

Mini-review

The microbiology of acidic mine waters

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Abstract

Acidic, metal-rich waters generated by the microbially accelerated dissolution of pyrite and other sulfide minerals, are frequently encountered in derelict mine sites, including many that have been long-abandoned. While these waters are major causes of environmental pollution and are toxic to the majority of prokaryotic and eukaryotic organisms, some life forms (mostly bacteria and archaea) thrive within them. “Acidophiles” comprise a surprisingly wide diversity (in terms of both physiology and phylogeny) of microorganisms. This article reviews current knowledge of the distribution and biodiversity of this group of extremophiles.

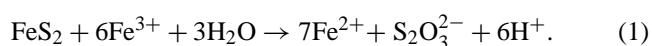
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Keywords: Acidophile; Acid mine drainage; *Acidithiobacillus*; *Acidiphilium*; Acid streamers; *Ferroplasma*; *Leptospirillum*; Iron; Mines; Sulfur

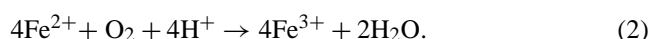
1. Acidic mine waters: origin and chemistry

In all but the most arid areas of the planet, deep mining and surface (opencast) mining of metal ores and coal results in the contamination of ground and/or surface waters. The nature and extent of this contamination is highly variable, depending on such factors as the nature of the ore body and associated geological strata, climate and mining engineering constraints. The most well documented type of water pollution associated with mining is that which results from the accelerated oxidative dissolution of exposed minerals, principally sulfides, giving rise to acidic, metal-enriched waters generally referred to as “acid mine drainage” (AMD) or “acid rock drainage” (ARD).

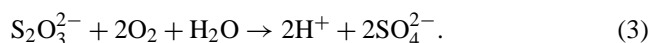
The origin and nature of AMD has been described in detail elsewhere (e.g., [20,31]). Briefly, minerals such as pyrite (FeS_2 ; the most abundant of all sulfide minerals) are chemically (and biologically) stable in situations where both oxygen and water are excluded. However, upon exposure to both moisture and air (e.g., following the fracturing and exposure of sulfide-containing mineral ores and sulfidic coals) sulfide minerals will oxidize spontaneously, with either molecular oxygen or ferric iron acting as the oxidant (e.g., reaction 1):



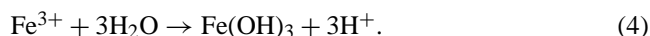
Whilst sulfide mineral oxidation may be abiotic, the rate of reaction is far greater, by many orders of magnitude, in the presence of certain lithotrophic (“rock eating”) prokaryotes. For acid-stable sulfides, such as pyrite, these microorganisms accelerate the oxidative dissolution of the mineral by re-generating ferric iron (reaction 2):



Reaction 1 is abiotic (and may occur in anaerobic as well as aerobic environments) while oxygen-requiring reaction 2 is primarily biological in acidic ($\text{pH} < 4$) waters. Some bacteria and archaea can also oxidize thiosulfate, the initial sulfur by-product of pyrite oxidation (reaction 1), and other reduced inorganic sulfur compounds (RISCs) and elemental sulfur, producing sulfuric acid (reaction 3):



Further acidity results from the hydrolysis of ferric iron (reaction 4):



Proton acidity resulting from reactions 3 and 4 may be counter-balanced by alkalinity resulting from the dissolution of basic (chiefly carbonate) minerals in the ore body or mine spoil, though this may be an important neutralization mechanism only in the short term. Water draining mines and mine spoils are therefore often acidic, and frequently contain elevated concentrations (relative to unpolluted streams) of aluminum and various heavy metals, such as copper,

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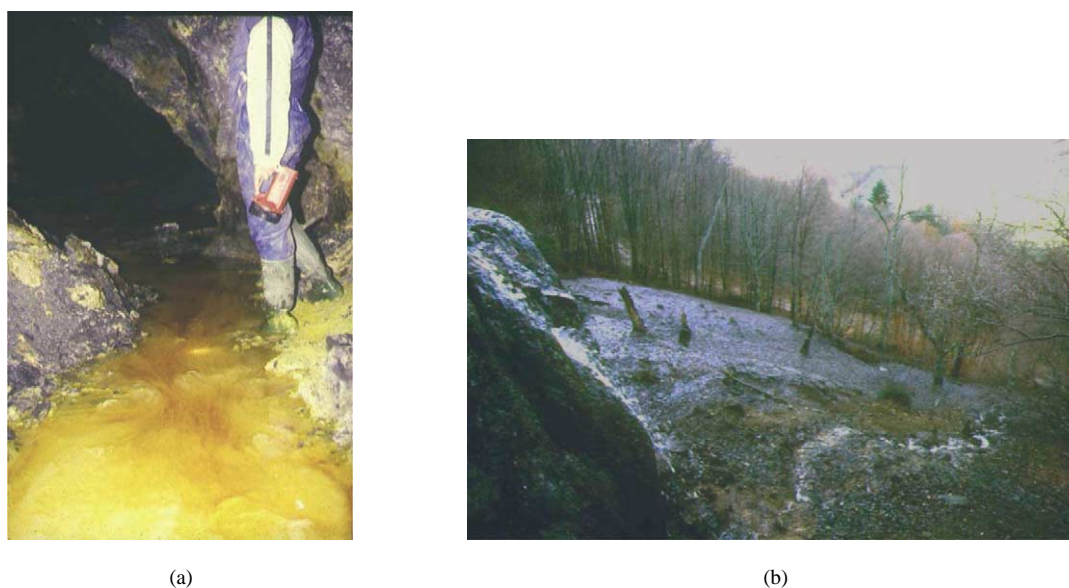


