Chapter 10 from:

Acidophiles

Life in Extremely Acidic Environments

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Distribution of Acidophilic Microorganisms in Natural and Man-made Acidic Environments

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Sabrina Hedrich and Axel Schippers

Abstract

Acidophilic microorganisms can thrive in both natural and man-made environments. Natural acidic environments comprise hydrothermal sites on land or in the deep sea, cave systems, acid sulfate soils and acidic fens, as well as naturally exposed ore deposits (gossans). Man-made acidic environments are mostly mine sites including mine waste dumps and tailings, acid mine drainage and biomining operations. The biogeochemical cycles of sulfur and iron, rather than those of carbon and nitrogen, assume centre stage in these environments. Ferrous iron and reduced sulfur compounds originating from geothermal activity or mineral weathering provide energy sources for acidophilic, chemolithotrophic iron- and sulfur-oxidizing bacteria and archaea (including species that are autotrophic, heterotrophic or mixotrophic) and, in contrast to most other types of environments, these are often numerically dominant in acidic sites. Anaerobic growth of acidophiles can occur via the reduction of ferric iron, elemental sulfur or sulfate. While the activities of acidophiles can be harmful to the environment, as in the case of acid mine drainage, they can also be used for the extraction and recovery of metals, as in the case of biomining. Considering the important roles of acidophiles in biogeochemical cycles, pollution and biotechnology, there is a strong need to understanding of their physiology, biochemistry and ecology.

Introduction

Environments inhabited by acidophiles can be of natural origin where acidic conditions have existed for many years, such as volcanic or geothermal areas, or environments where acidity has arisen due to human activities, such as mining of metals and coal. In such environments elemental sulfur and other reduced inorganic sulfur compounds (RISCs) are formed from geothermal activities or the dissolution of minerals. Weathering of metal sulfides due to their exposure to air and water leads to their degradation to protons (acid), RISCs and metal ions such as ferrous and ferric iron, copper, zinc etc. (see Chapter 1).

RISCs and metal ions are abundant at acidic sites where most of the acidophilic microorganisms thrive using iron and/or sulfur redox reactions. In contrast to biomining operations such as heaps and bioleaching tanks, which are often aerated to enhance the activities of mineral-oxidizing prokaryotes, geothermal and other natural environments, can harbour a more diverse range of acidophiles including obligate anaerobes.

Populations in natural acidic environments

Environments where acid is formed naturally without the influence of mining include geothermal terrestrial sites (solfatara) that occur at active volcanoes, deep-sea hydrothermal systems, and naturally exposed sulfide ore deposits. These sites are of great interest when searching for organisms to be used, for example in biomining operations, as it is often the case that these environments have existed for many years and therefore indigenous microorganisms are potentially adapted to high metal and salt concentrations, extreme temperature and low pH (Table 10.1).

Table 10.1 Physico-chemical parameters and prokaryotic diversity of selected natural environments where acidophiles have been detected

Site	pH	Temperature (°C)	Bacteria	Archaea	Reference
Geothermal sites					
Yellowstone National Park, Wyoming, USA	2.0–4.4	30–90	Fx. thermotolerans, Sulfobacillus-like, Am. ferrooxidans-like, Methylobacterium-like, Acidisphaera sp., Alicyclobacillus-like, H. acidophilus, Thiomonas sp.	Ad. brierleyi, Sulfolobales, Thermoproteales, Desulforococales, 'Geoarchaeota', Thaumarchaeota, Metallosphaera-like, Marinithermus sp.	Johnson et al. (2003), Inskeep et al. (2004, 2010), Kozubal et al. (2013), Beam et al. (2014)
Montserrat, Lesser Antilles	1.6–7.4	24–99	At. caldus, At. ferrooxidans. L. ferrooxidans, Am. ferrooxidans, Sulfobacillus spp., Acidiphilium-like, Alicyclobacillus spp., Desulfosporosinus sp.	Sulfolobus spp., Acidianus spp., Ferroplasma-like	Atkinson et al. (2000), Burton and Norris (2000)
St. Lucia, Lesser Antilles	2.0	42.6	At. caldus, Sulfobacillus spp.	Acidianus spp., unknown Eukaryota	Stout et al. (2009)
Palaeochori Bay, Milos	2.0	50–60	At. caldus, 'Acidithiomicrobium', Acidithiobacillus spp., Sulfobacillus spp.	Acidianus spp., Ferroplasma-like	Norris (2011)
Vulcano, Italy	2.0–3.5	35–75	Acidithiobacillus spp., T. prosperus	Tp. volcanium, Ad. brierleyi, Ad. inferno	Simmons and Norris (2002)
Mutnovsky volcano, Kamchatka	3.5–4.0	70	At. caldus, Thermotogae	Thaumarchaeota, Acidilobus sp., Vulcanisaeta sp., Thermoplasma sp.	Wemheuer et al. (2013)
Hveragerji, Iceland Salar de Gorbea	2.5–3.5 1.1–4.8	81–90 8–20	NR Sulfobacillus spp., Alicyclobacillus spp., Acidisoma sp., Alkalibacter sp., Proteo- and Actinobacteria, Cyanobacteria	Sulfolobales, Thermoproteales, crenarchaeotal group I.1b Thermoplastales, Thermococcales, Halobacteriales, Methanosarcinales and Methanobacteriales, Thermoproteales	Kvist et al. (2007) Davis-Belmar et al. (2013), Escudero et al. (2013)
Copahue volcanic area including Rio Agrio, Argentina	<1.0–4.0	8–85	At. ferrooxidans, At. thiooxidans, Leptospirillum spp., At. caldus-like, Sulfobacillus spp.	Ferroplasma spp., Ad. copahuensis, Sulfolobus spp.	Urbieta et al. (2012, 2014a)
Deep sea hydrothermal vents					
deep sea vent, Eastern Lau Spreading Centre	3–6	50–80	NR	'Aciduliprofundum boonei'	Reysenbach et al. (2006)
Caves					
Frasassi cave, Italy	0–1.5	13	At. thiooxidans, Sulfobacillus spp., Am. ferrooxidans	Ferroplasma spp., 'G-plasma'	Macalady (2007), Jones (2012)
Cueva de Villa Luz, Mexico	1.4	28	Acidithiobacillus spp., Am. ferrooxidans	NR	Hose et al. (2000)

Table 10.1 Continued

Site	pH	Temperature (°C)	*Bacteria*	*Archaea*	Reference
Gossan					
Citronen Fjord, Greenland	1.9–2.5	2–5	*Acidithiobacillus*-like, heterotrophs	NR	Langdahl and Ingvorsen (1997)
Antarctica	3.2–4.5	0–4	*T. plumbophilus*, *At. ferrivorans*	NR	Dold *et al.* (2013)

Abbreviations: *Acidianus* (*Ad.*), *Acidilobus* (*Al.*), *Acidimicrobium* (*Am.*), *Acidiphilium* (*A.*), *Acidisphaera* (*As.*), *Acidithiobacillus* (*At.*), '*Acidithiomicrobium*' (*Atm.*), *Alicyclobacillus* (*Alb.*), *Ferrithrix* (*Fx.*), *Ferroplasma* (*Fp.*), *Hydrogenobaculum* (*H.*), *Leptospirillum* (*L.*), *Metallosphaera* (*M.*), *Picrophilus* (*P.*), *Sulfisphaera* (*Ss.*), *Sulfobacillus* (*Sb.*), *Sulfolobus* (*S.*), *Thermoplasma* (*Tp.*), *Thiobacillus* (T.).
NR, not reported.

Hydrothermal environments

Solfatara are characterized by high temperatures (up to 100°C) and elevated concentrations of sulfur (and RISCs), hydrogen and often also soluble metals and arsenic. When elemental sulfur, formed by the condensation of volcanic gases such as H_2S and SO_2, is oxidized by acidophilic prokaryotes, sulfuric acid is formed which results in low-pH environments. Owing to the low pH and high temperatures in volcanic areas, acid-labile minerals are dissolved and release elevated concentration of transition metals and metalloids. The nature of hydrothermal sites is dictated by the subterranean geology and water flow. In particular, the degree of mixing of deep hydrothermally heated water (buffered in the neutral–alkaline region by CO_2/HCO_3^-) with cold shallow groundwater (strongly acidified by microbial and chemical oxidation of sulfur compounds) determines pH and temperature of the environment (Atkinson, 2000). The microbiology of acidic geothermal areas has been studied for more than four decades (e.g. Johnson *et al.*, 2003; Brock, 2001; 1978) and microbial communities in these environments host a variety of deeply rooted known and unknown *Archaea*, *Bacteria* and *Eukarya*.

One of the most intensively studied natural acidic hydrothermal environment is Yellowstone National Park (YNP; Wyoming, USA) which houses the most numerous and diverse geothermal terrestrial systems on Earth with widely varying geochemical properties, such as temperature, pH, and concentrations of dissolved ions and oxygen.

