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ORIGINAL ARTICLE

Characterization of Fe(II) oxidizing bacterial activities and communities at two acidic Appalachian coalmine drainage-impacted sites

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We characterized the microbiologically mediated oxidative precipitation of Fe(II) from coalmine-derived acidic mine drainage (AMD) along flow-paths at two sites in northern Pennsylvania. At the Gum Boot site, dissolved Fe(II) was efficiently removed from AMD whereas minimal Fe(II) removal occurred at the Fridays-2 site. Neither site received human intervention to treat the AMD. Culturable Fe(II) oxidizing bacteria were most abundant at sampling locations along the AMD flow path corresponding to greatest Fe(II) removal and where overlying water contained abundant dissolved O_2. Rates of Fe(II) oxidation determined in laboratory-based sediment incubations were also greatest at these sampling locations. Ribosomal RNA intergenic spacer analysis and sequencing of partial 16S rRNA genes recovered from sediment bacterial communities revealed similarities among populations at points receiving regular inputs of Fe(II)-rich AMD and provided evidence for the presence of bacterial lineages capable of Fe(II) oxidation. A notable difference between bacterial communities at the two sites was the abundance of Chloroflexi-affiliated 16S rRNA gene sequences in clone libraries derived from the Gum Boot sediments. Our results suggest that inexpensive and reliable AMD treatment strategies can be implemented by mimicking the conditions present at the Gum Boot field site.
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Introduction

An estimated 10 000 km of streams in the Appalachian coal mining region of the United States are adversely impacted by acidic mine drainage (AMD) that emanates from abandoned and operating coalmines (Herlihy *et al.*, 1990). The AMD arises when metal sulfides (primarily coal deposit-associated pyrite) come in contact with oxygenated water resulting in sulfuric acid production by the biogeochemical oxidation of reduced sulfur species (Baker and Banfield, 2003). These acidic fluids contain high concentrations of dissolved metals due to leaching of metal-containing minerals. The dissolved metal in Appalachian coalmine drainage of greatest concern is Fe(II), and the most prominent feature of AMD-impacted surface waters is the appearance of 'yellowboy,' which is an Fe(III) (hydr)oxide precipitate produced when Fe(II)-rich AMD waters enter circumneutral streams (Pennsylvania Department of Environmental Protection (PA-DEP), 1999). The relatively high pH (5.5–8.0) and dissolved O_2 concentration in surface waters allows rapid abiotic oxidation of dissolved Fe(II) and hydrolysis of Fe(III) (Stumm and Morgan, 1996) as depicted in Equations 1 and 2, respectively:

$$Fe^{2+} + 0.25\,O_2 + H^+ \rightarrow Fe^{3+} + 0.5\,H_2O \quad (1)$$

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

Yellowboy coats algae, plants, macroinvertebrates and sediments in the stream beds, leaving long stretches of 'dead' streams. Therefore, prior to its entry into nearby surface waters, two distinct and critical treatment objectives for Appalachian coalmine-derived AMD are (1) neutralization of low pH waters and (2) removal of Fe.

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'Active treatment' technologies require diversion and collection of water followed by addition of alkaline material before release (Pennsylvania Department of Environmental Protection (PA-DEP), 1999). While these approaches are quite reliable, they involve high energy costs and require feedback control for chemical addition, operator supervision and metal sludge collection and disposal. These requirements make active treatment quite expensive and impractical: the treatment of AMD that may emanate from abandoned coalmines for hundreds of years. Therefore, 'passive treatment' technologies are preferred due to the low cost of maintenance and materials. A common strategy for passively treating AMD from Appalachian coalmines is to divert AMD through limestone beds or channels (Cravotta and Trahan, 1999; Pennsylvania Department of Environmental Protection (PA-DEP), 1999; Nengovhela *et al.*, 2004; Johnson and Hallberg, 2005). Limestone dissolution neutralizes acidic fluids, which enhances the kinetics of Fe(II) oxidation (Equation 1) and Fe(III) hydrolysis (Equation 2) so that Fe removal is accomplished by precipitation of Fe(III) (hydr)oxides (Cravotta and Trahan, 1999; Pennsylvania Department of Environmental Protection (PA-DEP), 1999; Johnson and Hallberg, 2005). Although such precipitation helps remove dissolved Fe(II), these Fe(III) (hydr)oxide precipitates also coat limestone surfaces (commonly called 'armoring'), limiting further limestone dissolution and neutralization capacity and may also clog limestone beds (Pennsylvania Department of Environmental Protection (PA-DEP), 1999; Rose *et al.*, 2004; Weaver *et al.*, 2004).

To limit armoring and clogging, dissolved Fe may be removed from AMD before waters are neutralized with limestone. In the pH range typical of Appalachian AMD (2.5–4.5) (Cravotta *et al.*, 1999), the abiotic oxidation of Fe(II) (Equation 1) is kinetically limited, but hydrolysis and precipitation of Fe(III) (Equation 2) will still occur (Stumm and Morgan, 1996). Under such conditions, acidophilic Fe(II) oxidizing bacteria (Fe(II)OB) may catalyze Fe(II) oxidation, allowing for the oxidative precipitation of Fe from AMD effluents at low pH (Olem and Unz, 1977; Unz *et al.*, 1979; Kirby *et al.*, 1999; Nengovhela *et al.*, 2004; Johnson and Hallberg, 2005; Nicormat *et al.*, 2006a, b). This Fe-free water may then be neutralized using limestone before it is released into nearby streams (Nengovhela *et al.*, 2004). We have identified an AMD impacted system in north-central Pennsylvania (called Gum Boot), in which Fe(II) is effectively removed from AMD at low pH with no human intervention, and we hypothesized that this was due to Fe(II) oxidizing bacterial activity.

A better understanding of geomicrobiological processes in the Gum Boot system may be used to develop technologies suitable for stimulating microbiologically mediated oxidative precipitation of Fe at low pH in other AMD-impacted systems. To gain insight into the mechanisms by which this process occurs, we characterized AMD water chemistry, microbial activities and microbial communities in Gum Boot Run system and compared them to those of an AMD discharge system (called Fridays-2) that exhibits similar chemical and flow characteristics, but where little Fe is removed from the water.

