

Microbial Ecology in Extreme Acidic Environments: Use of Molecular Tools

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14.1 INTRODUCTION

Exploration of the Biosphere has led to continued discoveries of life in environments that were previously considered uninhabitable. Thus, life can survive and sometimes thrive under what seem to be harsh environmental conditions. Extreme environments (defined from our anthropocentric view) usually possess various factors incompatible with most life forms. However, in spite of the apparent hostility of such habitats, they may contain a higher level of biodiversity than expected. The environment that we are familiar with is full of oxygen, never too cold nor too hot (most of the visible organisms live in a relatively limited range of temperatures from 5 to 40°C), and is protected from most damaging atmospheric radiation. This familiar world defines what we consider normal, and the organisms that live at the edges of this world or beyond it are the “extremophiles.”

The number of different organisms known to reside and thrive in these environmentally extreme conditions has grown rapidly in recent years. For example, we find robust microbial communities at high temperature ranges, i.e., the hot springs acidophilic algae (*Cyanidiaceae*), grow at 45–56°C, while the hyperthermophilic archaea tolerate a temperature range above the boiling point (>100°C) (Seckbach, 1994). Other organisms live in cold polar zones, such as the psychrophiles of the Antarctica, which are able to live in briny waters, with several times the salinity of seawater and temperatures below –10°C, beneath 20-m thick ice (Price, 2000). Similarly, there are microbes living in very alkaline environments (as high as pH 12) like the rose chafer larvae host (Curds et al., 1986). On the other end of the pH scale, there are the acidophilic archaea (i.e., *Thermoplasma acidophilum*) or the unicellular alga *Cyanidium caldarium* thriving in very acidic habitats (pH ranges from 0 to 4) (Seckbach, 1994). Some microbial communities have been isolated from hypersaline areas, lakes, or mines containing saturated salt solutions such as those present in the Dead Sea or are able to grow under potent ionizing radiation fields in nuclear reactors. We can also find some microbes that grow under extremely dry conditions and we find others that grow in the deepest parts of the oceans and require 500–1,000 bars of hydrostatic pressure. To survive, organisms can assume forms that enable them to withstand freezing, complete desiccation, starvation, high levels of radiation exposure, and other physical or chemical challenges. Furthermore, they can survive exposure to such conditions for weeks, months, years, or even centuries (Rothschild and Mancinelli, 2001).

Interest in discovering extreme environments and the organisms that inhabit them has grown over the past years due to both basic, the idea that extreme environments are believed to reflect early Earth conditions in which prokaryotes originally evolved and adapted, and applied aspects, i.e., extremophiles as sources of enzymes and other cell products. Although at first the extremophile organisms were only considered as scientific curiosities, their potential in the field of biotechnology is nowadays one of the main reasons for research studies. Extremophiles have made it possible to carry out numerous advances in different fields within biotechnology. These include the search for new bioactive compounds useful in industry, agriculture, environment, and pharmacology (Raddadi et al., 2015; Dumorné et al., 2017). In the past few years, a great range of bacteria, cyanobacteria, algae, and fungi have been isolated from these habitats. This is due to their large potential both in the biotechnical level and as a method of researching biomolecular stability.

Besides, extreme habitats are large sources of biodiversity and new adaptation mechanisms. In these habitats, evolution works with a special intensity. These are extreme, greatly selective, and confined habitats, which constitute a

favorable environment for the creation of a unique type of biodiversity and specific adaptation mechanisms. Extreme ecosystems are real sources of biological uniqueness. This makes these ecosystems especially interesting for the study of biodiversity and for the protection of the biological patrimony. Uniqueness and specificity also make these extreme habitats especially fragile. Two factors can end with this type of biodiversity specifically linked to the environment: breaking the balance in ecosystems which are already under extreme conditions and reestablishing the normal or nonextreme conditions in the environment.

On the other hand, microbiology has experienced a transformation during the last 25 years that has altered microbiologists' view of microorganisms and how to study them (Handelsman, 2004). The realization that most microorganisms cannot be grown readily in pure culture forced microbiologists to question their belief that the microbial world had been conquered. The development of molecular technologies and their application to microbial ecology has increased our knowledge in many different environments (Zhang et al., 2010). The advent of genome sequencing has opened new windows into the global microbiome. The genomes of >30,000 bacteria and >700 archaea (Genomes OnLine Database; <https://gold.jgi-psf.org>; December 2014) have provided key insights into, for example, their evolution and role in biogeochemical cycles, their modes of pathogenesis and antimicrobial resistance, and the genetic determinants underlying their biotechnological potential (Cowan et al., 2015).

Among the methods designed to gain access to the physiology and genetics of uncultured organisms, meta-“omics” techniques, the genomic analysis of a population of microorganisms, have emerged as a powerful centerpiece. Direct isolation of genomic DNA from an environment circumvents culturing the organisms under study, and cloning of it into a cultured organism captures it for study and preservation. Advances have derived from sequence-based and functional analysis in samples from environmental water and soil samples (Handelsman, 2004). This is particularly relevant in extreme environments, generally more difficult to replicate in the laboratory (Cowan et al., 2015). This chapter summarizes the current status of our knowledge of extreme acidic environments microbial ecology through the use of meta-“omic” molecular techniques.