The first sulfur-metabolizing archaeon, subsequently named as *Acidianus brierleyi*, was isolated by James Brierley in 1965 from geothermal sites in Yellowstone (Brierley, 1973). Further cultivation-based studies on acidophiles from YNP resulted in the enrichment of novel thermophilic iron- and/or sulfur-metabolizing and heterotrophic bacteria (Johnson *et al.*, 2003), including the isolation of the type strain of the acidophilic, moderately thermophilic species *Ferrithrix thermotolerans* (Johnson *et al.*, 2009) and *Acidicaldus organivorans* (Johnson *et al.*, 2006).

While a number of described acidophilic archaeal species have been detected in samples of YNP, several studies have shown that the hot springs of YNP are a source of novel and deeply branching archaea (e.g. Beam *et al.*, 2014; Inskeep *et al.*, 2010, 2004; Table 10.1). Archaea in an acidic iron oxide geothermal spring were further analysed by Kozubal *et al.* (2013) and proposed as a new candidate phylum within the domain *Archaea* referred to as '*Geoarchaeota*' or 'novel archaeal group 1 (NAG1)'. These organisms were found to contain pathways necessary for the catabolism of peptides and complex carbohydrates and genes involved in the metabolism of oxygen. Furthermore Beam *et al.* (2014) applied metagenome sequence analysis to study thermophilic populations (65–72°C) at acidic iron oxide- and sulfur-rich sediment environments of YNP. These deeply rooted *Thaumarchaeota* were proposed to be chemo-organotrophic and couple growth to the reduction of oxygen or nitrate in iron oxide habitats, or sulfur in hypoxic (low-oxygen) sulfur sediments. Possible carbon sources for these

archaea include aromatic compounds, complex carbohydrates, oligopeptides and amino acids or carbon dioxide. No evidence for the oxidation of ammonia was obtained from the *de novo* sequence assemblies.

Some of the geothermal springs within YNP often contain elevated concentrations of arsenic (1–150 mg/l) which occurs predominantly as arsenious acid [As(III)] when the water is discharged, but is rapidly oxidized downstream from the spring source. The oxidation of As(III) to arsenate (As(V)) can occur through abiotic and biotic processes and, although biological oxidation is typically much faster (Cullen and Reimer, 1989), only a few studies have examined the organisms that carry out this process. Studies on the microbial communities of acid-sulfate-chloride springs within YNP (pH ~3.1, $T = 53$–74°C) indicated the dominance of hydrogen-oxidizing *Hydrogenobaculum acidophilum*, *Desulphurella* and smaller numbers of *Acidimicrobium* and *Thiomonas* (Inskeep *et al*., 2004). Archaeal sequences of characterized members in these springs were related to thermophilic, metal-mobilizing *Metallosphaera* and *Marinithermus*, but the majority were unknown crenarchaeotes and euryarchaeotes, most closely related to sequences from marine hydrothermal vents and which are proposed to be responsible for the oxidation of As(III) in the spring (Jackson *et al*., 2001). D'Imperio *et al*. (2007) described the isolation of a novel arsenite-oxidizing *Acidicaldus* species and documented arsenite oxidation inhibition by hydrogen sulfide and its influence on the distribution of arsenite-oxidizing chemolithotrophs.

Another interesting and intensively studied hydrothermal site is located on the Caribbean island of Montserrat (Atkinson *et al*., 2000; Burton and Norris, 2000; Table 10.1), the topography of which is dominated by the active Chances Peak volcano (914 m) and the eroded peaks of three extinct volcanoes, Centre Hills (676 m), Silver Hill (403 m) and South Soufrière Hills (756 m).

Microbial studies on Montserrat samples identified the thermophilic sulfur-oxidizer *Acidithiobacillus caldus*, which is known to grow over a wide temperature range, as the dominant organism at moderate to higher temperature (Burton and Norris, 2000). Cultivation studies furthermore indicated that iron-/sulfur-oxidizing *Acidithiobacillus ferrooxidans* dominated over iron-oxidizing *Leptospirillum* spp. at moderate temperatures, while the latter was dominant in samples of 50–58°C. These low to moderate temperature site populations were also accompanied by heterotrophic *Acidiphilium*-like spp., *Sulfobacillus* spp., *Acidimicrobium ferrooxidans*, *Alicyclobacillus* species, and an unknown actinobacterium (RIV 14). 16S rRNA gene sequences of two potentially moderate sulfur-reducing genera (one of the *Desulfurella* group) were detected in the same pools, while sulfate-reducing bacteria could be isolated from moderate pools (30°C, pH 3.2) one of which (strain M1) was later assigned as the type strain of the novel species *Desulfosporosinus acididurans* (Sánchez-Andrea *et al*., 2015). Higher temperature pools of Montserrat contained clone sequences related to *Ferroplasma*-like organisms, while the dominant group clustered in the *Crenarchaeota*, distantly related to classified *Sulfolobus* spp. and *Acidianus* spp. Members of the thermoacidophilic crenarchaeotal genus *Sulfolobus* were isolated from sites of 65–98°C including one isolate representing a new species which has also been isolated from the Azores (P.R. Norris, unpublished data).

Two more sites with similar microbiological composition such as Montserrat have been studied. One is located on the island St. Lucia, where volcanic, acidic sulfur-springs occur (pH of 2.0 and 42.6°C) and the other one is a geothermal site at Palaeochori Bay of the island of Milos in the Aegean Sea ($T = 55$–60°C). Both microbial communities were dominated by *At. caldus* and *Acidianus* spp. and samples from St. Lucia also contained low numbers of *Sulfobacillus* spp. and unknown *Euryarchaeota* (Stout *et al*., 2009; Table 10.1). The Milos samples additionally harboured unknown *Ferroplasma/Acidiplasma*-like clone sequences as well as a novel sulfur-oxidizing actinobacterium 'Acidithiomicrobium' (also isolated from Milos) and a novel *Acidithiobacillus* sp. V1 (Norris *et al*., 2011).

Copahue-Caviahue, a geothermal area in Argentina, is dominated by the Copahue Volcano which is responsible for the thermal activity in this area. Several acid ponds, pools and hot springs occur in this region, some of which are the source of the acidic river Rio Agrio that discharges in the acidic Caviahue lake. The various habitats harbour a broad range of acidophilic, moderate to thermophilic microorganisms as they vary in their physico-chemical characteristics (mainly pH and temperature).

Molecular analysis of the microbial river community (Urbieta *et al.*, 2012) revealed the presence of moderate, sulfur-oxidizing bacteria (*Acidithiobacillus thiooxidans* and *Acidithiobacillus albertensis*) and moderately thermophilic iron- and sulfur-oxidizing bacteria (*Alicyclobacillus* spp. and *Sulfobacillus* spp.) ubiquitous along the river. Iron-oxidizing bacteria (*Leptospirillum* spp. and *Ferrimicrobium* spp.) and archaea (*Ferroplasma* spp.) were present at the source of the river where iron concentrations were much lower than further downstream. The biodiversity of various ponds studied in the Copahue area (Urbieta *et al.*, 2014a; Table 10.1) is determined by the temperature, resulting in archaea (novel *Sulfolobales* spp. and *Thermoplasmatales* spp.) colonizing higher-temperature ponds, whereas ponds of moderate temperature are colonized by sulfur-oxidizing bacteria, e.g. *Thiomonas* spp., *Acidithiobacillus* spp., *Hydrogenobaculum* spp. and *Acidiphilium* spp. Also a novel *Acidianus* species, *Acidianus copahuensis*, whose genome has recently been sequenced, has been isolated from hot springs of Copahue (Urbieta *et al.*, 2014b). The presence of sulfate-reducing bacteria in anaerobic sediments of an acidic hot spring of Copahue was confirmed by the isolation of several strains related to the sulfate-reducer 'Desulfobacillus acidavidus' strain CL4 from these sediments (Willis *et al.*, 2013). Several eukaryotes, such as yeasts and filamentous fungi, have also been detected in the geothermal area (Chiacchiarini *et al.*, 2009). In microbial terms, Copahue is a very diverse environment and analyses have shown that the distribution of microorganisms in this system is determined by pH, temperature and conductivity, rather than mineral chemistry as it is the case in Yellowstone National Park (Urbieta *et al.*, 2014a).

Numerous hydrothermal vents are also located on the Kamchatka Peninsula in the northeast of Russia. These vents constantly release geothermal gases and fluids dominated by N_2, CO_2 and H_2; CH_4 and H_2S also frequently occur. The hot springs found in this area have multiple origins, including meteoric and magmatic water. Active hydrothermal springs with a large variety in chemical composition, temperature, and pH are located in the area of the Uzon Caldera, Geyser Valley near the volcanoes Karymskii and Mutnovsky. Similar sites can also be found in Iceland, which is located on the Mid-Atlantic Ridge and harbours a zone of active rifting

and is characterized by active volcanoes, fissure swarms, numerous normal faults, and high-temperature geothermal fields. In the famous Hveragerði high-temperature geothermal field, located about 50 km southwest of Reykjavik, geothermal manifestations consist of fumaroles and hot springs, which have different values of temperatures and pH.