Materials and methods

Site description and sampling techniques

Two AMD-impacted systems were characterized in this study: Gum Boot (GB) and Fridays-2 (FR). Flow rates at the two systems are relatively low in comparison to flow rates at other sites in the Appalachian coal mining region of Pennsylvania, which can be as high as 133 000 l min^{-1} (Cravotta, 2008). The GB system is located in McKean County, Pennsylvania (41° 41′ 02″ N; 78° 29′ 37″ W). AMD at the GB system emerges at the crest of a hill with a flow rate of approximately 50 l min^{-1} and flows as a sheet (0.5 cm deep) over an Fe(III)-rich (approximately 60% Fe_2O_3 by mass) mound that contains goethite as the predominant crystalline mineral phase (X-ray diffraction (XRD) data not shown). After emergence, water flows approximately 18 m downhill before flowing underground, re-emerging at a point approximately 48 m downhill from the source and finally entering a pool at the foot of the hill 127 m from the source (Supplementary Figure S1). Water from the pool ultimately discharges into nearby Gum Boot run water and sediment samples were collected in October 2005, February 2006, May 2006 and July 2006 (fall, winter, spring and summer sampling events, respectively) at discrete sampling locations 0 m (AMD emergence point), 2, 9, 15, 60, 95 and 127 m (in the pool at the foot of the hill) downstream from the AMD emergence point (Supplementary Figure S1). Macroscopic photoeukaryotic organisms that are often observed in AMD-impacted systems (Brake *et al.*, 2002) were not present at the GB system. No soil was evident in the GB AMD flow path within the first 18 m from emergence (Supplementary Figure S1). Water temperatures across the AMD flow path varied seasonally, with temperatures as high as 29 °C observed in warmer months (Supplementary Figure S3).

The Fridays-2 system is located in Clearfield County, Pennsylvania (41° 14′ 34″ N; 78° 32′ 28″ W). AMD at the FR system emerges at a former mine entrance at a flow rate of approximately 136 l min^{-1} and flows as a 0.5 cm thick sheet in a manner similar to that of the GB system. The Fe(III) (hydr)oxide-rich mound at the FR system is also approximately 1 m thick, but the predominant mineral phase is schwertmannite (XRD data not shown). AMD flows over this mound approximately 10 m before entering an adjacent unnamed creek (Supplementary Figure S2). Yellowboy was apparent in creek sediments downstream of AMD entrance into the creek, but no Fe(III) was apparent upstream of the point of AMD

entry. Samples were collected in February, May and July 2006 (winter, spring and summer sampling events, respectively) at discrete sampling locations 0 m (in the AMD source), 3, 8 and 10 m from the AMD emergence point (Supplementary Figure S2). Samples were also collected from the unnamed creek upstream and downstream of AMD entry (Supplementary Figure S2). As with the GB system, macroscopic photoeukaryotic organisms were not observed. The water temperature of the FR system remained constant seasonally (Supplementary Figure S3).

Sediments for laboratory-scale incubations, bacterial enumerations and nucleic acid-based microbial community characterization were collected with sterile spatulas and placed in sterile centrifuge tubes or whirl-pak bags. Sediments were collected from the top 2 cm of sediment at each location. Water samples were filter-sterilized (0.2 μm) in the field. Samples intended for sulfate analysis were not preserved. Samples for analysis of soluble Fe were preserved with 0.5 M HCl, and samples for analysis of other metals were preserved with 0.5 M HNO_3. All samples were stored on ice for transport to the laboratory, except for samples intended for nucleic acid-based microbial community analysis, which were transported on dry ice. Dissolved oxygen concentrations (DO), temperature and pH were determined in the field using portable meters.

Enumeration of culturable Fe(II) oxidizing bacteria

After collection, samples intended for microbial enumerations were stored at 4 °C for no more than 4 days before initiation of enumeration studies. Fe(II) oxidizing bacteria (Fe(II)OB) were enumerated by a plate counting technique described by Johnson (1995). This medium (called FeTSBo) contained 14 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.25 g l^{-1} trypticase soy broth and the pH was adjusted to 3.5 with H_2SO_4. Agarose (20 g l^{-1}; high gel strength from EMD Chemicals Inc.; Gibbstown, NJ, USA) was used as a solidifying agent. $FeSO_4$ (25 mM) was provided as an electron donor. Since products of the hydrolysis of agarose in the acidic medium may inhibit the growth of Fe(II) oxidizing bacteria, plates were prepared with two layers of medium. The underlayer was inoculated (2.5% vol/vol) with the acidophilic organoheterotrophic bacterium *Acidophilium organovorum* (that was cultivated on the medium described above with galactose as an electron donor and carbon source) before pouring. The overlayer received no inoculum before pouring. The inclusion of *A. organovorum* served to minimize the accumulation of agarose hydrolysis products. Sediments were suspended in agarose-free medium (approximately 0.2 g sediment/ 5 ml medium; described above), homogenized by vortexing, serially diluted and spread on plates. Fe(II)OB colony forming units (CFU) were indicated by the formation of red–orange colonies.

To obtain Fe(II)OB cultures, colonies were transferred to agarose-free FeTSBo or American Type Culture Collection (ATCC) medium 2039 ('*Acidithiobacillus ferrooxidans* medium'; pH 2.3). One culture grew sufficiently on ATCC medium 2039 and was successfully transferred eight times, after which a cell pellet was obtained for DNA extraction with a Lysis Plus Mini Kit (Qiagen Inc., Valencia, CA, USA), and processed as described below.

Sediment incubations

Sediments collected from the sampling locations described above were incubated with synthetic acidic mine drainage (SAMD) in 160 ml serum bottles with air in the headspace and sealed with thin Teflon-coated stoppers. SAMD contained 7 mM $FeSO_4$, 5 mM $CaSO_4$, 4 mM $MgSO_4$, 1 mM Na_2SO_4, 0.5 mM $Al_2(SO_4)_3$, 0.4 mM $MnSO_4$ and 0.1 mM $(NH_4)_2Fe(SO_4)_2$. The pH of SAMD was adjusted to 3.5 with H_2SO_4. Where appropriate for abiotic controls, biological activity was deactivated by the addition of 1% formaldehyde, 0.1% sodium azide, or autoclaving. To measure Fe(II) oxidation kinetics, 8 g of sediments were combined with 20 ml of SAMD, leaving 140 ml of headspace and an estimated 1.2 mmol O_2/bottle, with approximately 0.14 mmol Fe(II)/bottle. The pH was measured at the beginning and end of the incubations in sacrificed sediment-SAMD slurries. In initial experiments to determine effective abiotic controls, 10 g of sediment were incubated with 50 ml of SAMD. For these experiments, a larger volume was used to allow larger sample sizes so that pH could be determined throughout the experiment. Bottles contained approximately 0.9 mmol O_2 and 0.35 mmol Fe(II).