Fig. 1. (a) An acidic (pH 2.3) iron-rich stream within an abandoned pyrite mine, north Wales. The stream is ramified with growths of “acid streamers”, (b) destruction of trees caused by the AMD stream on leaving the mine.

Table 1
Physico-chemical and microbiological characteristics of acid mine drainage from various sites worldwide

| | Storwartz mine, Norway ^a | Ynysarwed, Wales ^b | Bullhouse, England ^b | Wheal Jane, England ^b | Killingdal mine dump, Norway ^a | King's mine stream, Norway ^c | Parys mine, Wales ^b | Rio Tinto, Spain ^d | Iron Mountain, California ^c |
|-------------------------------------|-------------------------------------|-------------------------------|---------------------------------|----------------------------------|---|---|--------------------------------|-------------------------------|--|
| pH | 6.5 | 6.2 | 5.9 | 3.4 | 2.77 | 2.75 | 2.5 | 2.2 | 0.5–1 |
| Eh (mV) | – | +214 | +257 | +462 | – | – | +685 | +450 | – |
| Fe _{total} | 1.60 | 160 | 61 | 290 | 265 | 172 | 650 | 2.3×10^3 | $13\text{--}19 \times 10^3$ |
| Fe ²⁺ | – | 140 | 45 | 250 | – | – | 650 | 1.5×10^3 | $13\text{--}19 \times 10^3$ |
| Al | 0.03 | – | 1.2 | 27 | 38.3 | 22.5 | 70 | – | $1.4\text{--}6.7 \times 10^3$ |
| Mn | 1.35 | – | 15 | 8 | 4.24 | 0.78 | 10 | – | 17–120 |
| Cu | 0.06 | – | – | 1.2 | 5.65 | 15.8 | 40 | 109 | 120–650 |
| Zn | 2.13 | – | – | 132 | 61.1 | 25.4 | 60 | 225 | 700–2600 |
| Sulfate | 151 | 464 | – | 400 | 1219 | 668 | 1550 | 10×10^3 | $20\text{--}108 \times 10^3$ |
| Fe-oxidizers (moderate acidophiles) | $<10^2$ | $<10^2$ | 40 | 3.0×10^4 | 1.3×10^2 | 1.0×10^3 | 1.3×10^3 | – | – |
| Fe-oxidizers (extreme acidophiles) | $<10^2$ | $<10^2$ | $<10^2$ | 1.0×10^3 | 2.1×10^5 | 5.6×10^4 | 3.5×10^3 | 1.3×10^6 | – |
| S-oxidizers (extreme acidophiles) | $<10^2$ | $<10^2$ | $<10^2$ | $<10^2$ | $<10^2$ | $<10^2$ | $<10^2$ | 6.1×10^6 | – |
| Heterotrophic acidophiles | 7.0×10^2 | $<10^2$ | $<10^2$ | 3.0×10^2 | 2.4×10^5 | 1.6×10^4 | 2.0×10^3 | – | – |

Note. All concentrations of metals and sulfate are in mg/l and acidophiles are #/ml. –, not determined. Data are from: (a) Johnson et al., unpublished; (b) [23]; (c) [22]; (d) [28]; and (e) [32].

zinc and manganese. These metals originate from dissolved sulfides, and other associated minerals, and are held in solution because of their greater solubility in acidic liquors. However, iron, which may be present in either (or both) ferrous and ferric forms (depending on water pH) is, in most cases, the dominant metal present in AMD, soluble ferric iron imparting the characteristic red color to extremely acidic mine waters (Fig. 1) while orange-yellow ferric iron-rich sediments (“ochre” or “yellow boy”) are common in higher pH streams. The metalloid arsenic (origi-

nating primarily from the oxidation of arsenopyrite, FeAsS, or arsenic containing pyrite) may also be present at greatly elevated concentrations in AMD. While both As(III) and As(V) may be present, the more oxidized state (e.g., arsenate) tends to be more prevalent in AMD. In contrast, the concentrations of dissolved organic matter in AMD tends to be relatively low (<20 mg/l), favoring oligotrophic rather than copiotrophic heterotrophs. The chemical compositions of several representative AMD waters are given in Table 1.