Studies on the prokaryotic diversity of the sediment samples near the Mutnovsky volcano (pH 3.5–4.0, $T = 70°C$) revealed the dominance of sulfur-oxidizing *At. caldus* and *Thermotogae* (accompanied by small numbers of *Desulfurella*, *Kosmotoga* and *Hydrogenobaculum* spp.) and sequences clustering with the thaumarchaeal 'terrestrial hot spring group' as well as minor numbers of *Acidilobus* spp., *Vulcanisaeta* spp. and uncultivated *Thermoplasma* spp. (Wemheuer *et al.*, 2013; Table 10.1). The type strain of the hyperthermophile *Acidilobus aceticus* has previously been isolated from the same area and described as a strictly anaerobic acidophile with a heterotrophic lifestyle (Prokofeva *et al.*, 2000).

The archaeal composition of samples from Hveragerði (Iceland) hot springs (pH 2.5, $T = 81–90°C$) was more diverse, comprising members of the acidophilic, sulfur-oxidizing *Sulfolobales*, *Thermoproteales* and uncultured members of the crenarchaeal group I.1b (Kvist *et al.*, 2007). Further studies on archaeal communities of water and mud samples from hot springs in Kamchatka and Iceland detected archaeal ammonia monooxygenase (*amoA*) genes in agreement with considerable *in situ* nitrification rates (Reigstadt, 2010). The wide temperature and pH range ($T = 38–97°C$ and pH 2.5–7) at which ammonium-oxidizing archaea (AOA) have been detected, together with the geographically disparate sampling locations, provide evidence for an active role of hyperthermophilic AOA in nitrogen cycling in hot spring microbial communities throughout the world.

Natural acidic, saline lakes are relatively rare compared to alkaline salt lakes, but can be found in Australia and the Atacama region of northern Chile. The Salar de Gorbea and Salar Ignorado are basins in Chile where the oxidation of native volcanic sulfur occurrences leads to the release of sulfuric acid and strong hydrothermal alteration of the country rocks lowers the buffering capacity and thereby cause these habitats to be acidic. The water bodies of Salar de Gorbea are characterized by high salinity (100–300% total dissolved

solids) and acidic pH (1.1–4.8) with temperatures between 8 and 19.5°C. The dominant bacteria in these samples were members of the *Proteo*-and *Actinobacteria, Cyanobacteria* as well as *Firmicutes* (e.g. *Sulfobacillus, Alicyclobacillus*) (Davis-Belmar *et al.*, 2013; Escudero *et al.*, 2013). Cultivation based studies revealed the presence of novel strains of the genus *Acidisoma* (Davis-Belmar *et al.*, 2013). The archaeal community of Salar de Gorbea was dominated by various members of the *Euryarchaeota* (*Thermoplastales, Thermococcales, Halobacteriales, Methanosarcinales and Methanobacteriales*) and minor numbers of *Crenarchaeota* (*Thermoproteales*) (Escudero *et al.*, 2013).

Salt-tolerant acidophiles appear to be relatively uncommon. One such is the acidophilic iron-oxidizer '*Thiobacillus prosperus*' (Huber and Stetter, 1989), which was isolated from the Mediterranean island of Volcano (located to the north of Sicily; Table 10.1) and which can grow in media containing up to 6% NaCl. 16S rRNA gene clone libraries of samples from Volcano sites were dominated by a novel *Acidithiobacillus* sp. at 35–45°C, while novel iron-oxidizing bacteria related to '*T. prosperus*' were isolated but not detected in gene banks (Simmons and Norris, 2002). Acidophilic archaea at Volcano included *Acidianus* spp. and *Thermoplasma* spp., all of which displayed a broad tolerance (up to 4%) to sodium chloride (Simmons and Norris, 2002).

While acidophilic prokaryotic communities in hydrothermal areas have been studied quite intensively, eukaryotes in geothermal sites have received far less attention. In one study, eukaryotes observed in Nymph Creek, YNP (pH 2.5, $T = 40°C$), comprised *Cyanidium*-like algae, the acidophilic diatom *Pinnularia*, the amoeba *Tetramitus thermoacidophilus and Naegleria* spp., flagellates and novel members of the amoeba *Vampyrellidae* (Amaral-Zettler *et al.*, 2013).

Deep-sea hydrothermal vents

The walls of deep-sea hydrothermal vent deposits (chimneys) with their steep chemical and thermal gradients provide a wide range of microhabitats for microorganisms. *In situ* measurement of pH at the exterior of such a deposit wall on the East Pacific Rise reported pH values of 4.1 at 120°C (von Damm *et al.*, 1995). Calculated pH values based on various flow rates and exchange of fluids within the pores can range from 3 to 6 at temperatures of 50–80°C (Le Bris *et al.*, 2005). Acidity is also produced locally in the chimneys by the precipitation of minerals such as pyrite and chalcopyrite. The majority of thermophiles isolated from deep-sea vents are, however, neutrophiles or acid-tolerant organisms. A notable exception is the thermoacidophilic sulfur- and ferric iron-reducing heterotroph '*Aciduliprofundum boonei*', a member of the 'deep-sea hydrothermal vent *Euryarchaeota* 2' (DHVE2) group, which grows between pH 3.3 and 5.8 (Reysenbach *et al.*, 2006; Table 10.1). Further thermoacidophilic strains related to '*Acp. boonei*' could be isolated from vents along the East Pacific Rise and Mid-Atlantic Ridge, showing that the DHVE 2 are widespread in various hydrothermal vents and constitute a major part of the archaeal population (Flores *et al.*, 2012).

Cave systems

Another example of natural-acidic environments are cave systems, such as the Lechuguilla Cave (New Mexico, USA; Hill, 1995), Frasassi cave (Italy; Macalady *et al.*, 2007) and Cueva de la Villa Luz (Mexico; Hose *et al.*, 2000) (Table 10.1). These develop in subterranean environments containing sulfide-rich ground-waters where, under aerobic conditions, microbial oxidation of sulfide to sulfuric acid leads to extensive dissolution of carbonate rock strata. One of the most comprehensively studied sites of this type, in terms of microbiology and chemical parameters, is the sulfidic Frasassi cave in Italy where biofilms ('snottites') of pH 0–1 have been described (Macalady *et al.*, 2007). Community analysis of theses snottites revealed a limited biodiversity with sulfur-oxidizing *At. thiooxidans* as the most abundant bacterium, and smaller numbers of *Sulfobacillus* spp., *Acidimicrobium ferrooxidans* and archaea related to the uncultivated 'G-plasma' clade of the *Thermoplasmatales* (Jones *et al.*, 2012). Also some protists and fungal filaments were observed via microscopy in the Frasassi cave samples (Macalady *et al.*, 2007).

The hypogenic cave Cueva de Villa Luz in southern Mexico also comprises extremely acidic microenvironments (pH 0.1–3.0) and sulfur-rich springs and shows elevated hydrogen sulfide emission. The microbial composition of water drips from snottites is similar to the Frasassi community with sulfur-oxidizing *Acidithiobacillus* spp. as key players and smaller numbers of moderately thermophilic

Am. ferrooxidans mediating redox reactions within the cave (Hose *et al.*, 2000).

Acid sulfate soils and acidic fens

Acid sulfate soils (ASS) form predominantly when anoxic marine sediments or salt-marshes rich in pyrite and other sulfide minerals are aerated, e.g. when drained for agricultural or industrial purposes, and oxidation reactions generate proton acidity and mobilize metals. ASS are widespread in coastal regions and cause a serious environmental risk due to severe soil acidity and acid metal-rich run-off effecting adjacent flora and fauna. As described earlier, microorganisms catalyse the oxidation of sulfidic minerals and acid production and are therefore the key drivers in the formation of acid sulfate soils. Initial studies on the formation of ASS by Arkesteyn (1980) resulted in the isolation of iron-/sulfur-oxidizing *At. ferrooxidans* and sulfur-oxidizing *At. thiooxidans* from the acidifying soil material but were not attributed to contribute to the initial pH decrease to pH 4. Sulfur-oxidizing acidophiles were also detected in a buried potential acid sulfate soil layer of a Japanese paddy field which were proposed to contribute to ASS formation by oxidizing sulfur compounds to sulfate and thereby producing acidity (Ohba and Owa, 2005). Wu *et al.* (2013) studied the zones of the Risöfladan experimental field, Finland, which had been drained for more than 40 years. Bacteria present in the plough and oxidized layers were related to known acidophilic heterotrophs and iron- and sulfur-oxidizers previously found in acidic, metal- and sulfur-containing environments. Enrichment cultures of partial oxidized soil at pH 3.0 included iron-/sulfur-oxidizing *At. ferrivorans*, *At. ferrooxidans*, *Sulfobacillus* spp. and *Thiomonas* spp..