Incubations were periodically sampled using a needle and syringe. For experiments to determine the contribution of microbiological activity to oxidative precipitation of Fe, dissolved Fe(II) and Fe(III) were measured in the soluble fraction and total Fe(II) in the 0.5 m HCl-extractable fraction (Lovley and Phillips, 1987) by 1.10 phenanthroline and ferrozine assay (described below). We found that dissolved Fe(III) did not contribute to the total dissolved Fe detected and that adsorption of Fe(II) to sediments was minimal. Therefore, dissolved Fe(II) was quantified by ferrozine assay through the remainder of the study. First order Fe(II) oxidation rate constants (k) were determined by linear least squares regression fitting of ln[Fe(II)] versus time using the following equation:

$$\ln[\mathrm{Fe(II)}_t] = -kt + \ln[\mathrm{Fe(II)}_{initial}] \qquad (3)$$

Nucleic acid-based bacterial community characterization

All samples for nucleic acid-based microbial community analysis were stored at −80 °C until DNA was extracted. Direct extraction of DNA from

Fe(III)-rich sediments proved difficult, so Fe(III) was removed from samples with 0.3 M ammonium oxalate (pH 3.0) as described by Nicormat *et al.* (2006a). Samples were incubated in ammonium oxalate solution for 1 h at room temperature, centrifuged, the supernatant was decanted and fresh ammonium oxalate solution was added. Removal of Fe(III) was indicated by a lack of orange color in the supernatant and was generally achieved after six washes. The remaining pellet (400–1000 mg (wet) recovered from 6 g of wet sediment) was then washed three times with TE buffer (10 mM Tris-hydroxymethylaminomethane (Tris) and 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0). Fe(III) was removed from Fe(II)OB cultures using the same techniques. Fe(III)-free samples were stored at −80 °C before further processing. DNA was extracted from Fe(III)-free sediments using the MoBio UltraClean Soil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Ribosomal intergenic spacer analysis (RISA)-PCR was performed as described by Castillo-Gonzalez and Bruns (2005) using bacteria-specific primers based on *Escherichia coli* positions 16S-926f (5′-AAAGTYAAAKGAATT GACGG-3′) and 23S-115r (5′-GGGTTBCCCCATT CGG-3′) (Lane, 1991) purchased from Invitrogen Corp (Carlsbad, CA, USA). PCR mixtures contained 2 μl of a 1:5 dilution of sediment-derived DNA, 5 μl of 10 × HotMaster PCR buffer with 25 mM $MgCl_2$ (Eppendorf Corp., Westbury, NY, USA), 1 μl of 10 mM dNTPs, 3 μl (each) of 10 mM primer, 0.5 μl of 50 mg ml^{-1} bovine serum albumin, 0.25 μl of 5 u μl^{-1} HotMaster Taq polymerase (Eppendorf Corp., Westbury, NY, USA) and 35.25 μl of molecular biology grade water. PCR cycling in a 2400 Perkin-Elmer thermocycler consisted of an initial denaturation step for 5 min at 94 °C and 30 cycles of 94 °C for 0.5 min, 54 °C for 0.5 min and 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. RISA-PCR products were separated based on DNA fragment size by agarose gel electrophoresis (2% agarose in Tris-acetate-EDTA buffer (0.4 M Tris, 0.2 M acetic acid and 0.01 M EDTA), and 5 μg ml^{-1} ethidium bromide) at 85 V for 1.5 h. Ethidium bromide-stained DNA bands were visualized under UV illumination and photographed with EpiChemi II equipment (UVP Inc., Upland, CA, USA). For 16S rRNA gene-based identification of cultured Fe(II)OB, PCR products were cloned directly into TOPO-TA vector (Invitrogen) following the manufacturer's instruction.

To obtain partial 16S rRNA gene sequences from bands in the RISA gel, individual bands were excised from the gel, suspended in 50 μl of molecular biology grade water and macerated. The 16S rRNA gene portions of the RISA amplicons were amplified in 50 μl reaction mixtures as described above using bacteria-specific primers based on *E. coli* positions 16S-926f (5′-AAAGTYAAAKGAATT GACGG-3′) and 16S-1492r (5′-TACGGYTACCTTGT TACGACTT-3′). Fresh PCR products were directly cloned into TOPO-TA vector as described above. Six PCR insert-containing clones were selected per RISA band, grown to late log phase in Luria-Bertani broth (Atlas, 2004) that was supplemented with 75 μg ampicillin ml^{-1}, and stored in 12% glycerol at −80 °C before further processing. PCR insert-containing TOPO-TA vectors were prepared for sequencing using TempliPhi rolling circle amplification (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in 96-well plate formats according to the manufacturer's instructions. DNA sequencing was performed at The Pennsylvania State University's DNA sequencing facility using an ABI Hitachi 3730XL DNA Analyzer.

For phylogenetic placement, 16S rRNA gene sequences were initially analyzed using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). Sequences were checked for chimeras using the Ribosomal Database Project II's chimera detection function (Cole *et al.*, 2003). Sequences with more than 90% similarity were considered to belong to the same operational taxonomic unit (OTU). OTUs were assigned phylum-level classifications using the Ribosomal Database Project II's classifier function (Wang *et al.*, 2007). Relative frequencies of detected taxa at GB and FR were estimated based on combined pools of sequences from excised RISA bands for each site (two RISA lanes for GB and three lanes for FR). Sequences obtained in this work and those obtained from GenBank were downloaded into a Geneious 3.0 software environment (Drummond *et al.*, 2007). Sequences were aligned within the Geneious environment using the ClustalW algorithm (Thompson *et al.*, 1994), and evolutionary distance trees (neighbor-joining algorithm with Jukes–Cantor corrections) were produced using Geneious 3.0. Evolutionary distance trees were produced as described above using 16S rRNA gene sequences from the GB and FR systems and those obtained from GenBank with *Aquifex pyrophilus* (GenBank accession number M83548) or *Limnothrix redekei* (GenBank accession number AJ58007) as outgroups.