2. Acidophilic bacteria and archaea

Prokaryotic microorganisms that are metabolically active in extremely acidic environments (often described as having a pH < 3) are widely distributed in the domains *Bacteria* and *Archaea*. The physiological and phylogenetic diversity of acidophilic prokaryotes has been reviewed elsewhere (e.g., [16]). Acidophiles have been categorized using a number of different criteria, such as temperature (as mesophiles, moderate thermophiles and thermophiles) and pH optima, and also on the basis of cellular carbon acquisition; autotrophs assimilate CO₂, heterotrophs assimilate organic carbon and mixotrophs use both. In general, the most extremely thermophilic acidophiles are archaea rather than bacteria (Fig. 2), which might be expected since archaea frequently inhabit the most extreme niches on our planet. Thermo-acidophilic prokaryotes are particularly abundant in geothermal areas (e.g., [6]) and in anthropogenic environments such as self-heating coal spoils (Section 3.4).

Another useful subdivision of acidophiles differentiates those that can solubilize minerals and those that cannot. The first group includes prokaryotes that accelerate mineral dissolution by an oxidative route, and those that use a reductive mechanism. The role of iron- and sulfur-oxidizing bacteria and archaea in the dissolution of sulfidic minerals is well documented, and is utilized in “biomining” [34] where their activities facilitate the extraction of gold (which occurs as fine-grain occlusions in refractory ores) or the solubilization of base metals (such as copper and cobalt). Sulfide minerals differ in their susceptibility to oxidation. Some (such as pyrite) are oxidized by ferric iron (as above), and acidophilic iron-oxidizing prokaryotes (e.g., *Leptospirillum ferrooxidans*, *Acidithiobacillus* (“At.”) *ferrooxidans*, *Acidimicrobium* (“Am.”) *ferrooxidans*, *Sulfolobus metallicus*) have a central role in their dissolution in environmental and commercial contexts. Other sulfides, such as chalcocite

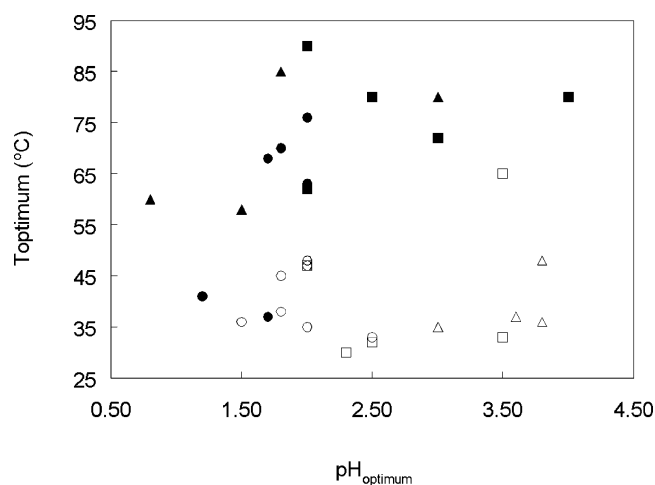


Fig. 2. Temperature and pH optima of acidophilic prokaryotes. Key: ○, iron-oxidizing bacteria; (●) iron-oxidizing archaea; (□) sulfur-oxidizing bacteria; (■) sulfur-oxidizing archaea; (△) heterotrophic bacteria; (▲) heterotrophic archaea.

(Cu₂S), are acid-soluble and can be oxidized by prokaryotes such as *Acidithiobacillus thiooxidans* and *Metallosphaera sedula* that generate sulfuric acid.

In contrast, iron-reducing bacteria have been shown to accelerate the reductive dissolution of ferric iron-containing minerals, such as jarosites and goethite. These include heterotrophic mesophilic bacteria, such as *Acidiphilium* spp., that do not oxidize ferrous iron [5], and moderate thermophiles, such as *Sulfobacillus* spp., that either oxidize ferrous iron or reduce ferric iron, depending on prevailing concentrations of dissolved oxygen [4]. Interestingly, all strains of *Acidiphilium* spp. appear to be able to cat-