14.2 EXTREME ACIDIC ENVIRONMENTS

Most of the best-characterized extreme environments on Earth correspond to geophysical constraints (temperature, pressure, ionic strength, radiation, etc.) in which opportunistic microorganisms have developed diverse adaptive strategies. However, extreme acidic habitats are unique in that they are actually produced by the metabolism activity of chemolithotrophic microorganisms and not a geophysical constraint of the habitat. Thus, one of the most interesting extremophile groups today is the one of the acidophile organisms, which can live with pH less than two. Under these conditions, the proteins become denatured and lose all their functional properties. For this reason, acidophilic organisms have developed systems to keep their cytoplasm to a neutral pH—this resistance to acids does not exclude extracellular proteins.

Most aquatic acidic habitats have two major origins, one associated to volcanic activities and the other to metal and coal mining (Johnson, 1998; Löhr et al., 2006) (Fig. 14.1). In the first case, acidity is mainly generated by the biological oxidation of elemental sulfur produced as a result of the condensation reactions among sulfur containing volcanic gases. In the second case, metal and coal mining expose sulfidic minerals to the combined action of water and oxygen, which facilitate microbial attack. Additionally, exposure of sulfide minerals to the combined action of water and oxygen produces acid mine drainage (AMD) or acid rock drainage (ARD), which are the cause of important environmental problems (Johnson and Hallberg, 2003). The most abundant sulfidic mineral, pyrite, is of particular interest in this context. Both habitats, volcanic and AMD/ARD areas, vary greatly in their physicochemical characteristics, and, as a consequence, in their microbial ecology. Acidic environments associated to mining operations are very recent on the geological and evolutionary scale, although some metal mining activities have a relatively long history (Edwards et al., 2000). The mechanism by which microbes obtain energy by oxidizing sulfide minerals, a process of biotechnological interest (biohydrometallurgy), was controversial for many years (Ehrlich, 2002) but the demonstration that the ferric iron present in the cell envelopes of leaching microorganisms is responsible for the electron transfer from insoluble sulfidic mineral substrates to the electron transport chain has done much to clarify the situation (Sand et al., 1995).

As we mentioned before, in the acidic habitats related to mining activity, the extreme conditions found in the environment are the product of the metabolic activity of chemolithotrophic microorganisms, mostly iron- and sulfur-oxidizing bacteria that can be found in high numbers in their waters. Their iron-oxidizing microorganisms are responsible for the solubilization of sulfidic minerals, mainly pyrite, and the correspondent high concentration of ferric iron, sulfate, and protons in the water column (Fernández-Remolar et al., 2003; González-Toril et al., 2003). Most of these chemolithotrophic prokaryotes are autotrophic, thus, in addition to promoting the extreme conditions of the habitat they are primary producers (López-Archilla et al., 2001; González-Toril et al., 2003). Acidophilic chemolithotrophs, especially