Chemical analytical techniques

Fe(II) was quantified with ferrozine (Lovley and Phillips, 1987) or by 1,10-phenanthroline assay (Tamura *et al.*, 1974). For the quantification of dissolved Fe(III), samples were incubated in an anoxic glovebag for approximately 16 h with 0.25 M hydroxylamine-HCl/ 0.25 M HCl (Lovley and Phillips, 1987) to reduce Fe(III) to Fe(II), which was then quantified by 1,10-phenanthroline. Sulfate was quantified by ion chromatography on a Dionex 100 system fitted with an AS4A column with conductivity detection (Dionex Corp., Sunnyvale, CA, USA). Dissolved Al, Ca, K, Mg, Mn, Na, Si, and Fe content of sediments were quantified by inductively coupled plasma emission spectrometry using a Leeman Labs PS3000UV ICP-AES (Teledyne Leeman Labs, Hudson, NH, USA). Total organic carbon

Table 1 Dissolved constituents and physical characteristics of emergent AMD at GB and FR

	Gum boot	*Fridays-2*
Al (μM)	52	4
Ca (μM)	344	232
K (μM)	82	18
Mg (μM)	313	103
Mn (μM)	51	BDL
Na (μM)	530	174
Si (μM)	436	129
Fe(II) (μM)	869	1150
Fe(III) (μM)	197	142
SO_4^{2-} (μM)	987	3961
D.O. (μM)	BDL	BDL
pH	4.10	4.50
Temperature (°C)	12	10
Flow rate ($l\,min^{-1}$)	50	136

BDL, below detection limit.
Dissolved species were quantified as described in the text.

(TOC) was measured using a Shimadzu TOC-V total organic carbon analyzer (Shimadzu Corp., Columbia, MD, USA).

Nucleotide sequence accession numbers
16S rRNA gene sequences obtained in this study have been deposited under GenBank accession numbers EU220838 to EU220922.

Results and discussion

To understand controlling factors on oxidative removal of Fe from AMD at low pH, we characterized water chemistry, microbial activities and microbial communities associated with two AMD discharges. Fe(II) has historically been observed to be effectively removed from AMD emanating from the Gum Boot (GB) discharge, but not from FR AMD, which led us to designate the GB system as a Fe removal (+) system, and the FR system as an Fe removal (−) system. Neither of these sites have received substantial human intervention to remove Fe from the AMD, and waters emerging from both sites exhibit roughly similar chemical and flow characteristics even though the AMD flow rate at the FR system is greater than that of the GB system (Table 1).

AMD chemistry at GB and FR
Dissolved Fe(II) was completely removed from GB AMD over a distance of approximately 15 m (Figure 1a). At sampling locations greater than 48 m from AMD emergence (Supplementary Figure S1), dissolved Fe(II) concentrations were low (Figure 1a). The lack of soil and the thick deposits of Fe(III) (hydr)oxide-rich sediments associated with the first 15 m of the GB AMD flow path led us to classify the sediments in this region of the flow path

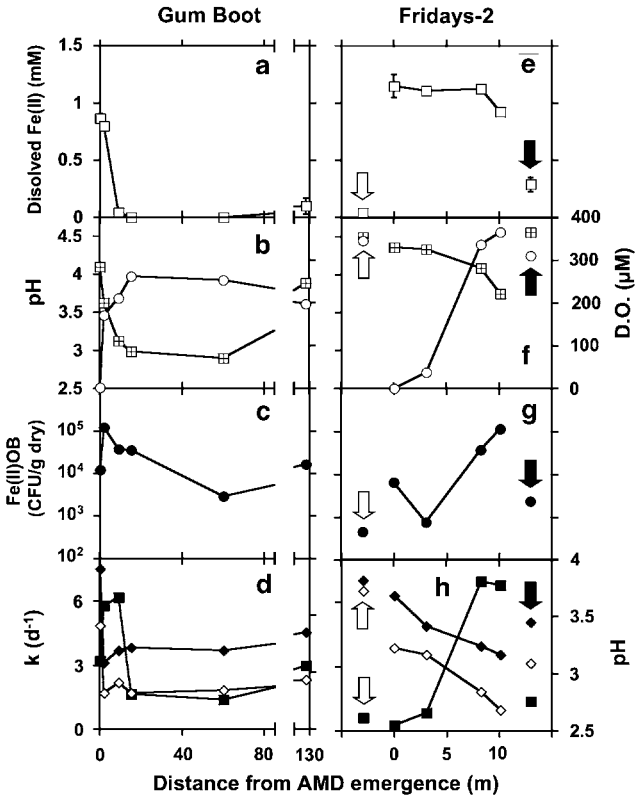

Figure 1 Characterization of water chemistry, abundance of culturable Fe(II) oxidizing bacteria (Fe(II)OB), and microbial activities from discrete sampling locations in the GB and FR systems. Dissolved Fe(II) concentrations (□) are shown in panels **a** and **e**; pH (⊠) and dissolved oxygen concentrations (○) are shown in panels **b** and **f**; numbers of culturable Fe(II)OB (●) (as indicated by colony forming units CFU) are shown in **c** and **g**; first order rate constants (k) of Fe(II) oxidation (■) observed in sediment incubations and starting (◆) and ending (◇) pH of the incubations are shown in panels **d** and **h**. In the Fridays-2 panels, upstream and downstream sampling locations in the unnamed creek are pointed out using white arrows and black arrows, respectively. Error bars represent one s.d.

as those that received regular inputs of unaltered (that is dissolved Fe(II)-rich and relatively low pH) AMD. After emergence, initially anoxic AMD was aerated within approximately 2 m of the AMD emergence point, and the pH of the AMD decreased concurrently with Fe(II) removal (Figures 1a and b), suggesting that Fe(II) was oxidized to Fe(III), with subsequent hydrolysis and precipitation. In the pool 127 m downstream from AMD emergence (Supplementary Figure S1), DO concentration was lower and Fe(II) concentration was higher (Figures 1a and b), suggesting that anaerobic processes were occurring in this pool.

Fe(II) removal was not as efficient in the FR system as it was in the GB system (Figure 1e), and AMD at the FR system traveled a greater distance (approximately 8 m) before it was well aerated (Figure 1f) compared to the GB system, perhaps due to the higher flow rate and/or other hydrodynamic factors. No soil was evident in the FR

system except for sediments in the unnamed creek (Supplementary Figure S2), leading us to designate AMD flowing over the first 10 m of the FR flow path as unaltered. The presence of the thick Fe(III) (hydr)oxide-rich sediments in the FR system suggested that Fe(II) was oxidatively precipitated there, but the persistence of dissolved Fe(II) suggested that this process does not occur as rapidly in the FR system as in the GB system.