Table 2
Acidophilic prokaryotic microorganisms

| Mineral-degrading acidophiles | Thermal classification* | Phylogenetic affiliation |
|---|-------------------------|--------------------------|
| 1a. Iron-oxidizers | | |
| <i>Leptospirillum ferrooxidans</i> | Meso | <i>Nitrospira</i> |
| <i>L. ferriphilum</i> | Meso | <i>Nitrospira</i> |
| <i>L. thermoferrooxidans</i> | Mod Thermo | <i>Nitrospira</i> |
| “ <i>Thiobacillus ferrooxidans</i> ” m-1 | Meso | β-Proteobacteria |
| “ <i>Ferrimicrobium acidiphilum</i> ” | Meso | Actinobacteria |
| <i>Ferropasma acidiphilum</i> | Meso | Thermoplasmatales |
| “ <i>Fp. acidarmanus</i> ” | Meso | Thermoplasmatales |
| 1b. Sulfur-oxidizers | | |
| <i>Acidithiobacillus thiooxidans</i> | Meso | β/γ-Proteobacteria |
| <i>At. caldus</i> | Mod Thermo | β/γ-Proteobacteria |
| <i>Thiomonas cuprina</i> | Meso | β-Proteobacteria |
| <i>Hydrogenobacter acidophilus</i> | Mod Thermo | Aquificales** |
| <i>Metallosphaera</i> spp. | Ext Thermo | Sulfolobales |
| <i>Sulfolobus</i> spp. | Ext Thermo | Sulfolobales |
| 1c. Iron- and sulfur-oxidizers | | |
| <i>Acidithiobacillus ferrooxidans</i> | Meso | β/γ-Proteobacteria |
| <i>Acidianus</i> spp. | Ext Thermo | Sulfolobales |
| <i>Sulfolobus metallicus</i> | Ext Thermo | Sulfolobales |
| 1d. Iron-reducers | | |
| <i>Acidiphilium</i> spp. | Meso | α-Proteobacteria |
| 1e. Iron-oxidizers/reducers | | |
| <i>Acidimicrobium ferrooxidans</i> | Meso | Actinobacteria |
| 1f. Iron-oxidizers/reducers and sulfur-oxidizers | | |
| <i>Sulfobacillus</i> spp. | Meso and Mod Thermo | Firmicutes |
| 2. Heterotrophic acidophiles (non mineral-degrading) | | |
| <i>Acidocella</i> spp. | Meso | α-Proteobacteria |
| <i>Acidisphaera rubrifaciens</i> | Meso | α-Proteobacteria |
| <i>Acidobacterium capsulatum</i> | Meso | Actinobacterium |
| <i>Acidomonas methanolica</i> | Meso | α-Proteobacteria |
| <i>Alicyclobacillus</i> spp. | Meso | Firmicutes |
| <i>Picrophilus</i> spp. | Mod Thermo | Thermoplasmatales |
| <i>Thermoplasma</i> spp. | Mod Thermo | Thermoplasmatales |
| 3. Obligate anaerobes | | |
| <i>Stygiolobus azoricus</i> | Ext Thermo | Sulfolobales |
| <i>Acidilobus aceticus</i> | Ext Thermo | Sulfolobales |

* Meso—mesophiles ($T_{\text{optimum}} < 40^{\circ}\text{C}$); Mod Thermo—moderate thermophiles ($T_{\text{optimum}} 40\text{--}60^{\circ}\text{C}$); Ext Thermo—extreme thermophiles ($T_{\text{optimum}} > 60^{\circ}\text{C}$).

** Inferred ability to oxidize minerals (via production of sulfuric acid).

| Acidophile | Detected in AMD from | | | | | | |
|--------------------------------------|----------------------|-----------|------------|-------------|------------|-----------|---------------|
| | Storwartz mine | Ynysarwed | Wheal Jane | King's mine | Parys mine | Rio Tinto | Iron Mountain |
| <i>At. ferrooxidans</i> | | | | | | | |
| <i>At. thiooxidans</i> | | | | | | | |
| <i>At. caldus</i> | | | | | | | |
| <i>L. ferrooxidans</i> | | | | | | | |
| <i>Acidiphilium</i> spp. | | | | | | | |
| <i>Acidocella</i> spp. | | | | | | | |
| <i>Acidisphaera</i> spp. | | | | | | | |
| " <i>Ferrimicrobium</i> " spp. | | | | | | | |
| <i>Acidimicrobium ferrooxidans</i> | | | | | | | |
| <i>Sulfobacillus</i> spp. | | | | | | | |
| <i>Thiomonas</i> spp. | | | | | | | |
| <i>Halothiobacillus neapolitanus</i> | | | | | | | |
| <i>Frateruria</i> spp. | | | | | | | |
| <i>Acidobacterium</i> spp. | | | | | | | |
| <i>Ferroplasma</i> spp. | | | | | | | |

Fig. 3. Block diagram showing the distribution of different genera of acidophilic microorganisms in acidic mine waters (location given in Table 1). Solid blocks indicate the presence while empty blocks indicate that a given acidophile was not detected in the mine water.

alyze the dissimilatory reduction of ferric iron to ferrous, though in some strains this is constitutive while in others it is inducible [21]. Other acidophilic microorganisms have not been shown to have a direct role in mineral breakdown. These include photosynthetic eukaryotes, such as *Cyanidium caldarium* (a moderately thermophilic rhodophyte) and the mesophile *Euglena mutabilis*, both of which can have an important role in primary production in acidic environments that receive light. Some heterotrophic prokaryotes (such as *Acidocella* and *Thermoplasma*) may contribute to mineral dissolution indirectly by forming commensal associations with iron- and sulfur-oxidizers, whereby they utilize organic compounds (cell exudates and lysates) originating from the autotrophic primary producers, thereby essentially "detoxifying" the environment for the more sensitive (e.g., to organic acids) latter group [19].

