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New perspectives in acid mine drainage microbiology

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ABSTRACT

Acid mine drainage varies significantly from site to site, and spans a wide range of pH, temperature and oxygen content. Such variations inevitably mean that a variety of acidophilic microorganisms with varying physiological properties can be found in AMD. With our increased understanding of the microbiology of AMD, better experimental design will lead to a greater understanding of the biogeochemistry of these environments. For example, even though it is readily accepted that at pH 4 and below abiotic oxidation of iron does not occur, it has been concluded that oxidation of iron in mine drainage of ~pH 3.5 was not due to microbial activity. This conclusion was based on the fact that no oxidation of iron occurred in culture medium typically used for *A. ferrooxidans* with a pH of 2, thereby excluding the growth of the moderate acidophiles that probably inhabited that mine drainage.

In addition, the detection of acidophiles with varying physiological capabilities allows for the further development of strategies for the remediation of this important pollution problem, and ultimately to the continued exploitation of minerals. Such emerging strategies include the use of acidophiles with varying pH optima for oxidation and precipitation of iron from AMD of varying water chemistry. Also, the exploitation of key phenotypes such as arsenite oxidation capacity of *Thiomonas* spp. allows for the removal of key pollutants in AMD. Similar approaches can also be taken for other toxic metals such as chromate. Lastly, the isolation and further understanding of anaerobic acidophiles has led to the proposal of methodology to selectively precipitate toxic metals from AMD, turning a pollution problem into a potential source of metals.

1. Introduction

Acid mine drainage (AMD) is one of the most pernicious forms of pollution in the world, and is widely accepted as responsible for costly environmental and socio-economic impacts. As implied by its name, AMD is characterized by a typically low pH, often from pH 3 to 2 (Banks et al., 1997). One case of extremely acidic AMD, with pH as low as − 3, as been observed at Richmond mine, California (Nordstrom et al., 2000). Due to the low pH of AMD, the solubility of transition metals is greater and so AMD often typically contains elevated concentrations of metals. While these chiefly include iron, aluminium and manganese, other toxic transition metals such as copper and cadmium and metalloids (e.g. arsenic) are also present depending on the mineralogy of the host rock. While it is difficult to estimate the worldwide impact of AMD, it has been suggested that over 12,000 km of watercourses in the UK are impacted by AMD. A critical consideration with AMD is the potential long-term threat of pollution, with the production of AMD continuing for many years, for decades or even on the order of centuries, after the closure of a mining operation (Younger, 1997).

AMD is toxic to receiving watercourses for several reasons. A major impact is due simply to the proton acidity of the AMD, which will lead to a decrease in pH of the recipient water should it have insufficient neutralization capacity. A further issue is related to mineral acidity, that is the oxidation and precipitation of metals contained by AMD will also lead to the generation of acidity. This mineral acidity will eventually lead to the depletion of neutralization capacity of recipient waters, and thus will extend the low pH far beyond the source of the AMD. As the pH in recipient waters is lowered, the solubility of the toxic metals contained in the AMD will be maintained at a high level, thus keeping them available to exert their toxic effects. Aside from the acute toxicity of the metals in solution, the precipitation of the metals, especially iron and aluminium, leads to their accumulation on the sediment surface of recipient waters where they inhibit the reproduction of benthic organisms, thus breaking food chains for aquatic organisms, as well as interrupting the life cycle of aquatic organisms that have breeding stages at the benthic surface. AMD is not only associated with surface and groundwater pollution, but is also responsible for the degradation of surrounding soil quality and allows the dispersion of heavy metals into the environment.

Acid mine drainage arises from the exposure of metal sulfide minerals, such as pyrite (FeS_2), to oxygen and water during the mining of metals and coals (Johnson, 2003). Many metals occur chiefly as sulphide ores (e.g. zinc in sphalerite), and these tend to be associated with pyrite, which is the most abundant sulphide mineral

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on the planet. Ferric iron is the main oxidant of the metal sulfides in an oxygen-independent reaction (Eq. (1)).

