the physiology of the Fe(III)-reducing micro-organisms is important. Due to their high phylogenetic diversity, a nucleic-acid based investigation is insufficient to characterize the iron-reducing microbial community, since the main protagonists may be unknown and overlooked by this approach.

Methods

In May 2003, a sampling campaign focusing on depth-oriented investigation of parameters related to iron and sulfur cycling was performed. Sediments were sampled by gravity coring, and pH was measured directly in sediment slices using conventional glass electrodes. Samples for total reduced inorganic sulfur (TRIS) determination were immediately frozen in the vapour of liquid nitrogen. In the laboratory, acid-volatile sulfur (AVS, e.g. monosulfides), chromium-reducible sulfur (CRS, e.g. pyrite) and elemental sulfur were determined polarographically according to Frömmichen et al. (2004). Porewater was obtained by centrifugation under an oxygen-free atmosphere and processed as described in Herzsprung et al. (2006). Iron in sediments (further referred to as reactive Fe) was determined in triplicate following a modification of Lovley and Phillips' (1987) protocol. Briefly, Fe(II) was extracted using 0.5 M HCl for 1 h, and hydroxylamine-reducible Fe was extracted for the same time in 0.25 M hydroxylamine hydrochloride in 0.5 M HCl (instead of 0.25 M HCl). Aliqots were transferred to ferrozine solution and clarified by centrifugation (16,000*g*) before photometry. Fe(III) concentrations were calculated as the difference between hydroxylamine-reducible Fe and Fe(II) in HCl extracts. Determinations of total microbial biomass and viable counts of Fe(III)-reducing bacteria as Most Probable Number (MPN) serial dilutions were performed as outlined in Wendt-Potthoff et al. (2002). The medium for *A. ferrooxidans*-like organisms (FeOB) had a pH of 1.6–1.8 and contained 80 mM $FeSO_4$. The medium for neutrophilic iron reducers (FeRB) was a modified medium for *Geobacter metallireducens* (DSM medium no. 579, www.dsmz.de) with a pH of 6.0, containing 50 mM ferric citrate, 10 mM sodium acetate, 10 mM ethanol, and 0.05 g l^{-1} yeast extract. For counting acidophilic iron reducers (acFeRB), the medium of Küsel et al. (1999) was used, which contained 35 mM ferric sulfate, 0.25 g l^{-1} tryptic soy broth (CASO bouillon, Merck, Darmstadt, Germany), 5 mM glucose and had a pH of 2.3. MPN enrichments were incubated at 20 °C in the dark for 6 weeks. To allow comparison with viable counts, lipid phosphate concentrations (biomass) were converted to cells ml^{-1} using the conversion factor of Balkwill et al. (1988). The variability of lipid phosphate concentrations was determined at the beginning of the enclosure experiment. From both untreated lake and enclosure sediments at three different depths, 14–16 samples each were analyzed, yielding relative standard deviations between 5% and 10% (Wendt-Potthoff, unpublished). Fe(III) reduction was measured as Fe(II) accumulation in closed vessels (batch assays) at in situ temperature (5 °C) and synthetic Fe(III) was added, so a potential rate and not a true in situ rate was determined (Wendt-Potthoff et al., 2002). Sulfate reduction rates were determined at 5 °C using ^{35}S sulfate core injection and passive diffusion (Meier et al., 2000).