Table 2 lists the various species of acidophilic microorganisms that can grow in extremely acidic (pH < 3) waters. Not all of these organisms have been officially classified (as indicated by names in quotation marks) and others require reclassification, such as *Thiobacillus ferrooxidans* m-1, which is phylogenetically distinct from *bona fide* *Thiobacillus* and *Acidithiobacillus* spp.

3. Acidophilic prokaryotes indigenous to mine waters

3.1. Moderately acidic and circum-neutral pH mine waters

Neutrophilic iron-oxidizing and iron-depositing bacteria were amongst the first prokaryotes to be observed and described. Stalked (e.g., *Gallionella ferruginea*) and filamentous (e.g., *Leptothrix discophora*) "iron bacteria" are, due to their characteristic accumulation of ferric iron deposits, amongst the most obvious microorganisms in freshwater environments. Due to the rapid abiotic oxidation of ferrous iron in aerated, circum-neutral pH waters, these bacteria tend to grow in the oxic/anoxic interface where dissolved oxygen

concentrations may be <10% saturation. Novel unicellular iron-oxidizing lithotrophic bacteria have recently been isolated from iron-rich groundwaters and the roots of aquatic macrophytes [30]. Again, these are "gradient" organisms and, since they grow best at pH's ca. 5.8–7.0, they are not acidophiles and will not be considered further in this review.

Further evidence of the importance of previously unknown bacteria mediating chemical changes (ferrous iron- and possibly sulfur-oxidation) in neutral pH and moderately acidic mine waters has emerged from work carried out with AMD discharges in the UK and Norway [17]. Bacteria have been isolated, either directly from AMD or following enrichment in liquid cultures, that oxidize ferrous iron at pH 3–5. Three phylogenetically-distinct groups of these "moderate acidophiles" have been described: one group is most closely related to the neutrophilic acetogen *Frateruria aurantia* (a γ -proteobacterium), a second appears to consist of novel *Thiomonas* spp. (β -Proteobacteria) and the third group is phylogenetically highly related to the sulfur-oxidizer *Halothiobacillus neapolitanus* (also a γ -proteobacterium). Interestingly, none of these genera has previously been reported to include iron-oxidizing species. Surveys of various mine waters have indicated that moderately acidophilic iron-oxidizers are widely distributed in all but the most acidic mine waters (Table 1 and Fig. 3). Oxidation of ferrous iron by these bacteria, and subsequent hydrolysis of ferric iron, causes AMD pH to fall, possibly to the level at which the more familiar extreme acidophiles (Section 3.2) become active.

3.2. Extremely acidic mine waters

The microbiology of extremely acidic (pH < 3) streams and rivers draining mining-impacted areas has been examined by relatively few research groups. The Afon Goch (a stream draining the former Parys copper mine in north Wales; pH 2.2–2.8) was found to contain about 10^3 /ml of both *At. ferrooxidans* and *L. ferrooxidans* [39]. The relative numbers of these iron-oxidizers changed with distance from

the discharge adit, and appeared to correlate with changing concentrations of ferrous iron; *L. ferrooxidans* has a higher affinity for Fe(II) and these bacteria were particularly numerous when ferrous iron concentrations were <10 mg/l. In contrast, *At. ferrooxidans* was noted to be the most numerous iron-oxidizer present in AMD draining the abandoned Cae Coch pyrite mine in north Wales, where ferrous iron concentrations were often >500 mg/l and the pH was 2.3–2.5 [29]. *At. ferrooxidans*-like bacteria were also found to be the dominant iron-oxidizer in a stream draining the former King's copper mine in Roeros, Norway [22]; moderately-acidophilic (*Thiomonas*-like) isolates have also been found in this AMD, though in far smaller numbers than the extreme acidophiles. Numbers of heterotrophic acidophiles in the same stream were similar to those of *At. ferrooxidans*; five different genera/species of obligate heterotrophs were isolated from the King's mine AMD, including *Acidiphilium cryptum*, *Acidiphilium rubrum*, and apparently novel species of *Acidocella*, *Acidisphaera* and *Acidiphilium*.