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ FeS^{2+} + 2\ SO_4^{2-} + 16\ H^+ \quad (1)$$

The regeneration of ferric iron (which is reduced to ferrous on reaction with pyrite) is the rate determining step in the ongoing oxidation of sulfide minerals (Singer and Stumm, 1970), and is a process that requires molecular oxygen. At pH values above 4, the oxidation of ferrous iron this may be mediated chemically (by O_2) or biologically (by iron-oxidising bacteria such as *Gallionella ferruginea*). Below pH 4, the rate of chemical oxidation of ferrous iron by O_2 is negligible (Stumm and Morgan, 1981) and, therefore the activities of acidophilic iron-oxidising microorganisms have a pivotal role in the genesis of acid mine drainage (Johnson and Hallberg, 2003).

This brief review will outline what is currently known about the biodiversity and physiologies of microorganisms that live in acidic waters that flow from deep mines and surface mine spoils and tailings. The microorganisms found in AMD include acidophilic prokaryotes, as well as eukaryotic microorganisms. In addition to the well known acidophiles, more recently discovered "moderate acidophiles", i.e. those that live in less acidic (pH 3–6) mine waters, will be highlighted. Finally, the perspectives for use of these new microorganisms for remediation of AMD will be discussed.

2. Variations in chemistry of acid mine drainage and associated microbial communities

As described above, AMD is typically a metal laden, acidic solution. There is, however, a wide range of variation in the physico-chemical properties of AMD, largely dependant on its origin (Table 1). In general, due to the relatively high carbonate content of the host rock, drainage from coal mines tends to be less acidic (in terms of proton acidity as opposed to mineral acidity) and thus metal poor. In contrast, drainage from metal mines and spoils tends to be more acidic and contains higher concentrations of metals. As would be expected, the more acidic the AMD the greater the concentration of metals it contains (c.f. Wheal Jane AMD compared with AMD at Richmond mine).

Given that ferrous iron is predominant in AMD, it is not surprising that the majority of the microorganisms found in such waters are capable of growth with ferrous as electron donor (Table 1). The majority of these iron-oxidizers act as primary producers in AMD waters, fixing CO_2 into organic matter (Johnson and Hallberg, 2008). This organic carbon can support a rather large population of heterotrophic acidophiles, though organic carbon for the heterotrophs may also arise from surrounding soils. While in general it is thought that the heterotrophs play little role in the geochemistry of AMD, it is generally accepted that they at minimum create a more suitable environment for growth of the iron-oxidizers by removing organic compounds that can be toxic to the primary producers (Johnson and Hallberg, 2008; Harrison, 1984). Surprisingly, few acidophiles that can oxidize only sulfur (e.g. *Acidithiobacillus thiooxidans*) are detected in AMD, though it must be remembered that some acidophiles, such as *Acidithiobacillus ferrooxidans*, can oxidize sulfur and reduced inorganic sulfur compounds in addition to ferrous iron. The low frequency of occurrence of sulfur-oxidizers in AMD may well be due to the fact that sulfur compounds released during the oxidation of pyrite can be oxidized to sulfate by ferric iron (Druschel et al., 2003) and are thus unavailable to support the growth acidophiles.

3. Phylogenetic and physiological diversity of acidophilic bacteria detected in AMD

Due to the acidic nature of AMD, as well as the elevated concentrations of dissolved metals, it is often considered to be devoid of life. While this is generally true for higher life forms, it has been known for over half of a century that microorganisms inhabit AMD (Colmer and Hinkle, 1947). The first microbe isolated from the AMD (Colmer et al., 1950) was an iron-oxidizing microbe called *Thiobacillus ferrooxidans* (now known as *Acidithiobacillus ferrooxidans*). Since then, the use of new media has allowed the isolation of a wide range of acidophilic microorganisms from AMD, and the recent introduction of molecular techniques to the study of mine drainage microbiology has led to an increased understanding of the diversity of acidophilic microorganisms (Johnson and Hallberg, 2003; Hallberg and Johnson, 2001; Baker and Banfield, 2003). The variety of acidophilic bacteria and archaea that have been detected in AMD sites world-wide is shown in Fig. 1.