Results and discussion

Compared to the sediments of the lake, in the treated enclosure pH values were elevated in the upper 12 cm, and microbial biomass, expressed as prokaryotic cell numbers, was roughly twice as high (Fig. 2a and b). Whereas reactive Fe in the untreated sediment consisted mostly of Fe(III), reactive Fe in the enclosure sediment was mainly Fe(II) with a maximum at 3.5 cm depth (Fig. 3), and the total reactive Fe concentration had roughly doubled. Dissolved ferrous iron was also higher in the enclosure sediment (Fig. 4a), and the profile indicated a diffusive flux of Fe^{2+} out of the sediment. However, dissolved Fe^{2+} accounted only for a fraction (6–23%) of reactive Fe(II), especially in the zone of elevated pH, showing the presence of ferrous minerals. Total reduced inorganic sulfur (TRIS) as a product of microbial sulfate reduction was only found in the upper cm of the enclosure sediment, above the Fe(II) maximum. In contrast to the experiment with Carbokalk alone (Wendt-Potthoff et al., 2002), the TRIS containing zone reached deeper into the sediment, and most of it was in the form of AVS, but CRS and elemental sulfur were also detected (Fig. 3). The shift towards AVS (monosulfides) corresponds to the roughly two-fold higher concentrations of HCl-soluble Fe(II) concentrations in this experiment compared to the Carbokalk-alone treatment. TRIS in the untreated lake sediment was close to zero in May 2002 (Fig. 3), and such values have been obtained before (Meier et al., 2004; 7 m site; Wendt-Potthoff et al., 2002; start of experiment), so there is little change with time. Therefore, the low reduced sulfur concentrations in the top layer of the untreated sediment in May 2003 (0.45, 7.12 and 2.84 μmol ml^{-1} for AVS, CRS and S^0, respectively) are background values due to erosion at the adjacent dumps and transport of eroded material within the lake.

At the sediment surface, potential microbial Fe(III) reduction was equal in the lake and enclosure, being at the high end of the range reported by Küsel (2003a). Below, potential Fe(III) reduction was up to six-fold higher in the enclosure, the maximum corresponding to the reactive Fe(II) maximum (Fig. 4b). Sulfate reduction (Fig. 4c) was only detected in the upper part of the enclosure sediment, corresponding with the AVS profile. Assuming the Fe(II) profile of the untreated lake corresponds to the intital state of the enclosure sediment, a net in situ Fe(II) production rate can be calculated from the two profiles by relating the differences in Fe(II) concentrations to the duration of the experiment. This gives net Fe(III) reduction rates of 25–149 nmol ml^{-1} d^{-1}, which are in the same order of magnitude as the sulfate reduction. However, as the rates are consistently higher and the Fe(III) reduction zone has a higher depth extension, area-related Fe(III) reduction still exceeded sulfate reduction. Fe(III) reduction rates obtained by closed vessel incubation of mine pit lake sediments without addition of Fe(III) (595 and 1405 nmol ml^{-1} d^{-1} for pH 3 and pH 5 sediments, Blöthe et al., 2008) lay between the potential and net rates observed in this study.

Bacterial viable counts (Fig. 5) in lake and enclosure were different. Counts in the enclosure sediment approximate those of the experiment with Carbokalk alone before re-oxidation occurred, although this experiment lasted for almost 2 years at the time of this sampling. Numbers of neutrophilic, acetate-utilizing Fe(III) reducers at pH 6 (FeRB) in the enclosure sediment were 10–100-fold higher at all depths in comparison to those of the lake, especially in the upper 4 cm. This agrees quite well with the zone of pH ≥ 6, their presumed habitat. Their presence in the other, more acidic layers indicates that some of these organisms might be acid-tolerant. Numbers of acFeRB were more similar between untreated lake and enclosure, except at 2 cm depth where they were roughly ten-fold higher in the enclosure.