The Rio Tinto, which flows from the Peña de Hierro in southwestern Spain and enters the Atlantic Ocean at Huelva, is a 100 km-long extremely acidic (pH 2.0–2.5), iron- (ca. 1–10 g/l) and sulfate- (ca. 12–27 g/l) rich river, and is probably the most famous water body of its kind. Mining at Rio Tinto has been in place for over five thousand years, and continues (albeit on a relatively small scale) to the present day. Interestingly, the river Tinto is acidic and metal-impacted even prior to flowing through the mine site, presumably due natural exposure of the Iberian pyritic belt close to its source. Microbial communities have been studied by Amils et al. [28] and acidophilic bacteria related to *L. ferrooxidans*, *At. ferrooxidans*, *At. thiooxidans* and *Acidiphilium* were identified. A combination of denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH) confirmed the presence of these acidophiles (~80% of total cells) and also showed that *Ferroplasma acidiphilum*/*Thermoplasma acidophilum*-like archaea were also present in the Tinto (Ricardo Amils, personal communication).

In the Lusatia region of eastern Germany, there are an estimated 200 acidic (pH < 3) lakes of a surface area >1 ha, which have arisen as a result of the infill of voids following opencast mining of open lignite coal. A large amount of research has been carried out on the geochemistry, microbiology and remediation strategies of these water bodies [13]. Photosynthetic (e.g., *Ochromonas*, *Chlamydomonas*) and lithotrophic (e.g., *Acidithiobacillus* spp.) as well as heterotrophic (e.g., *Acidiphilium* and “*Ferrimicrobium*” spp.) microorganisms have been identified in these acid mining lakes. Sulfate-reducing bacteria belonging to the genus *Desulfobacter* have been detected in mine lakes that have been amended with organic carbon [33].

The most dramatic evidence of microbial life in metal-rich, acidic waters lies in the often voluminous “acid streamers” growths (e.g., [9]). These are mixed communities of autotrophic and heterotrophic acidophilic bacteria (as well

as neutrophilic heterotrophs), some of which produce copious quantities of exopolymers while others are filamentous. Analysis of streamers from Parys mountain and Cae Coch (AMD pH 2.7–3.05) using FISH has indicated that they were composed predominantly of β -*Proteobacteria*, with smaller numbers of “*Ferrimicrobium*”-like bacteria and γ -*Proteobacteria* (Kimura et al., unpublished). Sulfate-reducing bacteria (SRB) have also been isolated from enrichment cultures of acid streamers [37]. In contrast, streamers and slimes from the hyperacidic mine waters at Iron Mountain, California (described in Section 3.3), appear to be very different from those in cooler, higher pH waters.

3.3. Hyperacidic mine waters

The Richmond mine at Iron Mountain, California, consists of a pyrite-rich ore body that was mined periodically for gold, silver, copper, zinc and pyrite until the 1960s, when mining ceased. In some areas of the mine, the AMD is the most acidic water recorded, with a pH as low as ~−4 [32]. In a series of papers, the microbiology of the AMD in the Richmond mine has been explored using mainly molecular techniques. Initial studies using FISH focused on the microbes commonly associated with AMD, namely *At. ferrooxidans* and *L. ferrooxidans*. In samples taken in January, 1997, both species were found in free-flowing mine water, mine water associated with the pyritic sediment and in slime (streamer) material in the mine [36]. Interestingly, in waters of pH < 1.3 and with a temperature >30 °C, *L. ferrooxidans* was found to constitute ~10% of the total population whilst *At. ferrooxidans* was not detected. Neither of these two species dominated the total microbial population in the Richmond mine, eukaryotes were generally minor components, but occasionally constituted up to 25% of the total population, and archaea made up less than 5%. The majority of the bacteria detected with the eubacterial probe, however, were unidentified at that time.

Seasonal variations in the microbial populations at Iron Mountain were also followed using FISH [10]. Again, in January bacteria dominated the microbial population, and *L. ferrooxidans* outnumbered *At. ferrooxidans*. During the summer, however, archaea increased to about 50% of the total population, with a concomitant fall in bacterial numbers. This increase in archaea was correlated with the low rainfall period in California, which was reflected by an increase in the conductivity of the mine water samples. Through enrichment with pyrite as energy source, a culture medium of pH 1.0 and mine water collected in July 1997 as inoculum, a culture developed that was dominated by archaea [11]. Amplification of the 16S rRNA gene and sequence analysis showed that this microbe, called “*Ferroplasma (Fp.) acidarmanus*”, was highly related (>98% sequence identity) to *Fp. acidiphilum*, an archaeon that had been isolated from a bioleaching reactor [15]. Using the sequence data obtained, a probe was designed that targeted “*Fp. acidarmanus*” and it was shown to be the dominant archaeon at Iron Mountain.