These include the well-known acidophiles such as *A. ferrooxidans* and *Leptospirillum ferrooxidans*. Other acidophiles that have been detected include iron-oxidizing heterotrophs, obligate heterotrophs, along with many microorganisms that have only recently been recognized as common to AMD. The latter include a group of bacteria that have been called moderate acidophiles (Johnson, 2007), and include *Thiomonas* and *Halothiobacillus* spp. While these bacteria have previously been considered to grow by sulfur oxidation only, they were isolated from AMD as iron-oxidizing bacteria on moderately acidic (~pH 4) solid media (Hallberg and Johnson, 2003). At around the same time, publications appeared describing the isolation of arsenite-oxidizing bacteria from arsenic-containing AMD where arsenic removal was occurring due to oxidation of As(III) to As(V) and the subsequent co-precipitation of the latter with Fe(III) (Bruneel et al., 2003; Battaglia-Brunet et al., 2002a). These isolates were also shown to be *Thiomonas* species, bacteria with apparently relatively considerable metabolic diversity.

Another bacterium that is frequently detected in AMD is an iron-oxidizing betaproteobacterium, for which the name "*Ferrovum*

Table 1
Physico-chemical characteristics of AMD from various world-wide sites and associated microbial populations. All concentrations of metals and sulfate are in mg/L, microbial counts are #/ml and "–" = not determined.

	Ynysawred, Wales	Wheal Jane, England	King's mine, Norway	Parys mine, Wales	Cantareras, Spain	Richmond mine, U.S.A
Mine type	Coal	Tin	Copper	Copper	Copper	Copper
pH	6.2	3.4	2.7	2.5	2.7	0.5–1
Eh (mV)	+257	+462	–	+285	+425	–
Fe_{total}	160	290	172	650	1130	$13–19\times10^3$
Fe^{2+}	140	250	–	650	915	$13–19\times10^3$
Cu	–	1	16	40	160	120–650
Zn	–	132	25	60	24	700–2600
Sulfate	464	400	668	1550	1190	$20–100\times10^3$
Moderate Fe-oxidizers	$<10^2$	3×10^4	1×10^3	1×10^3	$<10^2$	–
Extreme Fe–oxidizers	$<10^2$	1×10^3	6×10^4	3×10^3	1×10^5	–
S-oxidizers	$<10^2$	$<10^2$	<50	$<10^2$	$<10^2$	–
Heterotrophic acidophiles	$<10^2$	3×10^2	2×10^4	2×10^3	$<10^2$	–