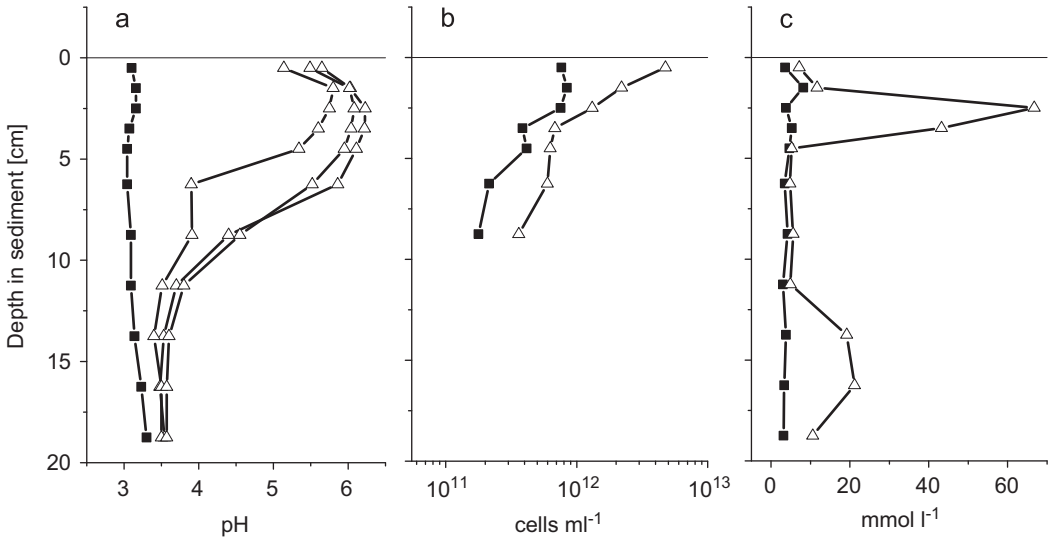

Fig. 2. Vertical profiles of pH (a), viable microbial biomass measured as lipid phosphate and converted to cells ml^{-1} (b) and porewater dissolved organic carbon concentrations (c) in untreated lake sediment (filled symbols) and enclosure sediment (open symbols). Three replicate pH profiles are shown for the enclosure sediment to illustrate its heterogeneity. Biomass values are means of duplicate determinations, and a relative standard deviation of 5–10% can be assumed (see Methods section).

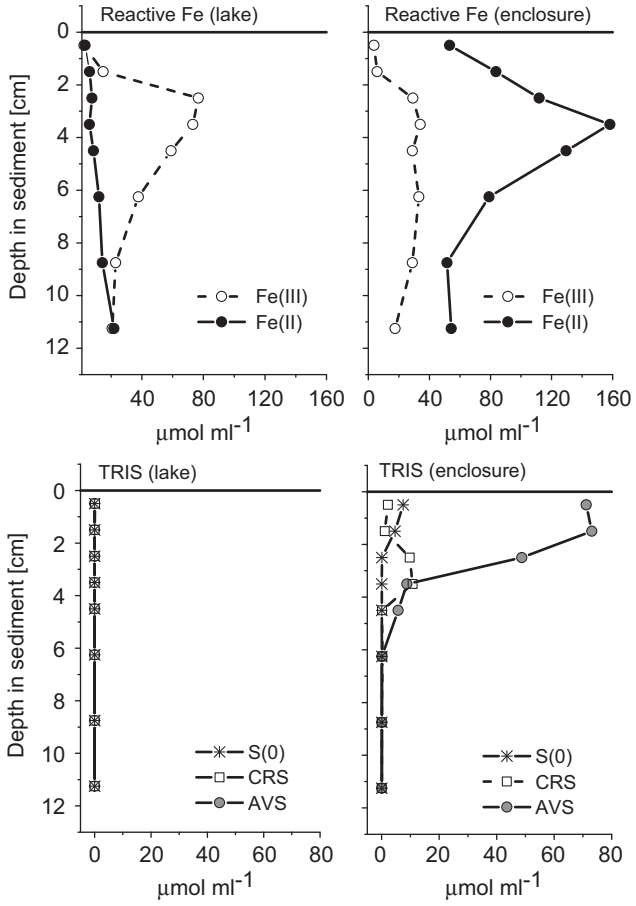

Fig. 3. Profiles of reactive iron in the lake and enclosure sediment (upper left and right), reduced inorganic sulfur species in the lake and enclosure sediment (lower left and right). S(0) elemental, CRS chromium-reducible, and AVS acid-volatile sulfur. Note that TRIS in the lake is from May 2002, as only surface layer values are available for May 2003. All symbols are means of triplicates.