Naturally exposed ore deposits

Acidophilic microorganisms also occur naturally in the uppermost weathered zones of sulfide ore deposits (gossans; Table 10.1). Such deposits have been mainly exploited by mining and remain only in remote areas e.g. in high altitudes. Langdahl and Ingvorsen (1997) studied the microbiology of gossan material at the Citronen Fjord in North Greenland, which was characterized by subzero temperatures and therefore a potential habitat for psychrophilic microorganisms. They successfully enriched *Acidithiobacillus*-like organisms and heterotrophic acidophiles from this environment. The bacteria assimilated ^{14}C-labelled bicarbonate and glucose below 0°C, but were found to be only cold-tolerant rather than psychrophilic.

Acid rock drainage (ARD), resulting from the microbially catalysed oxidation of sulfide minerals, becomes increasingly significant in polar regions as global warming causes increased glacier melting and thereby the exposing of more sulfide-rich rock strata to air. Schwertmannite present in glacier ice and icebergs of the Antarctica and Artic was the first indication of pyrite oxidation in this area. Dold *et al.* (2013) investigated the microbial community causing ARD on the Antarctic landmass, which was exclusively dominated by bacteria especially related to *Thiobacillus plumbophilus* and *At. ferrivorans*, both of which are psychrotolerant. Other bacteria in these Antarctic samples were species typically detected in acidic environments including *Frateuria*-like species, *Acidisphaera*, *Actinobacteria* and *Acidobacteria*.

Populations in mine-impacted environments

The best-studied low-pH environments are man-made and mostly associated with the mining of metals and coals. Pyrite becomes exposed to air (oxygen) and water during mining and oxidizes to sulfuric acid and ferric iron. However, the prime oxidant of pyrite is ferric iron, and sulfate is only the final product with the highest oxidation state, meaning that sulfur compound intermediates such as thiosulfate occur in the oxidation pathway (Vera *et al.*, 2013; see also Chapters 1 and 8).

Organisms in such habitats mainly catalyse carbon, iron and sulfur transformations and are widespread among the domains *Bacteria* and *Archaea*, and some *Eukarya*. In general, members of the *Proteobacteria* phylum are by far the most common representatives of *Bacteria* in mine-impacted environments followed by *Nitrospirae*, *Actinobacteria*, *Firmicutes* and *Acidobacteria*, while members of the *Bacteriodetes*, and Candidate division TM7 occur only in low numbers (Chapter 1).

In contrast to geothermal sites, which mainly comprise sulfur-oxidizing microorganisms that do not oxidize ferrous iron, few of these organisms are detected in AMD and one explanation might be that reduced sulfur compounds resulting from

pyrite oxidation can also be oxidized to sulfate by ferric iron (Druschel *et al.*, 2003) and are thus not available as substrate for acidophiles. The numerically dominant microorganisms in AMD sites are often iron-oxidizing microorganisms (many of which also oxidize sulfur), as iron is often the dominant metal in these environments. A well-known iron-oxidizer frequently found in acidic mine waters is *At. ferrooxidans* (Colmer *et al.*, 1950), which is also capable of oxidizing reduced sulfur compounds and hydrogen, and of ferric iron respiration under anaerobic conditions. *At. ferrooxidans* (and related iron-oxidizing acidithiobacilli) is mostly found in AMD-environments of low pH and moderate temperature. Another common iron-oxidizing bacterium in acidic environments is *L. ferrooxidans*, which has a higher affinity for ferrous iron and a greater tolerance of ferric iron than *At. ferrooxidans*, and therefore correlates with changing ferrous iron concentrations in the mine waters. Heterotrophic acidophilic bacteria, mostly belonging to the *Alphaproteobacteria* class (e.g. *Acidiphilium, Acidocella, Acidisphaera*), also play an important role in the carbon cycle of mine waters. Archaea have also been reported to be less abundant than bacteria in mine-impacted environments as most of them are (moderate) thermophiles and are more frequently found in geothermal areas. One archaeon, however, which is often found in association with AMD is the mesophilic, iron-oxidizer *Ferroplasma acidiphilum* (Golyshina *et al.*, 2000). Eukaryotes often only account for a minor portion of the microbial community in some AMD environments, but have been studied to a great extent e.g. in the Rio Tinto, Spain (e.g. González-Toril *et al.*, 2003) and to some extent at the Richmond Mine, California, USA (e.g. Edwards *et al.*, 1999).

Microorganisms in these environments often occur as biofilms on mineral surfaces, microbial stalactites, snottites, macroscopic growths in streams as well as in the planktonic phase of acidic water bodies, streams and pit lakes. Examples of the most intensively studied AMD environments are listed in Table 10.2 and some relevant and microbial diverse sites are further described below. Additional examples from extremely acidic environmental and industrial habitats are given in Chapters 9 and 17.

Microbial populations at various mine sites

Richmond Mine, Iron Mountain

One of the most intensively studied and most extreme acidic site is the Richmond Mine at Iron Mountain in California, comprising natural underground water pools of negative pH which are the lowest pH environments discovered yet (Nordstrom *et al.*, 2000). The Richmond Mine harbours a broad range of biodiversity and has been the source of several new species of iron-oxidizing prokaryotes (e.g. Tyson *et al.*, 2004; Edwards *et al.*, 1999; Table 10.2). Oxidative dissolution of pyrite and other sulfide minerals occurs at greatly accelerated rates within Iron Mountain causing increased temperatures within the mine (Nordstrom *et al.*, 2000).

The higher temperature sites of the mine have been found to be dominated by sulfur-oxidizing *Sulfobacillus* spp., while low-pH sites of the same temperature range were colonized by the iron-oxidizing archaeon 'Ferroplasma acidarmanus' (Bond *et al.*, 2000). Higher pH and cooler temperature areas are mainly dominated by *At. ferrooxidans*, while *Leptospirillum* spp. are widely distributed throughout the mine. All three groups of leptospirilli, *L. ferrooxidans* ('Group I'), *L. ferriphilum* and '*L. rubarum*' ('Group II'), '*Leptospirillum ferrodiazotrophum*' ('Group III'), are present in the Richmond Mine and recently a fourth group '*Leptospirillum* group IV UBA BS' has been detected (Aliaga Goltsmann *et al.*, 2013). Underground slime biofilms and acid streamer growths within the Richmond Mine were dominated by *Leptospirillum* spp. and '*Ferroplasma acidarmanus*', accompanied by the facultative heterotroph *Ferrimicrobium acidiphilum* and *Deltaproteobacteria* (Edwards *et al.*, 1999).

Genomic (Tyson *et al.*, 2004) and proteomic (Ram *et al.*, 2005) studies of biofilm communities within Iron Mountain carried out by the Banfield group have provided further insights into the nature of the microbial communities and motivated revision of the *Leptospirillum* and *Ferroplasma* taxonomy (e.g. *Leptospirillum* group IV, *Ferroplasma* group II). One particularly finding revealed by community genomic analysis was the presence of novel archaeal lineages designated as ARMAN (Archaeal Richmond Mine Acidophilic Nanoorganisms; Baker *et al.*, 2006), which form a deep branch within the *Euryarchaeota*. The cells of

Table 10.2 Characteristics of selected mine-impacted sites, and dominant microorganisms detected

	Richmond Mine, Iron Mountain	Parys Mountain (Mynydd Parys)	Cae Coch	Drei Kronen und Ehrt	Dyffryn Adda (draining Mynydd Parys)	Cantereras	Rio Tinto
Location	California (USA)	North Wales	North Wales	Germany	North Wales	Spain	Spain
Type of mine	Copper	Copper	Pyrite	Pyrite	Copper	Copper	Pyrite
Microbial growth	Mine water Biofilms Streamer growth	Underground lake Drapes	Water pools Water droplets Streamer growth Stalactites	Stalactites	Mine water Streamer growth	Mine water Streamer growth	Mine water Biofilms Streamer growth Sediments
pH	<0–2.0	2.3	1.8–2.3	2.6	2.5	2.5–2.8	2.2–4.7
Temperature (°C)	30–50	8–9	8–9	15	11	–	17.9
Dominant bacteria	*Leptospirillum* spp. *Sulfobacillus* spp. *At. ferrooxidans*	Iron-oxidizing acidithiobacilli *Acidobacteriaceae*	*At. ferrivorans* '*Fv. myxofaciens*' *Gallionella* sp.	*Leptospirillum* sp. '*Ferrovum*' sp.	*At. ferrivorans* '*Fv. myxofaciens*' '*Atx. ferrooxidans*'	*At. ferrivorans* *Acidobacteriaceae* *Acidiphilium* spp.	*L. ferrooxidans* *At. ferrooxidans* *Acidiphilium* spp.
Dominant archaea	'*Fp. acidarmanus*' ARMAN group	Euryarchaeota	Euryarchaeota	ARMAN-group *Thermoplasmatales*	Euryarchaeota	Euryarchaeota	*Ferroplasma* spp. *Thermoplasma* spp.
Eukarya	Algae Fungi	–	–	–	*Euglena mutabilis*	*Chlamydomonas acidophila*	Algae Protists Yeasts Fungi

ARMAN-members are of extremely small size and sequences have been detected in various locations of the Richmond Mine and also other habitats (e.g. the Drei Kronen und Ehrt mine; Ziegler *et al.*, 2013). The Richmond Mine, similar to the Rio Tinto in Spain, is also one of the best studied habitats of acidophilic eukaryotes, and harbours species of, for example, red algae, fungi and protists (*Vahlkampfiidae*, *Dothideomycetes* and *Eurotiomycetes*; Baker *et al.*, 2009, 2004). A more in-depth account of the microbiology of the metagenomics of the Richmond Mine site can be found in Chapter 14.