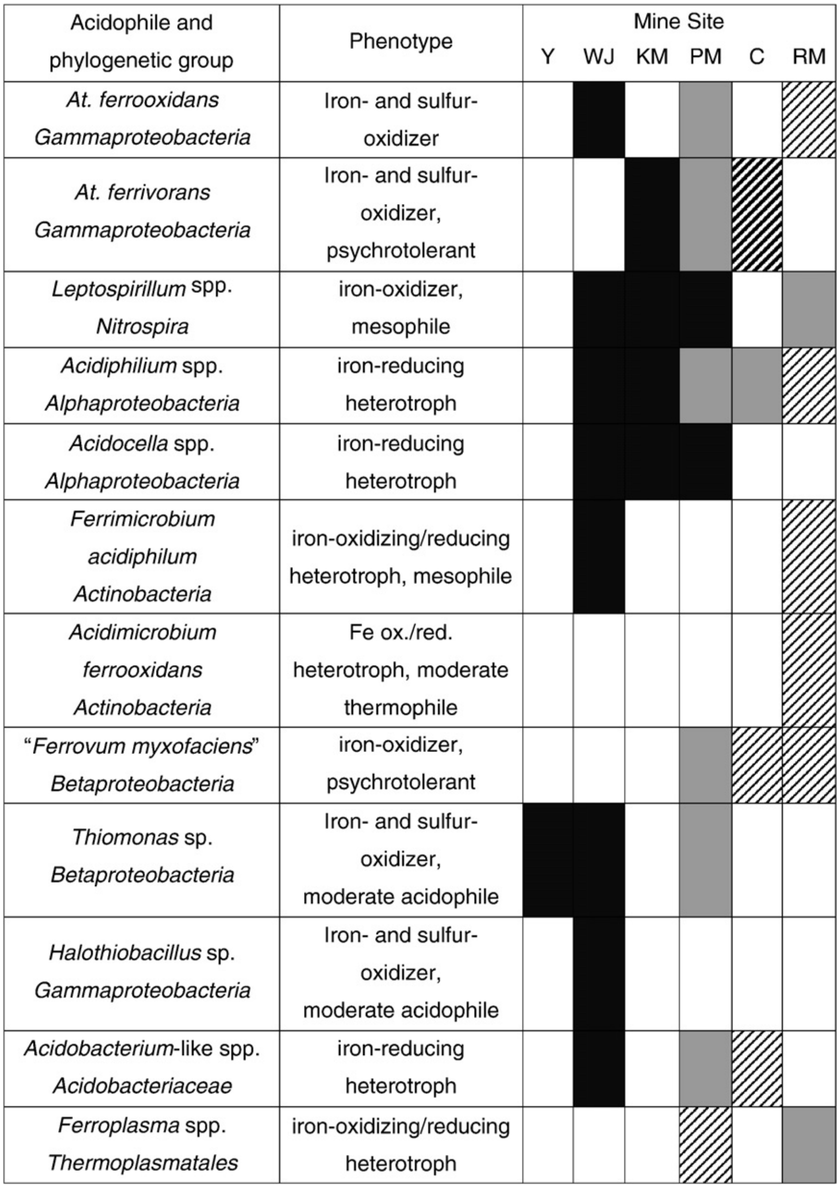

Fig. 1. Distribution of acidophilic prokaryotes in various AMD sites (mine site names are as in Table 1). Black boxes indicate that acidophiles were isolated from that AMD, striped boxes indicate detection by molecular methods, grey boxes indicate detection by both and empty boxes indicate that the respective acidophile was not detected in that AMD.

myxofaciens” (Johnson and Hallberg, unpublished) has been proposed to reflect the copious quantities of exopolysaccharides this bacterium produces. It was first detected by molecular means in macroscopic streamer growths found in two different mine sites in north Wales (Hallberg et al., 2006). Through modification of solid media used for the isolation of acidophilic bacteria, an iron-oxidizing isolate (PSTR) was obtained from one of these streamers, and 16S rRNA gene sequencing confirmed it to be this highly dominant (over 90% of total microbial counts) bacterium. Isolate PSTR was found to be capable of growth only by iron-oxidation, like *Leptospirillum* spp., and is unable to grow at pH below 2 (Johnson and Hallberg, unpublished). Since it was first detected in these streamers, “*F. myxofaciens*” has been found in acidic waters at other sites around the world (Heinzel et al., 2009; Rowe et al., 2007; Tan et al., 2009), where it often represents a major proportion of the microbial population. In one of the streamers where “*Ferrovum myxofaciens*” was originally detected, a second bacterium was found in similar abundance and was shown by 16S rRNA gene cloning to be related (~95% gene sequence identity) to the neutrophilic iron-oxidizing *Gallionella ferruginea* (Hallberg et al., 2006). Microscopic examination of this bacterium during fluorescent *in situ* hybridization revealed that this bacterium had a similar morphology to *G. ferruginea*, leading to speculation that this clone represents an acidophilic species of *Gallionella*, though isolation of this organism is required to confirm that it is an iron-oxidizing, acidophilic member of this genus.