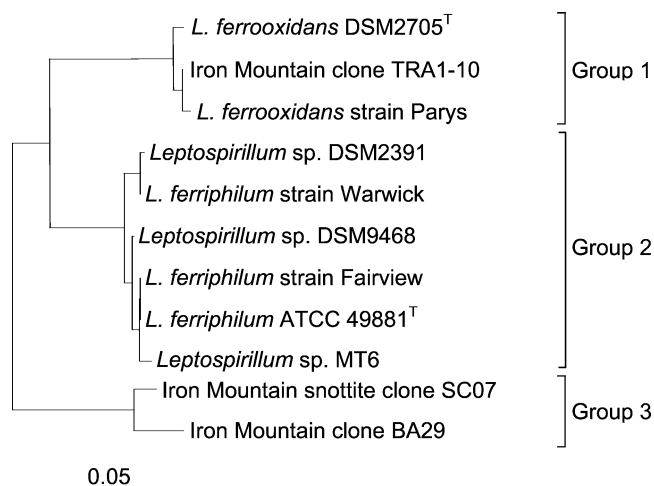


Fig. 4. The three phylogenetic groups of *Leptospirillum*. Group 1 contains the type species (^T) of *L. ferrooxidans*, group 2 consist of, amongst other strains, the type species (^T) of *L. ferriphilum* and group 3 consists solely of environmental clones. The bar shows 0.05 inferred nucleotide substitutions per 100 nucleotides for the horizontal branch lengths.

The above studies focused on the microbial population that included planktonic cells, cells attached to fine grained pyrite particles on the floor of the mine and on slime material in the mine water, and specifically targeted relatively few microbial species. 16S rRNA gene libraries made from DNA prepared from two different slime samples, a slime on the surface of the pyrite on the tunnel floor and “snottites” (slime growths hanging from the roof of the mine tunnel) revealed a greater degree of microbial diversity at Iron Mountain [3]. Although it is difficult to draw firm conclusions about the relative abundance of microbes based on such an approach, it was found that the two gene libraries produced were dominated (75 to 77%) by bacteria that were related to *Leptospirillum* spp. However, sequencing of the representative genes showed that, while a few were related to *L. ferrooxidans*, the major clone (BA29, representing ~70% of clones in the library) of each library was only remotely related to *L. ferrooxidans* and a second, recently designated species [7], *Leptospirillum ferriphilum* (Fig. 4). The sequence of this clone also revealed that the probe (LF581) used in the earlier studies at Iron Mountain would not detect group 3 leptospirilli. Other bacterial genes amplified from the slime library were nearly identical to that from “*Ferrimicrobium acidiphilum*” and another that was more remotely related to “*Fm. acidiphilum*”, but more closely related to clones from an acidic peat bog. Two other clones were obtained that belonged to the δ -*Proteobacteria* (which includes sulfate- and metal-reducing bacteria) but were not related to any known bacteria in this group. The “snottite” library also included 16S rRNA genes from archaea that were similar to *Thermoplasma acidophilum* and one that was identical to *F. acidiphilum* “*Fp. acidarmanus*”.

Quantitative studies on these same slime samples and streamer material were carried out using FISH [2]. Using the new sequence data, additional 16S rRNA probes were

utilized and, in general, confirmed results found by the gene library studies, i.e., in some slime material the group 3 leptospirilli were the predominant microbe present (accounting for nearly 100% of the eubacteria), while in other samples “*Fp. acidarmanus*” dominated (~100% of the total microbial population). Interestingly, the use of probes targeting other acidophiles revealed the dominance of *Sulfobacillus* species in some slime samples. The δ -*Proteobacteria* and “*Ferrimicrobium acidiphilum*” were detected only at low levels, or not at all. Other acidophiles that were not detected by FISH included *At. caldus* and heterotrophic *Acidiphilium* spp. That the streamer (and slime) material in Iron Mountain differs dramatically from the streamers found in Parys mountain and Cae Coch (see Section 3.2) is not too surprising given the dramatic differences in physico-chemical parameters of the three sites (see Table 1).

3.4. Mineral tailings, spoils and sediments

The source of waters draining working and abandoned mines may be underground workings, and/or surface deposits of waste materials (spoil heaps). Tailings (waste fine-grain minerals from ore processing by grinding and flotation) are frequently stored under water, in order to reduce exposure to oxygen and the ensuing oxidative dissolution of acid-generating sulfidic minerals. There are several reports on the microbiology of these ecosystems, including pioneering work by Tom Brock’s research team in the USA (e.g., [8]). Coal spoil heaps are susceptible to ignite spontaneously, and may smolder for many years. One of the first moderately thermophilic acidophiles to be discovered (*Thermoplasma acidophilum*) was isolated from such a site [8]. Schippers et al. [35] examined vertical heterogeneity (from 0–5 m) of microbial populations in uranium spoil heaps in Germany. Iron- and sulfur-oxidizing bacteria were detected, but their numbers declined dramatically below 1.5–2 m depth, corresponding with a marked decrease in oxygen concentrations. Earlier, Goebel and Stackebrandt [14] had analyzed microbial diversity in a chalcopyrite overburden heap in Australia, using a molecular approach (DNA extraction, construction of a clone library and analysis of 16S rRNA genes). Iron/sulfur-oxidizing bacteria (*L. ferrooxidans*, *At. ferrooxidans*, *At. thiooxidans*, *At. caldus*) and heterotrophic (*Acidiphilium* spp.) acidophiles were identified using this approach.