Cae Coch pyrite mine

In contrast to the relatively high temperature Richmond Mine site, two low-temperature and extreme acidic sites in north Wales, UK, Cae Coch mine and Mynydd Parys mine, have been extensively studied by Johnson and colleagues. The abandoned Cae Coch pyrite mine contains several water accumulations in form of several small pools and one single drainage stream. Microbial growth occurs as biofilms on the walls, stalactites, streamer growths within the drainage stream and in water droplets on the ceiling and stalactites. The microbial community of the water droplets is relatively simple and dominated by the iron-oxidizing *At. ferrivorans* and smaller numbers of iron-oxidizing *L. ferrooxidans* (Table 10.2). The streamer and slime communities, in contrast, are more diverse and vary between the different sampling locations, but are mostly dominated by the streamer-forming iron-oxidizer '*Ferrovum myxofaciens*', which has been detected as a dominant bacterium in a number of acidic environments (Johnson *et al.*, 2014). '*Fv. myxofaciens*' often occurs as macroscopic streamer growths due to its ability to produce copious amounts of extracellular polymeric substances (EPS). This bacterium, like *L. ferrooxidans*, appears to be only capable of growth by ferrous iron oxidation and fixes carbon dioxide and nitrogen (Johnson *et al.*, 2014). The dominance of '*Fv. myxofaciens*' in the stream correlates well with the occurrence of *At. ferrivorans* in the mine, as both species are psychrotolerant and capable of growth at low temperatures as is characteristic of the Cae Coch mine (9 +/− 1°C). One of the stalactites analysed was predominantly colonized by bacteria closely related to the neutrophilic iron-oxidizer *Gallionella ferruginea*. There have been a number of other reports on the occurrence of these proposed acidophilic/acid-tolerant iron-oxidizing *Gallionella* spp. in several mine-impacted environments (e.g. Fabisch *et al.*, 2013; Heinzel *et al.*, 2009). Minor numbers of *Alphaproteobacteria* (*Acidiphilium* spp. and a novel *Sphingomonas*-isolate), *Gammaproteobacteria* (*At. ferrivorans* and bacteria related to the unclassified species 'WJ2'), *Actinobacteria* (*Ferrimicrobium*-like bacteria), *Nitrospirae* (*L. ferrooxidans*) and *Firmicutes* were also detected in the streamer and slime growths. Archaeal 16S rRNA genes related to a single, novel euryarchaeotal species were detected in one pool characterized by lower pH and higher dissolved solutes than in the other sampled water bodies. With *At. ferrivorans*, '*Fv. myxofaciens*', *L. ferrooxidans* and the proposed iron-oxidizing *Gallionella* spp. identified as the dominant organisms, iron oxidation seems to be the main metabolism in this pyrite mine (Johnson, 2012).

Mynydd Parys copper mine

The flooded underground copper mine, Mynydd Parys (Parys Mountain), north Wales, represents a similar extreme acidic subsurface environment to the Cae Coch mine, though the underground lake is essentially anoxic. When the mine was partially dewatered, due to concerns that a concrete dam could fail and result in the flooding and severe pollution of a nearby coastal town, an opportunity to study the microbial community within the mine arose. The subterranean waters were dominated by iron-oxidizing *Acidithiobacillus* spp., and even so the exact species was not determined at this time, the dominance of *At. ferrivorans* is most likely due to the low temperatures and subsequent studies (Coupland and Johnson, 2004). As in the Cae Coch mine, *L. ferrooxidans*, *Gallionella*-related species, heterotrophic acidophiles (*Acidiphilium*, *Acidobacterium*, *Acidisphaera*) and *Fm. acidophilum* were present in the samples. Archaeal 16S rRNA gene sequences detected in the Parys Mountain underground lake were, however, very different to the Cae Coch archaea with clones related to potential methanogens and only minor numbers of *Thermoplasma*/*Ferroplasma*-like species (Coupland and Johnson, 2004; Table 10.2). Macroscopic growths hanging from pit props within the mine had a different microbial composition and were dominated by the heterotrophic iron-bacteria *Ferrimicrobium*, *Acidimicrobium* and the iron-reducing genus

Actinobacterium as well as a variety of archaea similar to those detected in the streamers (Coupland and Johnson, 2008). Owing to the limited oxygen ingress reported for Mynydd Parys, activities of iron-oxidizing microorganisms seemed to be low, but ferric iron reduction catalysed by autotrophic and heterotrophic bacteria, as well as due to reaction with pyrite and other residual sulfide minerals, led to ferrous iron being the dominant form in the subterranean lake (Johnson, 2012).

Other mine sites

Similar structures and microbial communities as described for the two former mine sites were also found in the Drei Kronen und Ehrt pyrite mine in the Harz mountains in Germany. Ziegler *et al.* (2009) studied small microbial stalactites and identified the iron-oxidizers *L. ferrooxidans* and '*Fv. myxofaciens*' as dominant bacteria and smaller numbers of *Acidiphilium* spp.. They also detected archaea related to the ARMAN-group, previously detected in the Richmond Mine, and uncultivated members of the *Thermoplasmatales* (Ziegler *et al.*, 2013; Table 10.2).

Mine sites with a more moderately acidic to circumneutral pH, as a habitat for moderate acidophilic and acid-tolerant microorganisms, have also been studied. One of these is the former Wheal Jane tin mine in Cornwall (England) which harbours similar microorganism as the Parys mine but is dominated by moderate acidophilic iron-oxidizing species closely related to *Halothiobacillus neapolitanus* (Hallberg and Johnson, 2003).

Various other mine sites across the world, which will not be discussed in detail, have been analysed for their microbial composition: e.g. metal mines in China (e.g. Hao *et al.*, 2010; He *et al.*, 2007), the Königsstein uranium mine in Germany (Seifert *et al.*, 2008) and the Los Rueldos mercury mine in Spain (Mendez-Garcia *et al.*, 2014).

Acidic streams draining abandoned mines

Dyffryn Adda

Closely connected to the Mynydd Parys copper mine microbiology described above is the microbial colonization of a stream bed, the Dyffryn Adda, into which AMD draining the abandoned mine was diverted in 2003. Kay *et al.* (2013) described a study of this stream carried out for 9 years following its inception (Table 10.2). A few months after it carried AMD, the stream became heavily colonized with macroscopic streamer growths, which continued to occupy a large part of the stream over the entire study period. The prokaryotic community of the streamer growths was dominated by the autotrophic iron-oxidizers *At. ferrivorans* and '*Fv. myxofaciens*' and a novel heterotrophic iron-oxidizer ('*Acidithrix ferrooxidans*'). As time progressed acidophiles, such as iron-reducing heterotrophs of the genus *Acidiphilium*, *Acidobacterium*, *Acidocella* and *Metallibacterium*, as well as the autotrophic iron-oxidizers *At. ferrooxidans* and *L. ferrooxidans*, were detected in the stream. Archaeal species found in the Dyffryn Adda were all distantly related to known *Euryarchaeota*. The surface of the stream becomes occasionally colonized by the algae *Euglena mutabilis*, which might be one cause of the varying abundance of heterotrophic bacteria within the stream as the algae provide organic carbon that can sustain their growth (Ñancucheo and Johnson, 2012). The relatively constant physico-chemical parameters supported a stable microbial community in the stream over the years; with most organisms originating from the underground mine water of Parys Mountain. The only notable difference between the Parys mine underground water and the Dyffryn Adda stream is the dominance of '*Fv. myxofaciens*' in the stream, which was not detected within the mine itself, possibly because it is an obligate aerobe.

Cantareras

Similar physico-chemical parameters to the waters draining Mynydd Parys was reported in a stream draining the small abandoned Cantareras copper mine in south-west Spain (Table 10.2). The microbial community inhabiting stream water draining the mine and the stream sediment was found to be dominated by the psychrophilic iron-oxidizer *At. ferrivorans* and heterotrophic bacteria (Rowe *et al.*, 2007). The microbial mat-like structures were populated by acidophilic algae (*Chlamydomonas acidophila*). Even so the mat was dominated by iron-oxidizing bacteria, high concentrations of ferrous iron could be detected, which is explained by the presence of heterotrophic iron-reducing microorganisms (*Acidiphilium* spp., *Acidobacteriaceae*) whose growth was postulated to be supported by

organic carbon released by the algae. Another acidophilic alga, *Chlorella protothecoides* var. *acidicola*, was isolated from the mat at Cantareras (Nancucheo and Johnson, 2012) and had an optimum growth pH of 2.5 tolerating elevated concentrations of various transition metals. Novel acidophilic sulfate-reducing bacteria (designated as 'Desulfobacillus acidavidus') were detected and isolated from the anaerobic, sulfate-rich bottom layer of the Cantareras stream (Rowe *et al.*, 2007). Archaeal sequences related to the *Euryarchaeota* could be detected in the mine water and the streamer growths of Cantareras.