Our increasing understanding of the physiological characteristics of acidophiles provides clues as to controls on microbiological populations. The temperature of AMD is an obvious controlling factor in the distribution of acidophiles in AMD. In general, mesophilic acidophiles are exclusively detected in AMD, though at Richmond mine, where the intensive pyrite oxidation leads to increased temperatures, moderate thermophiles dominate the microbial populations (Bond and Banfield, 2001). Conversely, in many AMD waters that flow from underground mines, the temperature is constantly low and thus bacteria of the newly described psychrotolerant species *Acidithiobacillus ferrivorans* (Hallberg et al., 2010) tend to dominate over *A. ferrooxidans*, or even *L. ferrooxidans* in acidic solutions with a high ferric iron concentration where the latter would be expected to dominate (Kimura et al., submitted). Another controlling factor of microbial populations is pH, with AMD of relatively high pH often being dominated by the moderate acidophiles as opposed to the more extreme acidophiles (e.g. see Table 1). When these two key factors are similar, more subtle factors control microbial populations in AMD, such as relative affinities for electron donors (e.g. Fe^{2+}) and electron acceptors (e.g. oxygen), as well as the propensity of a microorganism to attach to solid surfaces and form biofilms (e.g. "*Ferrovum myxofaciens*").

4. Archaea in acid mine drainage

While a great deal of information is known about acidophilic bacteria that are indigenous to AMD, little is known about archaea in AMD. Most known acidophilic archaea tend to be thermophilic (Colmer et al., 1950), with the exception of *Ferroplasma acidiphilum*. Though this archaeon was originally isolated from a mineral processing operation (Golyshina et al., 2000), it was also isolated from AMD of the Richmond mine (Edwards et al., 2000). Like other archaea of the order *Thermoplasmatales*, it is capable of growth at low pH (down to ~pH 0), though it is the only described archaeon of this order capable of growth at the relatively low temperatures commonly encountered in AMD. All of the archaea in this order are heterotrophic, and only archaea of the genus *Ferroplasma* are able to carry out oxido-reduction of iron. Aside from the Richmond mine AMD, *Fp. acidiphilum* has also been detected in mine waters throughout the world.

Other archaea have also been detected in AMD (Fig. 2), and while also phylogenetically related to the *Thermoplasmatales*, none are sufficiently related to any known archaeal species to be able to infer anything about their physiological traits. Aside from studies at the Richmond mine where fluorescence *in situ* hybridization has been used to assess both the abundance and activity of archaea, little information is known about the abundance of archaea relative to bacteria in AMD nor whether those unknown archaea are active. Clearly, this is an area of mine drainage microbiology that requires further exploration.

5. Iron and sulfur cycling catalyzed by acidophiles in acid mine drainage

Given that ferrous iron is often a major constituent of AMD, it is not surprising that iron oxidation is a key biogeochemical reaction catalyzed by acidophilic prokaryotes inhabiting AMD. The heterotrophic acidophiles are key to organic carbon turnover, which is either derived from the primary producers (e.g. the autotrophic iron oxidizers) or arises from the surrounding soils. Many of the heterotrophs are also capable of reduction of ferric iron (Johnson and Hallberg, 2008), and recently is was shown through the use of insoluble ferric iron (in the form of the mineral schwertmannite) that acidophilic heterotrophs other than *Acidiphilum* spp. can reduce ferric iron, including acidophilic isolates of the *Acidobacteriaceae* (Coupland and Johnson, 2008). While it was not shown that reduction of ferric iron supported growth of these bacteria in the absence of oxygen, acidobacteria were detected in sediment material of AMD in Spain where sulfate-reducing bacteria were also detected (Rowe et al., 2007).