One explanation for the more efficient removal of Fe(II) from AMD at the GB system compared to the FR system is the greater mass transfer of Fe(II) into the FR system than the GB system due to the 2.7-fold greater flow rate of AMD at FR. However, a flow rate-normalized comparison of Fe(II) removal efficiency suggests that this is not the case. At GB, Fe(II) is removed from solution at a rate of approximately 0.09 mM m^{-1} at a flow rate of 50 l min^{-1} (Figure 1a). If a similar rate of Fe(II) removal was occurring at the FR system (where water flows at a rate of 136 l min^{-1}), we would expect that approximately 0.7 mM Fe(II) would be removed from FR AMD within 10 m. This was not the case with the FR system, where we observed removal of only 0.2 mM Fe(II) from AMD within 10 m (Figure 1e). These considerations suggest that biogeochemical factors other than the mass transfer of Fe(II) into the FR system must influence the efficient removal of Fe(II) from AMD in that system.

Assessment of biological Fe(II) oxidation

We hypothesized that the oxidation of Fe(II) and subsequent precipitation of Fe(III) (hydr)oxides in these systems was predominantly mediated by Fe(II) oxidizing bacteria (Fe(II)OB), although the presence of Fe(III) (hydr)oxides may enhance the kinetics of abiotic Fe(II) oxidation (Dempsey *et al.*, 2001; Park and Dempsey, 2005). To assess the role of microbiological activity in the oxidative precipitation of Fe from AMD, we incubated non-sterile sediments from the GB system with synthetic AMD (SAMD). We compared rates of Fe(II) oxidation and precipitation by non-sterile sediments to Fe(II) oxidation rates by sediments in which biological activity was deactivated by formaldehyde or sodium azide poisoning or autoclaving. We tested a variety of methods for deactivating biological activity because each method had the potential to induce physicochemical changes to the sediments that could lead to improper interpretation of abiotic or microbiologically mediated Fe(II) oxidation (Tebo, 1991; Shiller and Stephens, 2005). Oxidative precipitation of Fe was not observed in microbiologically deactivated incubations, regardless of the deactivation technique (Figure 2). The addition of formaldehyde to incubations altered solution chemistry less than the addition of sodium azide, which increased the solution pH, or autoclaving, which induced the release of dissolved Fe and a decrease in pH (Figures 2a and b). Removal of Fe(II) from solution was only observed in non-sterile incubations (Figure 2a) and this occurred concomitantly with a decrease in pH (Figure 2b), suggesting that the oxidative precipitation of Fe in systems such as GB and FR is predominantly mediated by Fe(II)OB.

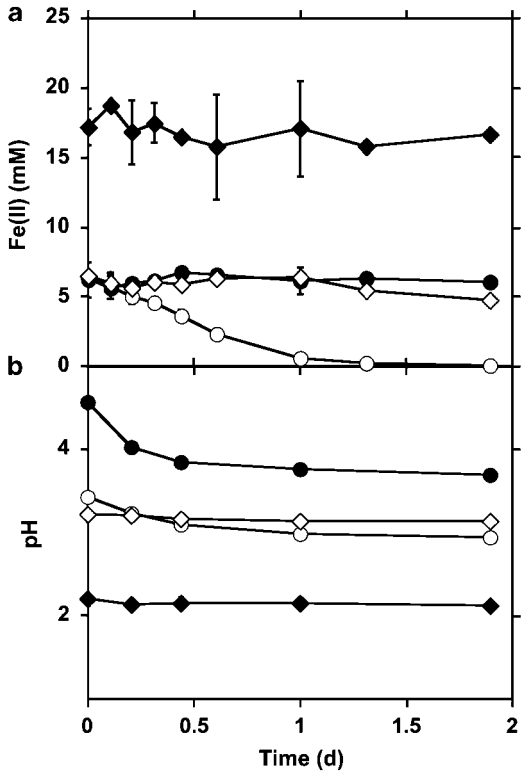

Figure 2 Fe(II) removal (**a**) and concurrent pH decrease (**b**) in non-sterile incubations containing sediments from the GB system (2 m sampling location) (○) compared to incubations that were biologically inactivated by autoclaving (◆) or the addition of 1% formaldehyde (◇), or 1 mg/l sodium azide (●). Error bars represent one s.d.

Abundance and activity of Fe(II)OB in the GB and FR systems

We hypothesized that Fe(II)OB would be most abundant in regions of the AMD flow path where oxidative precipitation of Fe was evident based on field measurements (that is close to the AMD emergence point at the GB system (Figure 1a), and distant from the AMD emergence point at the FR system (Figure 1e)), and that higher numbers of Fe(II)OB would correspond with faster rates of Fe(II) oxidation by sediments recovered from the respective sampling locations. Indeed, culturable Fe(II)OB were most abundant at locations in the GB and FR systems where most Fe removal was occurring as determined by field measurements (for example the GB 2 m and FR 10 m sampling locations; Figures 1c and g). Fe(II)OB were most abundant in the GB system immediately below the AMD discharge point, in contrast to relatively low numbers of Fe(II)OB at downstream locations where all Fe(II)

had been removed (Figures 1a and c). In the FR system, Fe(II)OB were most abundant below the AMD discharge at the two most downstream sampling locations where the highest levels of dissolved oxygen were measured (Figures 1f and g). The numbers of Fe(II)OB in the GB and FR systems (10^3–10^5 CFU g^{-1} in the AMD flow paths) are comparable to those reported in a similar AMD-impacted system (Hallberg and Johnson, 2005). Attempts to culture organisms present in colonies on medium used for plate counts in liquid FeTSBo medium (pH 3.5) were unsuccessful, but organisms present in colonies from GB 2 m could be cultured in ATCC medium 2039 ('*Acidithiobacillus ferrooxidans* medium'; pH 2.3). Sequencing of partial fragments of the 16S rRNA gene (*E. coli* positions 926–1542) of this culture revealed that it was 99% similar to *A. ferrooxidans* ATCC 27320. Thus, culturable Fe(II)OB closely related to *A. ferrooxidans* were recovered from GB sediments.

When we incubated sediments from each discrete sampling location in the GB and FR systems, we observed the highest first order rate constants (k) of Fe(II) oxidation in sediments that harboured the highest numbers of Fe(II)OB (Figures 1c, d, g and h). These results suggest that rates of Fe(II) oxidation are dependent on the abundance of Fe(II)OB and that the availability of dissolved oxygen may exert strong control on the abundance and activity of Fe(II)OB in AMD-impacted systems.