According to the pH profile, their zones of activity should be in the upper cm and below 7 cm, as long as other factors (e.g. availability of carbon sources) would not become limiting. *Acidithiobacillus*-like organisms (FeOB) showed identical abundance for untreated lake and enclosure at the sediment surface, but below the numbers were significantly lower in the enclosure. This can be interpreted as a reaction towards elevated pH and dissolved organic carbon concentrations (Fig. 2a and c), which are inhibitory for these bacteria. The surface layer was also the only part of the enclosure sediment where sulfur-driven iron-reducing activity of *Acidithiobacillus*-like organisms was possible, since in this zone an appropriate pH value and elemental sulfur occurred simultaneously. As this zone contains free H_2S (Koschorreck et al., 2007), there is little possibility of oxic respiration. At the same time, hydrogen (which is likely present in an organics-amended sediment) may have acted as electron donor for Fe(III) reduction ($H_2 + 2Fe^{3+} \rightarrow 2\,H^+ + 2Fe^{2+}$). Fe(III) reduction with hydrogen has been demonstrated both for *Acidiphilium* sp. (Küsel et al., 1999) and for *Acidithiobacillus* sp. (Ohmura et al., 2002), but their competitive abilities for this process are unknown. So, in the surface layer, there is competition between sulfur-driven, hydrogen-driven and heterotrophic Fe(III) reduction by acidophiles, as it can be expected in the surface layer of the untreated lake sediment, since potential Fe(III) reduction and numbers of acidophilic Fe(III) reducers are identical there. However, since this surface layer is thin and potential Fe(III) reduction rates were much higher in deeper layers of the enclosure sediment, the acid-generating ferric iron reduction by *Acidithiobacillus*-like organisms was unlikely to counteract alkalinity generation at the time of our study.

In the untreated lake, the maximum of Fe(III) reduction coincided with the maxima of microbial numbers, both being located near the sediment surface. In that upper cm, Fe(III)-reducing activity and viable counts of Fe(III) reducers were the same in the enclosure sediment, except for neutrophilic FeRB. Numbers of this group were significantly greater in the enclosure compared to the untreated lake sediment, i.e. 95% confidence limits did not overlap. However, FeRB numbers were still lower than those of the other groups and probably the organisms growing in this medium contributed less to the measured activity. This has been demonstrated by Meier et al. (2005) who calculated cell-specific Fe(III) reduction rates for MPN enrichments of FeRB and found them unrealistically high compared with activities of pure cultures. Moreover, in the enclosure the maximum of potential Fe(III) reduction was at 3.5 cm depth, where none

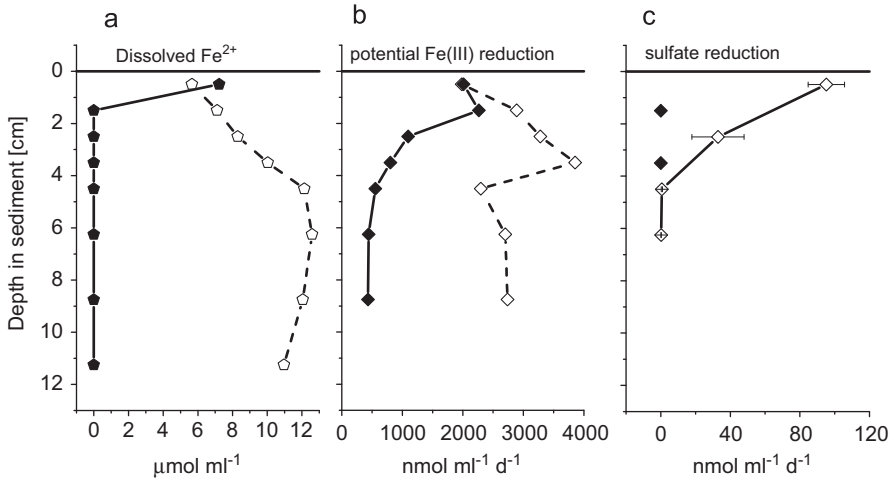

Fig. 4. Profiles of dissolved Fe(II) (a), potential ferric iron reduction, means of duplicates (b), and gross sulfate reduction (c) in the untreated lake sediment (filled symbols) and enclosure sediments (open symbols). Error bars indicate standard deviation of triplicates. Dissolved Fe(II) concentrations refer to ml sediment, not to ml porewater.