Sulfate is another important solute in AMD, and could serve as an important electron acceptor for anaerobic growth of microorganisms. Despite this, little is known about sulfate reduction in AMD, probably as most studies of microbial populations in AMD focus on the planktonic populations. Sulfate reduction in enrichment cultures inoculated with AMD sediments was shown to occur when glycerol was used as carbon source and electron donor in place of the commonly used lactate (Johnson et al., 1993). Later, sulfate reducing bacteria were isolated from mine sites in Wales that were capable of growth at pH of 3 (Sen and Johnson, 1999). These were inferred to be a new species of the genus *Desulfosporosinus* through 16S rRNA gene sequence analysis. The activity of acidophilic SRB in AMD was shown by the discovery of blackened sediment of AMD in Spain (Rowe et al., 2007), which was shown by energy-dispersive analysis of X-rays (EDAX) to consist of exclusively CuS and not FeS. That CuS was being formed and not FeS implied that the pH of these sediments was below 5. Similar results were found in AMD of ~pH 4 in the U.S., where CuS and CdS were formed but not ZnS or FeS (Church et al., 2007).

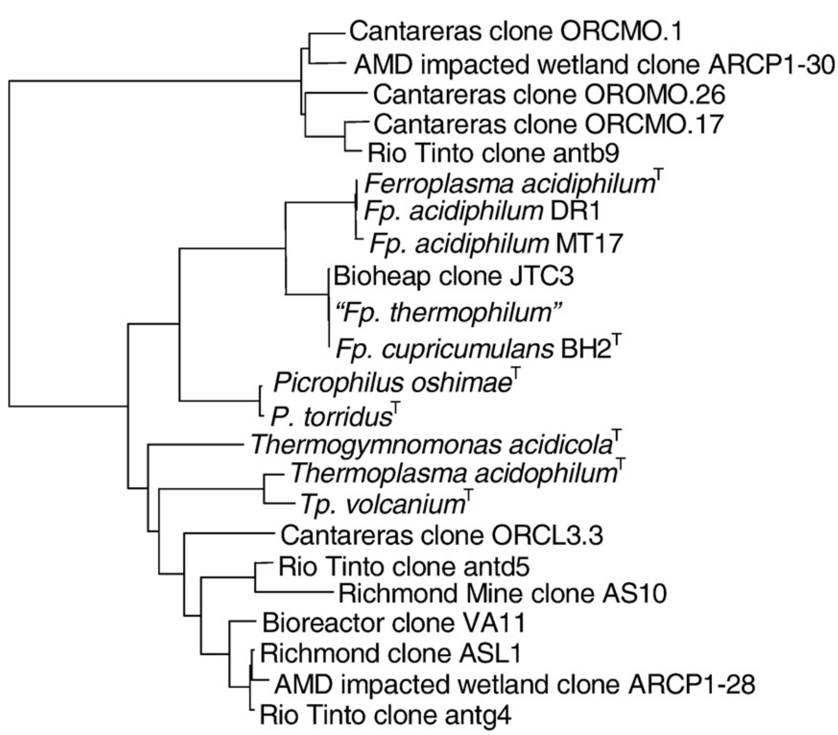

Fig. 2. Phylogenetic relationship of archaea detected in AMD as cloned 16S rRNA genes to isolated and classified euryarchaeotae (the type strain of each named species is designated with a superscript T).

Molecular analysis revealed that bacteria related to *Desulfosporosinus* and *Desulfitobacterium* (bacteria that reduce sulfite but not sulfate) inhabited the latter AMD, while bacteria related to *Desulfitobacterium* only were detected in the Spanish AMD. An isolate obtained from the Spanish AMD was shown to be that detected by molecular analysis, and has been shown to be capable of reducing sulfate at pH as low as 3 (Johnson et al., 2009).