Nucleic acid-based characterization of bacterial communities in the GB and FR systems

We hypothesized that bacterial communities present in Fe-rich sediments would exhibit community profiles dissimilar to those associated with sediments that did not receive regular inputs of unaltered AMD (that is in the 127 m sampling location at GB and within the unnamed creek at FR). We used RISA to assess bacterial diversity at discrete sampling locations at GB and FR. Bacterial communities associated with AMD sources and Fe(III)-rich sediments at both GB and FR exhibited considerably less diversity (as indicated by the number of bands observed in each lane) than those associated with sediments at the 127 m sampling location at GB and in the unnamed creek at FR (Figure 3). Similar RISA banding patterns of the GB and FR sources and Fe(III)-rich sediments suggested similarly simple community structures among these sampling locations. These observations are consistent with previous work suggesting that due to the relatively harsh chemical conditions of AMD, the diversity of microorganisms present in AMD systems is generally lower than that of less chemically 'extreme' systems (for example, soils or sediments of circumneutral pH) (Baker and Banfield, 2003; Hallberg *et al.*, 2006). Therefore, it appears that the regular input of unaltered AMD is the primary controlling factor on the structure of microbial

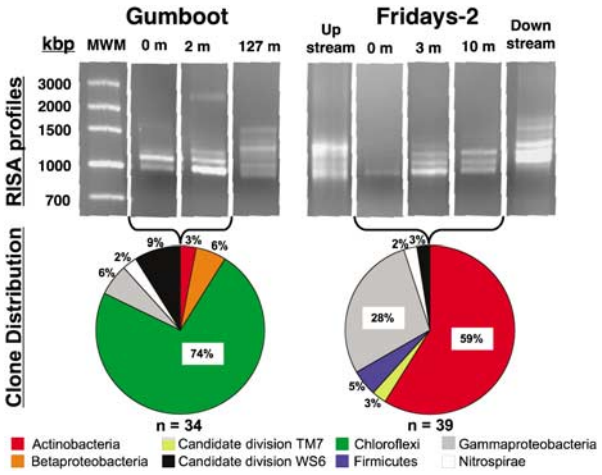

Figure 3 Ribosomal RNA intergenic spacer analysis (RISA) (top panels) and phylum-level distribution of partial bacterial 16S rRNA gene sequences observed in clone libraries recovered from the RISA gel (bottom panel) of microbial communities present in the sources and Fe(III)-rich crusts of the Gum Boot (0 and 2 m sampling locations) and Fridays-2 (0, 3, and 10 m sampling locations) systems. Percentages of clones obtained from excised RISA bands and affiliated with a given phylum are shown next to their respective segments in each pie chart. n indicates the number of clones in each 16S rRNA gene clone library.

communities in AMD-impacted systems. Microbial communities receiving unaltered AMD are similar and have the potential for rapid oxidative removal of Fe(II) from AMD, but numbers of Fe(II)OB and rates of Fe(II) oxidation may be suppressed by limited O_2 availability.

Owing to the similarities in RISA banding patterns of the Fe(III)-rich sediments at GB (0 and 2 m) and FR (FR 0, 3, and 10 m), partial 16S rRNA gene sequences from these sampling locations were recovered from the RISA gel and analyzed in more detail using the Ribosomal Database Project (RDP) II's classifier function. Phylotypes attributable to the Actinobacteria (59%) and Gammaproteobacteria (28%) were most abundant in the source and Fe(III)-rich sediments of the FR system (Figure 3). Phylotypes attributable to the Chloroflexi (74%) were most abundant in the source and Fe(III)-rich sediments of the GB system (Figure 3). Actinobacterial phylotypes recovered from the FR system (and one from the GB system) were members of the Acidimicrobiales subclass (Figure 4). The Acidimicrobiales consist of several acidophilic Fe(II)OB, including the heterotrophic Fe(II)OB *Ferrimicrobium acidiphilum* (Lane *et al.*, 1992; Clark and Norris, 1996; Johnson and Roberto, 1997; Johnson *et al.*, 2003; Druschel *et al.*, 2004; Hallberg and Johnson, 2005; Hallberg *et al.*, 2006). Gammaproteobacterial phylotypes recovered from the FR system were members of the orders Chromatiales, Legionellales and Xanthomonadales (Figure 5). Of these, phylotypes attributable to the Xanthomonadales were most abundant in the FR system and were also detected in the GB system (Figure 5). Xanthomona-

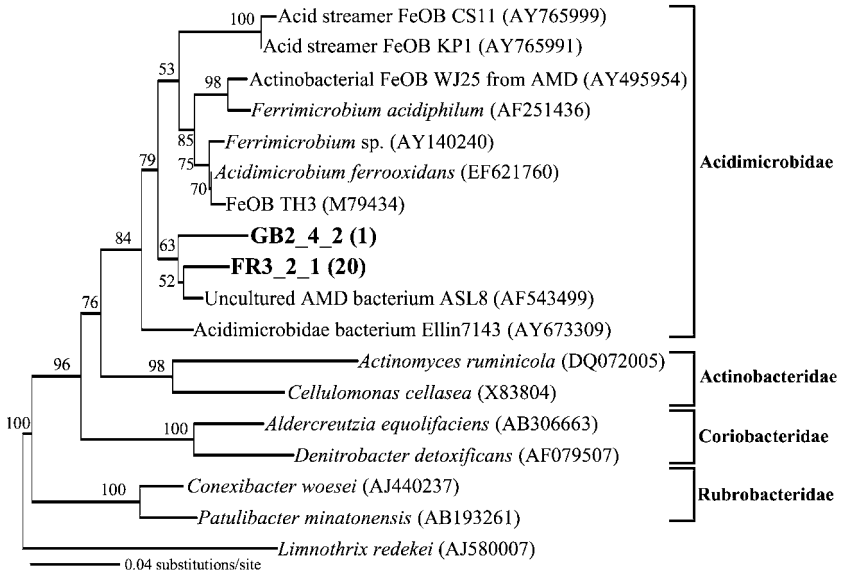

Figure 4 Neighbor-joining tree showing phylogenetic relationship between Actinobacterial sequences recovered from RISA (Figure 4) and selected Actinobacterial sequences from GenBank. Sequences recovered from the RISA gel are in bold font. Numbers in parentheses (besides GenBank accession numbers) represent the frequency of occurrence of a given OTU. Bootstrap values (%) were determined on the basis of results for 1000 replicates and are shown for branches with more than 50% bootstrap support.