Rio Tinto

The Rio Tinto is a long (ca. 100 km) and extremely acidic (pH 2.0–2.5), river, which originates from the Peňa de Hierro in south-west Spain and enters the Atlantic Ocean at Huelva (Table 10.2). The microbial communities of the Tinto river have intensively been studied by Amils and colleagues, and are described in more detail in Chapter 17. The biogeochemical transformations of iron dominates the microbial ecology of the Tinto river, acting as both electron donor and electron acceptor for chemoautotrophic (*L. ferrooxidans*, *At. ferrooxidans*) and heterotrophic (*Acidiphilium* spp., *Fm. acidiphilum* and a moderately acidophilic iron-oxidizer related to strain WJ2) acidophiles (Gonzalez-Toril *et al.*, 2003). Bacteria involved in the sulfur redox cycle in Rio Tinto include *At. ferrooxidans*, *At. thiooxidans* and the sulfate-reducing *Desulfosporosinus* spp.. Macroscopic growth occurring in some parts of the river are devoid of iron-metabolizing acidophiles, predominantly colonized by *Alphaproteobacteria* (including *Sphingomonas*-like bacteria) with smaller numbers of *Betaproteobacteria*, *Actinobacteria* and *Firmicutes* (Lopez-Archilla *et al.*, 2001). Archaea related to the genera *Ferroplasma* and *Thermoplasma* have also been detected in Rio Tinto, although they account for only a small proportion of the total prokaryotic population. The most remarkable trait of the Tinto river is, however, the unexpectedly high diversity of eukaryotes detected (Aguilera, 2013). The algae are the main primary producers in the river and are mainly represented by diatoms and *Euglena mutabilis* (Lopez-Archilla *et al.*, 2001). Further eukaryotes detected in the Rio Tinto are other photosynthetic algae, protists, yeasts and filamentous fungi (Aguilera, 2013), which are described in detail in Chapter 7.

Acidic pit lakes

Pit lakes form as a result of opencast mining of coal or metals when the abandoned voids become filled with groundwater. Oxidation of sulfide minerals in adjacent rocks or dumps leads to acidification of these lakes and release of sulfate, iron and other metals (Geller *et al.*, 1998). Due to the high iron content of pit lakes, ferric minerals are precipitated and form sediments which often leads to the adsorption of phosphorus and lead to oligotrophic conditions developing in most acid pit lakes (Kleeberg and Grüneberg, 2005). Acidic pit lakes can be found in mining areas of e.g. China, Australia, Poland, Spain and Germany, whereby the two later areas have been studied most intensively.

Many pit lakes in Germany resulting from lignite coal mining have an acidic pH (2.0–4.0) and are rich in sulfate and iron with minor amounts of toxic metals (Geller *et al.*, 1998), while concentrations of nutrients such as nitrate, phosphate, and ammonium, are very low. The maximum depth of the lakes is 11 m and they receive most of their inflow by a ditch connecting the lakes and only secondarily from the aquifer. Most of the pit lakes have an anoxic hypolimnion during summer stratification and a complete mixture of the water column occurs during winter (Blodau *et al.*, 1998). The phytoplankton community commonly observed in these lakes is dominated by the phytoflagellates *Ochromonas*, *Chlamydomonas* and *Gymnodinium* (Beulker *et al.*, 2003) which is influenced by the availability of inorganic carbon. Microbial oxidation of iron occurring in the oxic zones of these lakes results in the sedimentation of ferric iron minerals (e.g. schwertmannite, goethite) serving as electron acceptor for acidophilic iron reducing bacteria (*Acidiphilium* spp., *Acidobacterium* spp.) under oxygen-limited conditions. The pH of the lakes increases with depth causing the microbial communities (including Fe(III)-reducing microorganisms) to be more heterogeneous than those in the surface waters. The acidic sediments of 'lake 77' in the Lower Lusatia area (Germany) were found to be dominated by moderately acidophilic *Acidobacterium* spp., which were able to catalyse the reductive dissolution of ferric iron minerals (Blöthe *et al.*, 2008). *Acidithiobacillus* spp. and *Acidocella* spp. were more dominant in the acidic sediments, while *Bacillus* spp. and *Alicyclobacillus* spp. were isolated from moderate acidic sediments of the

lake and *Acidiphilium* spp. could be found in almost all sediment depths (Lu *et al.*, 2010; Blöthe *et al.*, 2008). Acidic conditions in the upper sediments of the lakes are stabilized by the cycling of iron which restricts fermentative and sulfate-reducing activities, but as reactive iron decreases and pH increases with sediment depth, sulfate-reducers (e.g. *Desulfosporosinus* spp.) have been detected (Koschorrek, 2008). Enhanced sulfate-reduction occurred in the sediments of the acidic mining lake '*Gruenewalder Lauch*' where the sediment was covered by a thick layer of periphytic algae separating the sediment (pH 6) from the acidic water (pH 3.1) and provided large amounts of organic carbon as electron donors (Koschorreck *et al.*, 2007). Sulfate-reduction in lake sediments below pH 4.5 has also been reported for other environments (e.g. Koschorreck *et al.*, 2007; Gyure *et al.*, 1990; Satake, 1977) and is usually related to increased influx of organic matter and the inhibition of competing iron-reducing bacteria and methanogens (Koschorreck, 2008).

Pit lakes located in Spain are the consequence of metal mining rather than coal mining, and are biochemically more complex as they can contain various transition metals (e.g. Zn) and metalloids in addition to iron and sulfate. There are more than 25 pit lakes in the Iberian Pyrite Belt (IPB), which hosts one of the world's largest accumulations of mine wastes and AMD, including the Rio Tinto. The lakes in the IPB are characterized by low pH and high concentration of heavy metals, but each lake presents unique characteristics. The microbiology of two acidic, sulfate- and metal-rich pit lakes in the IPB, Nuestra Señora del Carmen and Concepción, was described by Santofimia *et al.* (2013). In Nuestra Señora del Carmen, a chemically and thermally stratified pit lake consisting of two different layers, they detected typical AMD microorganisms involved in iron redox-cycling. The upper layer of the lake was dominated by heterotrophic *Acidiphilium* spp. and accompanied by iron-oxidizing *Leptospirillum* spp. and *Planctomycetes*. Eukaryotic sequences detected in the upper layer belonged to the genus *Chlamydomonas* and filamentous species and diatoms were observed by microscopy. The lower layer (15 m) of the lake, which exhibited a slightly reduced environment and equal concentrations of ferrous and ferric iron, was more diverse and mainly represented by *Acidithiobacillus* spp., *Acidimicrobiaceae*, both facultative iron-reducers,

leptospirilli, *Actinobacteria*, and archaeal members of the *Thermoplasmata*. The microbial diversity in the Concepción pit lake was more diverse, but dissolved iron concentrations were low and therefore less typical AMD microorganisms could be detected. In the lower layers, however, where dissolved iron concentrations increased, iron-oxidizing '*Fv. myxofaciens*' and potential iron-reducing *Acidiphilium* spp., *Acidimicrobaceae* and *Acidobacteria* were present. Possibly because of the low concentrations of sulfate and transition metals in this lake, members of AMD atypical genera (*Erwinia*, *Legionella* and *Halomonas*) were also detected.

Falagan *et al.* (2014) studied two other meromictic stratified pit lakes in the IPB, Cueva de la Mora and Guadiana, which were claimed to have the most dramatic vertical pH gradients and water chemistry of pit lakes in the region. Cueva de la Mora has three layers, the oxygenated mixolimnion, the chemocline and the more anoxic monimolimnon. Most interesting was the observed enhanced accumulation of algal biomass well below the lake surface, which was thought likely due to the highly variable bioavailability of phosphorus in these water bodies. Acidophilic micro-algae were reported to be the primary producers in the lake, providing oxygen and organic carbon and thereby supporting growth of heterotrophic acidophiles (e.g. *Acidobacteriaceae*), which were dominant in the mixolimnion and the chemocline. The chemocline was quite diverse and populated by iron-oxidizing *L. ferrooxidans*, iron-/sulfur-oxidizing and facultative iron-reducing *At. ferrooxidans* and sulfate-reducing *Desulfomonile* sp. Based on chemical parameters and the various microbial metabolisms present, the chemocline seems to be the zone where dynamics of carbon, iron and sulfur redox transformations were most dynamic. Archaeal sequences detected in the water column and sediments of this lake belonged to the *Eukaryota* and few sequences to the recently proposed *Thaumarchaeota*, possibly mediating the transformation of nitrogen.

Mine water treatment systems

Mine waters pose serious threats to the environmental, owing to their high metal and sulfate loads and (often) their low pH, and therefore require treatment prior to release in receiving water courses. The ability of bacteria found in acidic environments

to mediate iron and sulfur redox reactions can be advantageous in mine water treatment to remove toxic metals and sulfate and raise pH.