6. Remediation of acid mine drainage: the solution from the pollution

The remediation of organic pollutants usually leads to their disappearance as they are mineralized to carbon dioxide. The remediation of metal pollutants, on the other hand, usually only involves their removal from solution. This occurs either by transformation of redox state, e.g. oxidation of ferrous to ferric which precipitates, or by the formation of insoluble minerals, e.g. precipitation of chalcophilic metals as insoluble metal sulfides (Fig. 3). In AMD, oxidative reactions are generally carried out by autotrophic iron-oxidizing acidophiles using inorganic carbon, while reductive reactions are fuelled by organic carbon as electron donor and carbon source.

Natural attenuation of transition metals in AMD is known to occur (Sánchez España et al., 2005), due mainly to the oxidation and precipitation of iron, and the adsorption of other metals and metalloids to the ferric minerals formed. In an attempt to elucidate the role acidophiles in the natural attenuation process, a survey was carried out on the acidophile population inhabiting the stream water and sediments of AMD draining the Cantareras mine, Spain (Rowe et al., 2007) (Rowe et al., 2007). The planktonic phase of the AMD consisted mainly of the newly described species *A. ferrivorans*, with increasing numbers of heterotrophic acidophiles in samples taken downstream of the mine adit. The benthic zone of the AMD channel was heavily populated by microorganisms, which formed mat-like community structures. The surface of the mats was populated by a range of acidophilic microorganisms, including acidophilic algae. In spite of the fact that the AMD quickly became oxygenated, as would be expected from the presence of the algae, and that it was populated by an iron-oxidizing bacterium, little oxidation of ferrous iron occurred. This was explained by the presence of heterotrophic acidophiles that were capable of reduction of ferric iron. It was shown that the dissolved organic carbon produced by one of the algae could support growth of both *Acidiphilium* spp. and an *Acidobacteriaceae* isolate, and that the mat communities were capable of catalyzing the reduction of iron in micro- and anaerobic conditions. Thus, on the one hand the algae should promote iron oxidation by generating oxygen in the anoxic water that exited the underground mine, yet they also appeared to inhibit net iron oxidation by providing electron donors for the heterotrophs to reduce ferric back to ferrous. When the algae abruptly disappeared from the mats, rapid oxidation of the iron in the AMD ensued. As described in the previous section, evidence was also obtained for the attenuation of copper as a copper sulfide due to sulfate-reducing bacteria in the mat community.

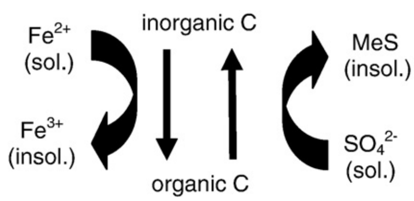

Fig. 3. Major oxidative and reduction reactions carried out by acidophilic prokaryotes in AMD, which lead to metal removal from solution as insoluble products. These occur naturally or can be harnessed in AMD remediation schemes.

Such information is useful for the design of AMD remediation strategies. Although it appears that algae can potentially inhibit the oxidation of iron (and thus removal of iron and other metals from solution), it is difficult to envision a management strategy that would exclude the growth of algae from AMD. A simple method, then, to enhance iron oxidation is to increase the numbers of iron-oxidizing acidophiles through the use of fixed bed bioreactors. A recent study surveyed the utility of various iron-oxidizing acidophiles in such systems (Rowe and Johnson, 2008). Variations were found in the ability of these acidophiles to oxidize ferrous iron to low levels, with *L. ferrooxidans* and "*F. myxofaciens*" achieving the lowest concentrations of Fe^{2+} in bioreactor effluents, even though all four strains used exhibited similar oxidation rates. It was proposed that the differences of ferrous iron in effluents from the various bioreactors was due to variations in affinities of those acidophiles for ferrous iron. Furthermore, it was suggested that bioreactors of mixed communities of these microorganisms could be used to achieve complete oxidation of ferrous iron, and would be better suited for treatment of AMD of varying chemistries than a bioreactor of a single organism.