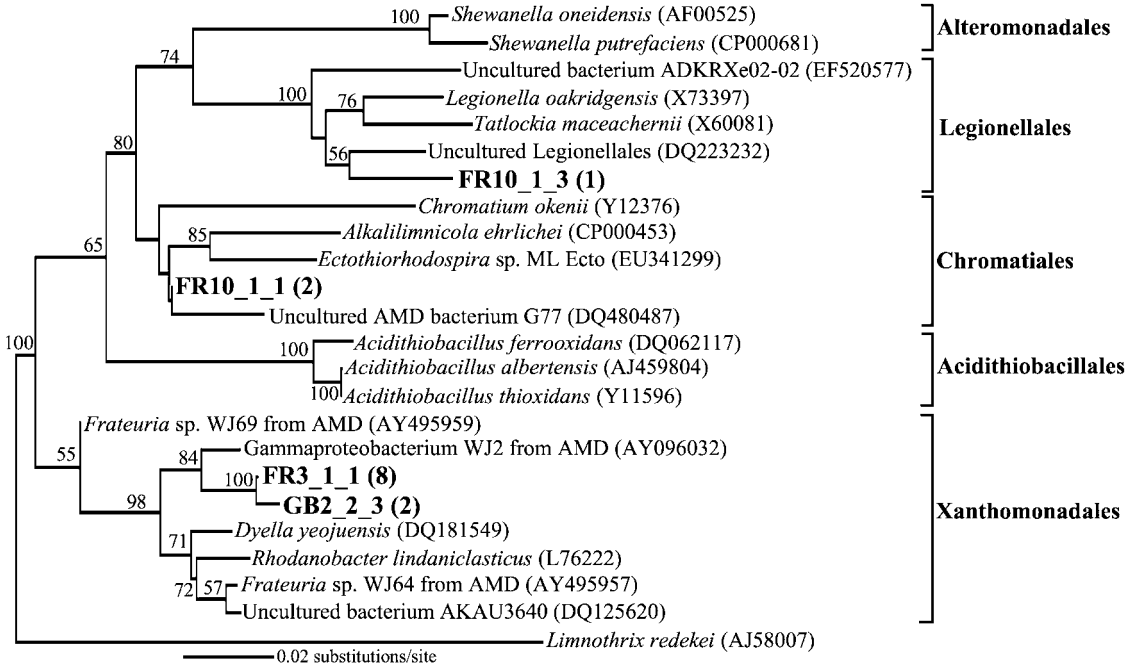

Figure 5 Neighbor-joining tree showing phylogenetic relationship between γ-proteobacterial sequences recovered from RISA (Figure 4) and selected γ-proteobacterial sequences from GenBank. Sequences recovered from the RISA gel are in bold font. Numbers in parentheses (besides GenBank accession numbers) represent the frequency of occurrence of a given OTU. Bootstrap values (%) were determined on the basis of results for 1000 replicates and are shown for branches with more than 50% bootstrap support.

dales-affiliated 16S rRNA gene sequences were most closely related to strain WJ2 (Figure 5), a moderately acidophilic (isolated at pH 4.5), heterotrophic Fe(II)OB (Hallberg and Johnson, 2003). It is notable that none of the Gammaproteobacterial phylotypes that we recovered from the RISA gel were affiliated with the Acidithiobacillales, the order that includes the relatively well-characterized acidophilic, autotrophic Fe(II)OB *Acidithiobacillus ferrooxidans* (Figure 5), despite the fact that this organism was cultured from GB sediments. The lack of Acidithiobacillales-like sequences in the RISA bands is likely

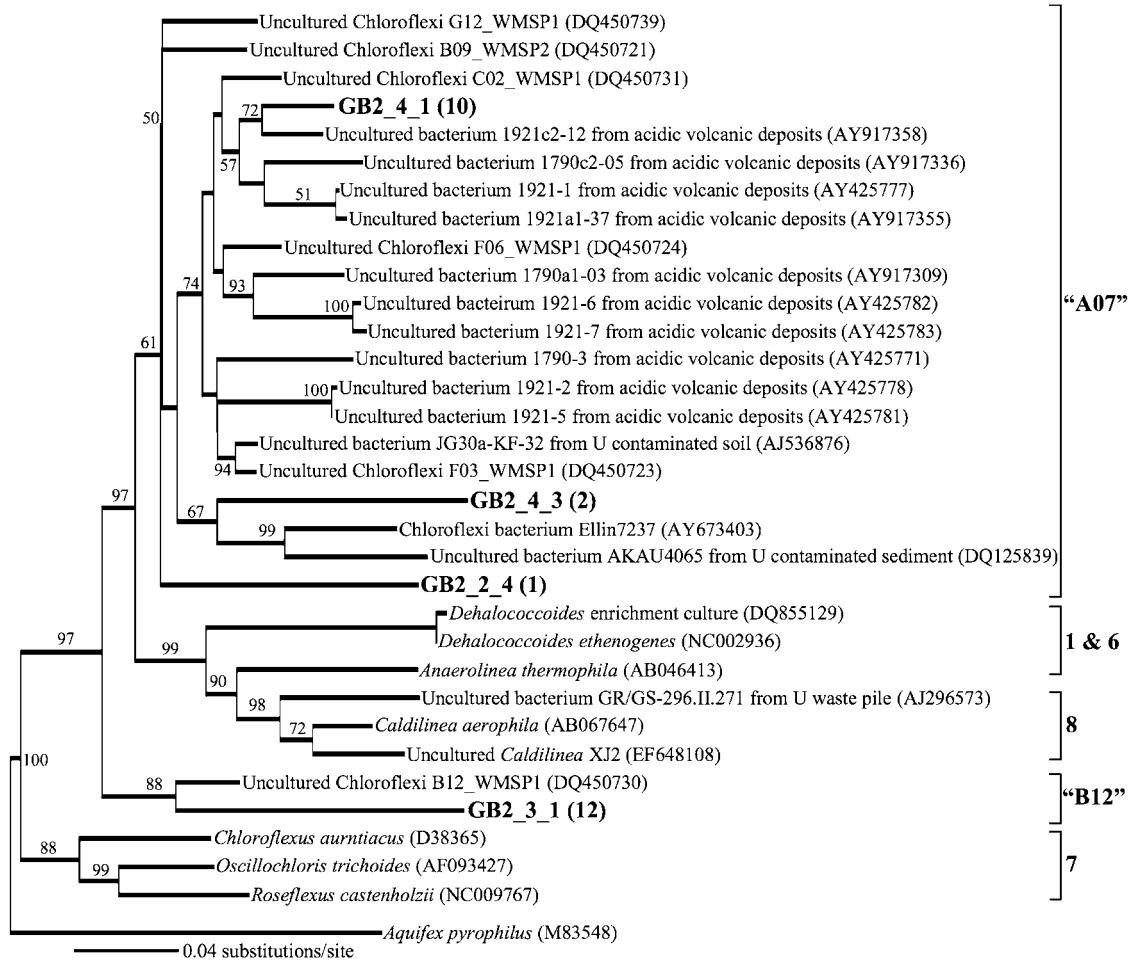

Figure 6 Neighbor-joining tree showing phylogenetic relationship between Chloroflexi bacterial sequences recovered from RISA (Figure 4) and selected Chloroflexi bacterial sequences from GenBank. Sequences recovered from the RISA gel are in bold font. Numbers in parentheses (besides GenBank accession numbers) represent the frequency of occurrence of a given OTU. Bootstrap values (%) were determined on the basis of results for 1000 replicates and are shown for branches with more than 50% bootstrap support.