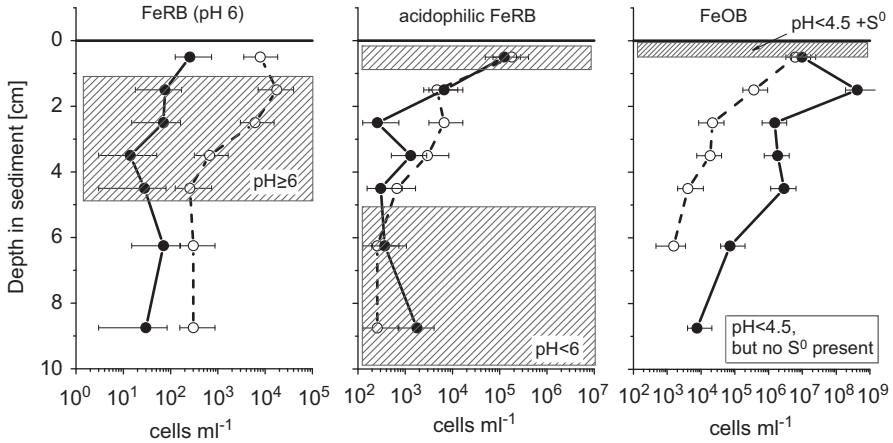

Fig. 5. Most Probable Numbers of neutrophilic (FeRB) and acidophilic (acFeRB) heterotrophic Fe(III)-reducing bacteria and of acidophilic ferrous iron oxidizing (*Acidithiobacillus*-like) bacteria (FeOB) in the untreated lake sediment (filled symbols) and enclosure sediments (open symbols). Error bars indicate 95% confidence intervals. The shaded areas mark zones in the enclosure sediment with certain pH ranges (and S^0 in the case of FeOB) where the organisms are supposed to be active.

of the cultivated bacterial groups showed a maximum. Abiotic Fe(III) reduction by H_2S is unlikely here, as AVS and S^0 concentrations as well as sulfate reduction rates were low. An especially strong artifact due to Fe(III) addition in the Fe(III) reduction assay of this layer is also unlikely, as the in situ concentrations of reactive Fe(III) were similarly low between 2 and 9 cm depth (Fig. 3) and the same amount of Fe(III) was added to all assays. Either microbial abundance and activity were not correlated in this sediment or the activity was predominantly mediated by micro-organisms not detected with our culture-based approach. The limitations of selective cultivation are well known, and our selective counts for Fe(III)-reducing prokaryotes made up less than 1% of the estimated total cell count, although Fe(III) reduction is an important electron transport process in the anoxic part of these sediments (Figs. 3 and 4b; Koschoreck et al., 2007), and nitrate and manganese are only present in very low concentrations (Wendt-Potthoff et al., 2002). Some sulfate- and sulfur-reducing bacteria may also reduce Fe(III) (Tebo and Obraztsova, 1998; Roden and Lovley, 1993), and viable counts of sulfate-reducing bacteria are high in the enclosure sediment (around 10^7 cells ml^{-1} in the upper 2.5 cm; Meier et al., 2005). However, not all of the presumptive Fe(III) reducers among them may grow well in the FeRB medium, e.g. citrate has an inhibitory effect on *Desulfuromonas acetoxidans* (Roden and Lovley, 1993). Finally, it has not been tested if *Ferribacterium* sp., which might be important in our system and have been reported to utilize acetate (Cummings et al., 1999; Cardenas et al., 2008), are really able to grow in our FeRB medium. A larger variety of substrates, Fe(III) forms and gas phase compositions might have resulted in much higher FeRB counts.