The isolation of acidophilic (or acid tolerant) sulfate-reducing bacteria has allowed for the development of a novel treatment strategy of AMD (Johnson et al., 2006), whereby the metals are selectively recovered as metal sulfides by controlling the concentration of the reactant S^{2-} through control of bioreactor pH in relation to their respective solubility products (see Fig. 4). This approach not only achieves effective removal of toxic metals from AMD, but it also changes a waste into a useful product. Furthermore, the recovery and use of the base metals would reduce the amounts of toxic wastes produced by processes currently used to remediate metal-rich waste effluents. In an interesting and novel variation of this approach (Jameson et al., submitted), sulfidogenesis by *A. ferrooxidans* and *A. ferrivorans* was exploited to achieve more efficient selective precipitation of CuS and ZnS, as these bacteria can generate sulfide in medium of lower pH (<3) than those aSRB isolated to date, and where both metal sulfides can form.

7. Future perspectives

Acid mine drainage varies significantly from site to site, and spans a wide range of pH, temperature and oxygen content. Such variations inevitably mean that a variety of acidophilic microorganisms with varying physiological properties can be found in AMD. With our increased understanding of the microbiology of AMD, better experimental design will lead to a greater understanding of the biogeochemistry of these environments. For example, even though it is

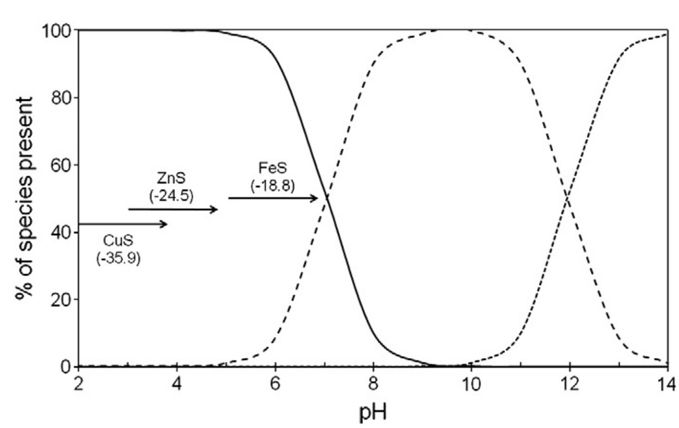

Fig. 4. Proportion of the ionic forms of sulfide present at various solution pH, based on the dissociation constants $pK_{a1} = 7.04$ and $pK_{a2} = 11.96$, and the respective pH range over which the indicated metal sulfides are formed (arrows). H_2S is represented by the solid line, HS^- by the dashed line, and the reactant S^{2-} by the dotted line. The solubility products ($\log K_{sp}$ at 25C) of the metal sulfides are given in parentheses.

readily accepted that at pH 4 and below abiotic oxidation of iron does not occur, it has been concluded that oxidation of iron in mine drainage of ~pH 3.5 was not due to microbial activity (Kirby et al., 1999). This conclusion was based on the fact that no oxidation of iron occurred in culture medium typically used for *A. ferrooxidans* with a pH of 2, thereby excluding the growth and activity of the moderate acidophiles that probably inhabited that mine drainage.

In addition, the detection of acidophiles with various physiological capabilities allows for the further development of strategies for the remediation of this important pollution problem, which is critical to the continued exploitation of mineral resources world-wide. Such emerging strategies include the use of acidophiles with varying pH optima for oxidation and precipitation of iron from AMD of varying water chemistry. Also, the exploitation of key phenotypes such as arsenite oxidation capacity of *Thiomonas* spp. allows for the removal of key pollutants in AMD (Battaglia-Brunet et al., 2002b). Similar approaches can also be taken for other toxic metals such as chromate. Lastly, the isolation and further understanding of anaerobic acidophiles has led to the proposal of methodology to selectively precipitate toxic metals from AMD, turning a pollution problem into a potential source of metals.

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