due to the fact that RISA bands were generated with 'universal bacterial' primers (Lane, 1991), which would not be likely to amplify genomes from populations representing <1% of the total bacterial community (Borneman *et al.*, 1996).

Although members of evolutionary lineages of organisms known to be capable of Fe(II) oxidation (that is Actinobacteria and Gammaproteobacteria) were present in sampling locations receiving unaltered AMD at the GB site (GB 0 and 2 m), Chloroflexi phylotypes outnumbered them in the clone library from this site (Figure 3). The Chloroflexi are a phylum that to our knowledge does not contain acidophilic or Fe(II) oxidizing members, and their potential role in the GB system is unclear. None of the phylotypes recovered from the GB system were affiliated with the Chloroflexi subgroups proposed by Rappé and Giovannoni (2003) (Figure 6). Rather, they were affiliated with the B12 and A07 subgroups proposed by Costello and Schmidt (2006) (Figure 6). These new Chloroflexi subgroups were first observed in saturated tundra soil (Costello and Schmidt, 2006), and subsequently identified in young, acidic volcanic deposits (Gomez-Alvarez *et al.*, 2007), and uranium-contaminated soils and sediments (Selenska-Pobell *et al.*, 2001; Brodie *et al.*, 2006). The lone cultured representative of the A07 group of Chloroflexi (Chloroflexi bacterium Ellin7237) is an organoheterotrophic bacterium isolated from pasture soil and is characterized by slow growth (up to 12 weeks for colony formation; Davis *et al.*, 2005). The reason for abundance of Chloroflexi phylotypes in the GB system, but not in the FR system is unclear, but may be attributed to physical differences between the two sites such as water temperature (Supplementary Figure S3), flow rate (Table 1) or other factors.

Implications for low pH Fe(II) removal from AMD

Our results suggest that when systems are challenged with AMD, bacterial communities develop which have the potential to mediate efficient oxidative precipitation of Fe from AMD. While the

role of Chloroflexi in the GB system is uncertain, other phylotypes belonging to evolutionary lineages known to be capable of Fe(II) oxidation were detected in both the GB and FR systems. Previous studies have implicated the autotrophic Fe(II)OB *A. ferrooxidans*, *Leptospirillum ferrooxidans* and related organisms in the oxidative precipitation of Fe(II) in AMD treatment systems (Coupland and Johnson, 2004; Nicormat *et al.*, 2006a, b). In studies similar to the ones we present here, *A. ferrooxidans* has been cultured from AMD-impacted sediments (Casiot *et al.*, 2003), but was subsequently shown in culture-independent studies to comprise a minor fraction of Fe(II)OB-related phylotypes (Bruneel *et al.*, 2006). The work presented here and by others suggest that organisms other than *A. ferrooxidans* and *L. ferrooxidans* may play a prominent role in oxidative precipitation of dissolved Fe(II) from AMD (Casiot *et al.*, 2003; Hallberg and Johnson, 2003; Morin *et al.*, 2003; Bruneel *et al.*, 2006). Hallberg and Johnson (2003) point out that the pH of the of AMD-impacted systems may influence the composition of Fe(II)OB communities, with relatively low pH (<3.0) favoring the growth and activity of *A. ferrooxidans* and *L. ferrooxidans*, and relatively high pH (>3.0) favoring the growth and activity of 'moderately acidophilic' Fe(II)OB that are phyolgenetically related to organisms that we detected in the GB and FR systems. Although the Actinobacteria and Gammaproteobacteria (including *A. ferrooxidans*) that we have detected in the GB and FR systems are closely related to known acidophilic Fe(II)OB (Johnson and Roberto, 1997; Hallberg and Johnson, 2003; Johnson *et al.*, 2003; Druschel *et al.*, 2004; Hallberg and Johnson, 2005; Hallberg *et al.*, 2006), we definitively cannot assign Fe(II) oxidizing activity in the GB and FR systems to these organisms. However, this work does suggest that oxidative precipitation of Fe(II) may be mediated by diverse groups of organisms, several of which have only recently been identified as Fe(II)OB and are poorly characterized. We also point out that we have not assessed the role of archaea in the GB and FR systems, though previous studies have suggested that bacteria predominantly mediate Fe(II) oxidation in moderately acidic systems (Baker and Banfield, 2003; Hallberg *et al.*, 2006; Nicormat *et al.*, 2006b).

Bacterial communities capable of efficient removal of Fe(II) from AMD are present in both the GB and FR systems. However, the lack of O_2 availability appears to limit the abundance of Fe(II)OB and rates of Fe(II) oxidation, leading to inefficient removal of Fe(II) at the FR system. To efficiently remove Fe(II) from AMD, we envision treatment systems called 'aeration terraces' that could maximize the O_2 concentration in AMD, mimic the sheet-like water flow of the GB and FR systems, and maximize residence time. Wetlands are a traditional method for oxidative precipitation of Fe from AMD, and are attractive because wetland vegetation enhances residence time and retains Fe(III) (hydr)oxides (Kirby *et al.*, 1999; Johnson and Hallberg, 2005). However, due to the inherently high productivity of wetlands (with the subsequent degradation of organic matter), anoxic zones develop where Fe(II) oxidation is limited and the reduction of Fe(III) (hydr)oxides by anaerobic bacteria may lead to the re-release of Fe(II) (Tarutis *et al.*, 1992; Vile and Wieder, 1993; Tarutis and Unz, 1995; Johnson and Hallberg, 2002). By designing AMD treatment systems that mimic the GB system, the O_2-dependent limitations on Fe(II) oxidation may be alleviated.

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