Iron- and sulfur-oxidizing acidophiles have also been found in mine tailings (including samples taken below the water table) by a number of research groups (e.g., [38,40]). Aerobic *Acidiphilium*-like heterotrophic acidophiles have also been isolated and characterized from mine tailings [1]. Fortin and Beveridge [12] detected up to 10⁶ sulfate reducing bacteria/g (dry wt) in mineral tailings at a copper/zinc mine in Ontario, Canada. Bacteria resembling spore-forming *Desulfotomaculum* spp. and vibrioid, motile *Desulfovibrio* were observed, though these were not formally identified. Interestingly, SRB were found in oxygen-containing as well

as anaerobic zones in the tailings, and also in acidic (pH 2.5) as well as circum-neutral pH samples.

Anaerobic acid-tolerant bacteria have also been isolated from sediments in AMD streams and lakes. Küsel et al. [25] isolated an iron-reducing bacterium from anoxic acidic (pH 3.2) sediment from a mining lake that coupled the oxidation of glucose to the reduction of iron(III). The isolate was found by 16S rRNA gene sequence analysis to be a strain of *Acidiphilium cryptum*, and later work confirmed that, like other *Acidiphilium* species, this isolate was more effective at reducing iron when small amounts of oxygen were present [5,24]. Sen and Johnson [37] obtained mixed cultures containing SRB from Afon Goch (Parys copper mine) that were able to reduce sulfate in batch culture at pH > 2.4. Two SRB isolates were obtained, both of which formed endospores and were shown to be related to *Desulfosporosinus orientis* (~95% identity of 16S rRNA genes). One of the isolates grew as distinctly large (>5 µm long) motile cells, and appeared to ferment mannitol as well as oxidize glycerol (Sen et al., unpublished data). Küsel et al. [26] also isolated a fermentative anaerobe from AMD sediment. This isolate, which was also capable of acetogenic growth on hydrogen, was found to very closely related (99.6% sequence similarity of its 16S rRNA gene) to *Clostridium scatologenes*. In other work, Küsel et al. [27] carried out a series of mesocosm experiments using sediment samples taken from a coal mining-impacted acid lake in Lusatia. Bacteria that were able to couple the oxidation of elemental sulfur to the reduction of ferric iron were estimated to be 1% of the total microbial population. In addition, a SRB was isolated from a sample with an *in situ* pH of 5.2. Phylogenetic analysis revealed that this was again most closely related to the spore-former *Desulfosporosinus orientis* and that it had a pH optimum of pH 5.5, but could not grow at pH < 4.9.

4. Overview

Acid mine drainage worldwide is highly variable, with notable differences in pH, which ultimately affects the dissolved metal concentration. In addition to the mine water chemistry, the diversity of microbes detected can also vary significantly. In some instances the factors controlling microbial diversity are obvious, as in the case of the higher temperatures (up to 40 °C) and lower pH waters of Iron Mountain as compared to water draining mines in North Wales or Norway. In other cases, however, the factors controlling microbial populations are not so obvious.

The study of the biogeochemistry of AMD is important due to the impact it has on the environment. A better understanding of the microbial processes that lead to the generation of AMD may enable the development of technology to prohibit the formation of this significant form of pollution. Additionally, a better understanding of the microbes that inhabit AMD will allow the manipulation of the

redox reactions that these microbes carry out to achieve a desired remediation goal (e.g., iron oxidation for iron removal from AMD, and sulfate reduction for chalcophilic metal removal and alkalinity generation). The topic of remediation of AMD pollution has been thoroughly reviewed elsewhere [41].

The improvement of solid media [18] and the recent application of molecular techniques to the study of AMD has led to a thorough re-evaluation of the microbial diversity of this extreme environment. Only until about a decade ago, relatively few acidophiles were recognized, and enrichment/cultivation based studies have led to erroneous conclusions about the significance of *At. ferrooxidans* and *At. thiooxidans* in AMD. Recent advances in molecular biology allow the positive identification of microbes to be made easily through 16S rRNA gene sequence analysis, rather than simply making a presumptive identification of a microbe based on physiological characteristics (many traits are now known to be shared amongst various groups of acidophiles). Continued media development guided by information gathered through cultivation-independent (molecular) studies of AMD will lead to the ability to cultivate even more acidophiles, allowing a greater understanding of these important microbes.

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