A pilot passive treatment plant was constructed at the Wheal Jane tin mine in Cornwall (England) to remediate acidic (pH 3.4), metal-rich water draining the mine utilizing naturally occurring microorganisms (Hallberg and Johnson, 2005). The treatment plant consisted of three separate composite systems, comprising a series of aerobic wetlands for iron oxidation and precipitation, a compost bioreactor for removing chalcophilic metals and to generate alkalinity, and rock filter ponds for removing soluble manganese and organic carbon. Moderately acidophilic iron-oxidizing bacteria (related to *Halothiobacillus neapolitanus*) and later heterotrophic acidophiles were the dominant cultivatable bacteria in the Wheal Jane AMD. Heterotrophic acidophiles (*Acidiphilium* spp., *Acidobacterium*-like) and smaller numbers of moderately and extremely acidophilic iron-oxidizing bacteria (*Acidithiobacillus* spp., *Leptospirillum* spp.) were isolated from the surface waters and sediments of the constructed aerobic wetlands. The dominant microbial isolate in waters draining the anaerobic compost bioreactors was an iron- and sulfur-oxidizing moderate acidophile closely related to *Thiomonas intermedia*. Acidophiles enumerated at the Wheal Jane plant only accounted for up to 25% of the total microbial population.

The Carbondale constructed wetland in Waterloo Township (Ohio, USA) was constructed to treat coal mine drainage of more or less constant pH (2.0–3.9), metal and sulfate concentrations. Each wetland cell was layered with mushroom or manure compost followed by a limestone layer. The microbial community of the oxidized surface of the wetland was dominated by iron–sulfur oxidizing *Acidithiobacillus* spp. and accompanied by iron-oxidizing *Actinobacteria* and '*Fv. myxofaciens*' (Nicomrat *et al.*, 2008).

Another example is an acid mine water treatment plant in the Lusatia area (Germany) operated at pH 3.0 and continuously fed with mine water pH 4.5 containing an elevated concentration of iron (and sulfate) which is microbially oxidized to form schwertmannite. The iron-oxidizing community in the plant, which proofed to be very stable (Heinzel *et al.*, 2009), was dominated by the iron-oxidizing betaproteobacterium '*Ferrovum*' and often accompanied by an acidophilic organism related to the neutrophilic iron-oxidizer *Gallionella ferruginea* (Heinzel *et al.*, 2009). Despite being inoculated with more familiar iron-oxidizing acidophiles (*At. ferrooxidans* and *L. ferrooxidans*) at the beginning of the plant operation, '*Ferrovum*' and the *Gallionella*-related bacterium prevailed in the plant.

Mine heaps and waste rock dumps

From an industrial viewpoint, sulfidic mine deposits arrangements can be divided into commercial heap and dump bioleaching operations, and waste rock dumps and tailings which may generate acid mine drainage (AMD). Heap and dump bioleaching is used extensively around the world for copper extraction with an estimated 15% of the world's copper produced (Brierley and Brierley 2013; Schippers *et al.* 2014; see also Chapter 17).

Waste rock originates from mining and comprises of rocks of different size in which the metal content is too low for an economically feasible ore processing. Tailings are the fine grained remains of ore processing which are dumped as a slurry (Fig. 10.1). Both kinds of mine waste often contain high amounts of iron sulfide minerals (pyrite or pyrrhotite) and often produce AMD due to chemical and microbial metal sulfide oxidation. Over a period of several years or decades, an oxidized zone with depleted sulfide content, low pH, and enrichment of secondary minerals develops above an unoxidized zone with unaltered material in tailings dumps (Fig. 10.2; Korehi *et al.*, 2013; Schippers *et al.*, 2010).

The biogeochemical processes in sulfidic waste rock dumps and tailings are similar to those in commercial heap bioleaching operations. At low pH < 4, the biological metal sulfide oxidation by acidophilic, chemolithoautotrophic iron- and sulfur-oxidizing bacteria and archaea, dominates over chemical oxidation. These prokaryotes can dissolve pyrite very efficiently, and the biological pyrite oxidation rate has shown to be two orders of magnitude or greater than the chemical rate. At pH below 2.5, ferric iron is soluble and serves as a more efficient oxidant for metal sulfides than molecular oxygen. By reacting with a metal sulfide, ferric iron is being reduced to ferrous, the substrate for Fe(II)-oxidizing acidophiles. In addition, ferric iron can act as electron acceptor for acidophiles that oxidize RISCs (or organic carbon) in the absence of oxygen. Elemental sulfur and RISCs are formed

Figure 10.1 Depositing sulfidic mine tailings as slurry on top of the tailings dump (ore processing plant in the background).
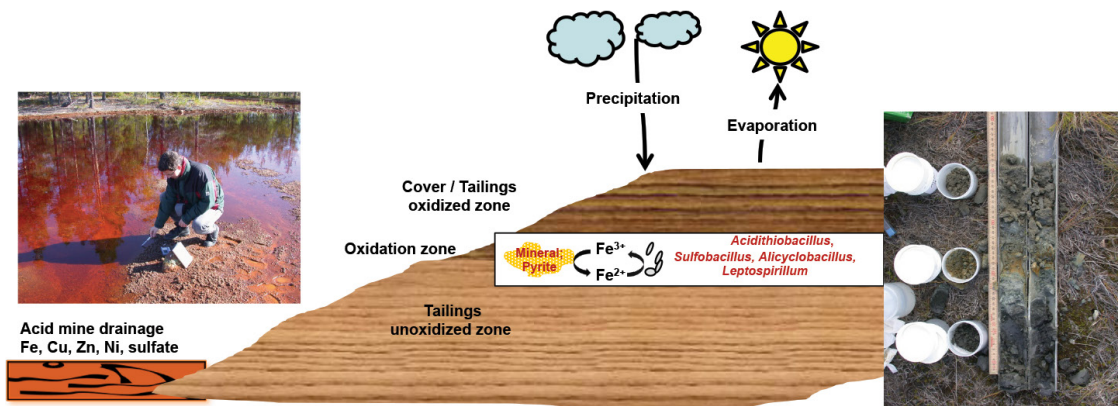
Figure 10.2 Acid mine drainage releasing sulfidic mine tailings with developed different zones due to microbially driven pyrite oxidation (modified from Korehi *et al*., 2013).

as intermediates in the metal sulfide oxidation process with sulfate as the most oxidized sulfur form (Schippers *et al.,* 2010, 2014; Vera *et al.,* 2013).

Since concentrations of dissolved organic carbon are usually very low in mine heaps and dumps, microbial metal sulfide oxidation is the dominant biogeochemical process at low pH and chemolithotrophic, acidophilic iron- and sulfur-oxidizing bacteria are often the dominant members of the microbial community. In addition, ferric oxyhydroxides can be dissolved by ferric iron-reducing

microorganisms, with the consequent release of adsorbed metals and metalloids (such as As). In contrast, if sulfate reduction is occurring, metals may precipitate as metal sulfides within the dump (Schippers *et al.,* 2010).

As the main geochemical processes in sulfidic mine dumps and heaps are catalysed by microorganisms, analysis of microbial communities is vital to understand the scale and nature of these transformations. Microbial analysis helps understand which organisms are responsible for these processes,

where in a dump or heap a particular process takes place, where zones of high or low microbial activity occur, and how the kinetics of particular processes, e.g. pyrite oxidation or sulfate reduction, can be influenced by stimulating or inhibiting microbial activity. Monitoring microbiology is also useful for evaluating the success of AMD countermeasures (Sand *et al.*, 2007) or heap leaching operations (Remonsellez *et al.*, 2009).

In the Escondida mine in northern Chile, the extensive industrial copper heap leaching operation has been monitored by analysing 16S rRNA gene copy numbers of acidophiles in the pregnant leach solution over time. *At. ferrooxidans* was the most abundant organism during the first part of the leaching cycle, while the abundance of *L. ferriphilum* and *Fp. acidiphilum* increased with age of the heap. *At. thiooxidans* remained at similar levels throughout the leaching cycle, and *Firmicutes* group showed a low and a patchy distribution in the heap. *Acidiphilium*-like bacteria reached their highest abundance corresponding to the amount of autotrophs (Remonsellez *et al.*, 2009).

The microbial diversity in sulfidic mine dumps and heaps comprises aerobic and anaerobic species which are autotrophic (CO_2-fixation) or heterotrophic (C_{org} as carbon source) as well as lithotrophic (inorganic compounds as energy source) or organotrophic (C_{org} as energy source). A comprehensive list of microorganisms on the genus level from the three domains of life, *Bacteria*, *Archaea* and *Eukarya*, which have been detected in mine dumps or heaps by cultivation and molecular biological techniques, is shown in Tables 10.3 and 10.4. These lists are based on the results of published papers. Important physiological properties relevant to biogeochemical oxidation and reduction processes are given for each genus.