Generally, the maxima of bacterial numbers were within the zones of their presumed activity, indicating that the culture-based approach gave reasonable results. The fact that ferric iron reduction exceeded sulfate reduction under all observed circumstances may be partly due to energetic or thermodynamic reasons. However, the combined addition of organic matter and lime from the Carbokalk fostered the desired development of a Fe sulfide accumulating type of sediment, in which Fe(III) reduction and sulfate reduction coexist (Blodau and Peiffer, 2003). Even more important may be that ferric iron-reducing micro-organisms as a physiological group are nutritionally as versatile as sulfate-reducing bacteria, but tolerate a broader spectrum of pH values and oxygen concentrations and have extended capabilities of utilizing alternative electron acceptors (Johnson and Hallberg, 2003; Lovley, 2000). This makes them ecologically more versatile and competitive. Thus, ferric iron reduction and the

micro-organisms that carry out this process deserve further study, especially in environments with shifting biogeochemical conditions and intermediate pH values.

Until now, not much is known about the identity of the organisms that mediate ferric iron reduction at moderately acidic pH around 5. First studies in peatlands exhibiting Fe(III) reduction have indicated the presence of *Acidiphilium*, *Geothrix*, and *Geobacter* among many sequences not closely related to known Fe(III) reducers (Küsel, 2003b), but phylogenetic and physiological details of these communities are not yet resolved. Acidic Fe(III)-reducing enrichments (pH 4–5) from subsurface material revealed an increased proportion of spore-forming (Gram-positive) organisms and *Anaeromyxobacter* strains, which can also produce spore-like bodies (Petrie et al., 2003). Moreover, an *Anaeromyxobacter* strain has been found to be capable of dissimilatory Fe(III) reduction (Treude et al., 2003). In a recent long-term ethanol injection experiment at a contaminated subsurface site, the pH rose from values around 3.8 to 5.7–6.5, and *Ferribacterium* and *Geothrix* were found to be the dominant Fe(III) reducers in sediment clone libraries (Cardenas et al., 2008). Unfortunately, the authors did not test if these organisms were also well represented in their MPN dilution cultures, which gave high FeRB counts. Blöthe et al. (2008) obtained 16 S rRNA gene-based clone libraries from the sediment of a mine pit lake with a zone of naturally elevated pH below 10 cm and found that *Acidobacteria* dominated both in that zone and in the acidic upper layer. Dilution PCR analyses with specific primers yielded products for the genera *Acidiphilium* and *Geobacter*. The enrichments of Porsch et al. (2009) revealed a high frequency of *Acidithiobacillus*-related sequences in the enclosure sediments, which were accompanied by sequences related to *Fulvimonas* and *Clostridium* in the top cm. In the layer with pH $\geq$ 6, enrichments showed a high diversity with frequent Rhodocyclaceae and few *Geobacter*-related sequences. Enrichments of the lower layer with pH $<$ 6 were rich in sequences affiliated with *Sulfobacillus* sp. and *Trichococcus* sp.. Since some of these organisms are not yet known as Fe(III) reducers, it will be interesting to try to isolate them and find out more about their competitive abilities and their substrate spectrum rearding both electron donors and utilized Fe(III) forms.

Conclusions

All of the three groups of well-known ferric iron-reducing bacteria defined by using selective media were detected in the enclosure sediment, and their depth distributions were affected by the treatment. For each of them a zone could be defined where the respective group most likely has been active and contributed to the measured Fe(III) reduction. The acidity-producing, sulfur-driven Fe(III) reduction by *Acidithiobacillus*-like organisms seems to be restricted, both spatially and by competition with other processes, so a negative effect on biological neutralization is unlikely under the prevailing conditions. Other ferric iron-reducing prokaryotes not growing in our culture media might have also contributed significantly to Fe(III)-reducing activity. Especially the neutrophilic or acid-tolerant Fe(III) reducers were underestimated. The presence of such organisms could be detected by combining more sophisticated enrichment procedures, which better reflect in situ conditions with the generation of clone libraries or the use of a comprehensive set of PCR primers. In such an effort, both specific activities and community composition should be monitored over time to better judge the relevance of detected sequences. Depending on the experimental setup, these methods may give at least semi-quantitative results. In addition, culture techniques and media should be refined to be able to isolate and study the physiology of novel Fe(III) reducers.

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