Most microbial genera detected in dumps and heaps belong to the domain *Bacteria* and comprise acidophilic species. The *Archaea* are mainly thermophiles and all of them are acidophiles. *Eukarya* detected in these environments include algae, fungi, yeasts and protozoa. The iron- and sulfur-oxidizing bacteria or archaea are responsible for metal sulfide oxidation. These microorganisms have been widely detected in mine waste tailings located in different climate zones. Ferric iron and oxidized sulfur produced by these *Bacteria* or *Archaea* may be used as terminal electron acceptors by obligately and

facultatively anaerobic acidophiles under anoxic conditions (Schippers *et al.*, 2010).

In addition to exploring the microbial diversity of sulfidic mine dumps and heaps, the quantification of particular organisms, e.g. acidophilic iron-oxidizers, in different areas and depth layers of a dump is of interest especially for monitoring purposes. Primarily cultivation techniques, i.e. solid media and most-probable-number (MPN), have been used to enumerate prokaryotes involved in oxidation and reduction processes in sulfidic mine dumps (Schippers *et al.*, 2010).

Far more studies have been carried out for mine tailings than for waste rock dumps. A study by Schippers *et al.* (2010) compared the numbers of relevant groups of microorganisms in selected waste rock and tailings dumps with different metal contents, pH values and located in eight countries in different climate zones. The maximum number of total cells ranged between 10^6 and 10^8 cells per gram waste rock or tailings material, and acidophilic iron- and sulfur-oxidizing bacteria represented a significant proportion of these. Large numbers of these organisms were detected in waste rock dumps and tailings all over the world. Neutrophilic sulfur-oxidizing bacteria oxidize the sulfur compounds formed by chemical sulfide oxidation at circumneutral pH. These bacteria were detected in most of the cases in similar numbers to acidophilic representatives. Acidophilic heterotrophic bacteria can facilitate the continued growth of autotrophic iron- and sulfur-oxidizing bacteria, and have also been detected in some cases. In waste rock dumps, maximum numbers of heterotrophic acidophiles were in the range of 10^3–10^6 cells/g dry sample, and tailings contained lesser numbers.

A quantification of the microbial communities in four different mine tailings was carried out by Kock and Schippers (2008) and Korehi *et al.* (2013) using both cultivation and molecular techniques. Depth profiles of cell numbers showed that the compositions of the microbial communities are significantly different at the four sites, and varied greatly between zones of oxidized and non-oxidized tailings. qPCR data indicated that *Bacteria* dominated over *Archaea* and *Eukarya* and that the genus *Acidithiobacillus* represented the most abundant iron- and sulfur-oxidizers detected. *Leptospirillum* and *Sulfobacillus* were detected in smaller numbers and not in all tailings dumps. Anaerobes such

Table 10.3 Bacteria detected in mine dumps or heaps and their physiological properties. Genus names are listed in alphabetical order. Genus names in bold means that the genus comprises acidophilic species

Genus	Oxidation of					Reduction of	
	Pyrite	Other MS	Fe(II)	Sulfur	C_{org}	Fe(III)	Sulfate
Bacteria							
Acidimicrobium	+		+		+		
Acidiphilium				+	+	+	
Acidisphaera					+		
Acidithiobacillus	+	+	+	+		+	
Acidobacterium					+		
Acidovorax					+		
Acinetobacter					+		
Alicyclobacillus	+	+	+	+	+	+	
Amycolatopsis					+		
Arthrobacter					+		
Bacillus					+	+	
Bradyrhizobium					+		
Brevibacterium					+		
Caulobacter					+		
Cellulomonas					+		
Chryseobacterium					+		
Comamonas					+		
Corynebacterium					+		
Desulfobacter					+	+	+
Desulfobacterium					+	+	+
Desulfobotulus					+		+
Desulfobulbus					+	+	+
Desulfococcus					+		+
Desulfonema					+		+
Desulfosarcina					+		+
Desulfosporosinus					+		+
Desulfotomaculum					+	+	+
Desulfovibrio					+	+	+
Enhydrobacter					+		
Ferribacterium					+	+	
Ferrimicrobium	+	+	+		+	+	
Ferritrophicum			+				
Gallionella			+				
Gemmatimonas							
Geobacter					+	+	
Hydrogenophaga							
Hyphomicrobium					+		
Legionella					+		
Leptospirillum	+	+	+				
Leptothrix					+		
Meiothermus					+		
Methylobacterium					+		

Table 10.3 Continued

Genus	Oxidation of					Reduction of	
	Pyrite	Other MS	Fe(II)	Sulfur	C_{org}	Fe(III)	Sulfate
Methylococcus					+		
Microbacterium					+		
Microvirgula					+		
Nocardiopsis					+		
Ochrobactrum					+		
Paenibacillus					+		
Peredibacter					+		
Pseudomonas				+	+	+	
Ralstonia					+		
Rubrobacter					+		
Sedimentibacter					+		
Sphingomonas					+		
Subtercola					+		
Sulfobacillus	+	+	+	+	+	+	
Thiobacillus		+		+	+		
Thiomonas		+		+	+		
Thiovirga		+		+			

MS, metal sulfide other than pyrite; sulfur, inorganic sulfur compounds; +, physiological property shown (modified from Schippers *et al*., 2010).

Table 10.4 Archaea and Eukarya detected in mine dumps or heaps and their physiological properties. Genus names are listed in alphabetical order. Genus names in bold means that the genus comprises acidophilic species

Genus	Oxidation of					Reduction of	
	Pyrite	Other MS	Fe(II)	Sulfur	C_{org}	Fe(III)	Sulfate
Archaea							
Acidianus	+	+	+	+	+		
Ferroplasma	+	+	+		+		
Metallosphaera	+	+	+	+	+		
Sulfolobus	+	+	+	+	+		
Sulfurisphaera				+	+		
Thermogymnomonas					+		
Eukarya							
Amoeba					+		
Cladosporium					+		
Euglena					+		
Eutrepia					+		
Hormidium							
Penicillium					+		
Rhodotorula					+		
Trichosporon					+		
Ulothrix							

MS, metal sulfide other than pyrite; sulfur, inorganic sulfur compounds; +, physiological property shown (modified from Schippers *et al*., 2010).

as sulfate-reducers or the neutrophilic iron- and manganese-reducing *Geobacteraceae* were detected in lesser abundance.

Several tailings studies have reported the predominance of iron- and sulfur-oxidizing acidophiles at low pH, but microbial communities at the moderately acidic oxidation stage (between the initial circumneutral to alkaline pH and the strong acidic final stage) have only been studied for few mine tailings sites (Liu *et al.*, 2014; Chen *et al.*, 2013). Microbial communities detected were found to be very different to those in tailings of < pH 3. For example microbial communities in lead/zinc mine tailings samples in China with a pH range of 1.8–7.5 revealed a predominance of *Proteobacteria*, including the hydrogen- and sulfur-oxidizing genera *Hydrogenophaga*, *Thiovirga* and *Thiobacillus*, respectively, in the circumneutral samples at the initial weathering stage, while gene sequences related to the acidophilic, iron-oxidizing genera *Ferroplasma* (*Euryarchaeota*), *Acidithiobacillus* (*Proteobacteria*), *Leptospirillum* (*Nitrospira*) and *Sulfobacillus* (*Firmicutes*) were mainly detected in samples of lower pH and more intense weathering and iron precipitations (Chen *et al.*, 2013). The finding that the composition of microbial communities in sulfidic mine tailings is mainly controlled by pH was also shown for copper mine tailings (Liu *et al.*, 2014) and supported by experimental evidence (Chen *et al.*, 2014).

Conclusions

Acidophiles can thrive in both natural and man-made environments. The most ancient sites where acidophilic life has developed over many decades represent geothermal areas which display high temperatures and are rich in sulfur compounds. Due to these physico-chemical parameters, such sites are mostly colonized by organisms that metabolize reduced sulfur compounds, and archaea dominate over bacteria at high temperatures. Mine-impacted environments, in contrast, often contain more soluble iron than reduced sulfur compounds and are therefore predominantly inhabited by microorganisms that can catalyse redox transformations of iron. Microbial growths, occurring as filamentous 'acid streamers' – growths in flowing waters, thick and often dense 'acid mats' – and 'pipes'/'snotites' – pendulous growths attached to mine roofs, are

characteristic of acidic environments. The microbial composition of these growths is determined by the physico-chemical parameters of the site, resulting in warm and extremely acidic streams being dominated by iron-oxidizing bacteria (*Leptospirillum* spp.) and archaea ('*Fp. acidarmanus*'), while in higher pH and more moderate mine waters other iron-oxidizing bacteria (*Acidithiobacillus* spp. and '*Fv. myxofaciens*') are more abundant. Numbers of heterotrophic species are often relatively low in acidic environments, as dissolved organic carbon levels are usually extremely low. However, extraneous inputs of dissolved organic carbon and that originating from indigenous acidophilic algae in acidic streams and pit lakes promotes the growth of heterotrophic bacteria, which may then the outnumber chemolithotrophic bacteria. The microbial ecology of extremely acidic environments can therefore be both complex and dynamic, and provides an exciting area for microbiological and molecular-based research.

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