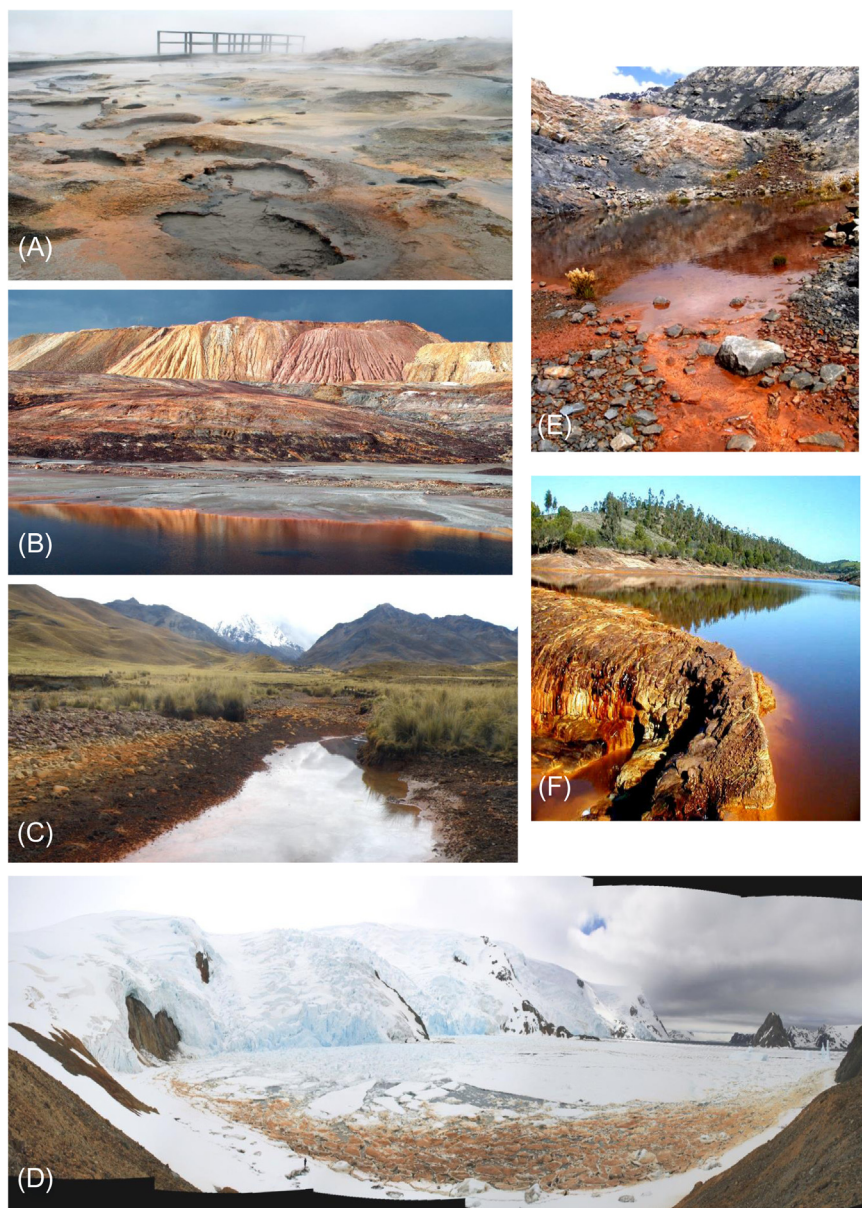


FIGURE 14.1 Examples of acidic extreme environments. (A) Geothermal area in Reykjanes Peninsula (SW, Iceland), (B) acidic mining drainage area in Río Tinto (SW, Spain), (C) acidic rock drainage area in Huascarán National Park (Perú), (D) acidic rock drainage area in Almirantazgo Bay (Shetland Islands, Antarctica), (E) acidic rock drainage area in Pachacoto river (Huascarán National Park, Perú), and (F) Río Tinto at Nerva area.

Acidithiobacillus ferrooxidans and *Leptospirillum* spp. accelerate the rate of pyrite oxidation (Edwards et al., 1999; González-Toril et al., 2003). At the same time, low pH facilitates metal solubility; therefore, acidic water tends to have high concentrations of heavy metals (Johnson, 1998).

14.3 MICROBIAL DIVERSITY IN ACIDIC ENVIRONMENTS

The advent of high-throughput sequencing technologies has allowed sampling microbial diversity more deeply and widely than ever before (Sogin et al., 2006) and thus affords new opportunities for comprehensively examining broader trends of microbial distribution with larger numbers of ecological samples (Huang et al., 2016). Acidic extreme environments are unique ecological niche for acid- and toxic-metal-adapted microorganisms. These low-complexity systems offer a special opportunity for the ecological and evolutionary analyses of natural microbial assemblages. The last decade has witnessed an unprecedented interest in the study of acidophilic communities using 16S rRNA high-throughput sequencing and community genomic and postgenomic methodologies, significantly advancing our understanding of microbial diversity, community function, and evolution in acidic environments (Huang et al., 2016).

14.3.1 Prokaryotic Microbial Diversity

The combined use of conventional and molecular microbial ecology methodologies has led to the identification of the most representative microorganisms in acidic environments (Johnson, 1998; González-Toril et al., 2003, 2006, 2011). Highest percent of the water column diversity corresponds to microorganisms belonging to three bacterial genera: *Leptospirillum*, *Acidithiobacillus*, and *Acidiphilium*, all members of the iron cycle (González-Toril et al., 2003). *Leptospirillum* are aerobic iron oxidizers while *At. ferrooxidans* can oxidize ferrous iron aerobically and reduce ferric iron in anaerobic conditions (Malki et al., 2006) (Fig. 14.2) *Acidiphilium* can oxidize organic compounds using ferric iron as electron acceptor. Interestingly enough, some *Acidiphilium* isolates can do so in the presence of oxygen (Coupland and Johnson, 2008; Malki et al., 2008). Although other iron-oxidizers (like the archaea *Ferroplasma* spp. and *T. acidophilum*) or iron reducers (*Ferrirobium* spp.) have been detected (González-Toril et al., 2003; González-Toril et al., 2010), their low numbers suggest that they play a minor role in the operation of the iron cycle, at least in the water column. Concerning the sulfur cycle, only *At. ferrooxidans* is found in significant numbers in the water column. This bacterium can oxidize both ferrous iron and reduced sulfur compounds. Reduced sulfur compounds can be oxidized aerobically and anaerobically. Certain sulfate-reducing microorganisms have been detected in the sediments in some locations along the river (Malki et al., 2006; García-Moyano et al., 2009; Sánchez-Andrea et al., 2011) (Fig. 14.3).

Using 16S rRNA pyrosequencing, Kuang et al. (2013) recently examined the biogeographic patterns of microbes from diverse AMD sites across. By analyzing 59 natural acidophilic assemblages across a broad range of spatial and physicochemical gradients, this study demonstrated that solution pH is the strongest predictor of community composition in these harsh environments. More importantly, this pH-dependent distribution was identified as a general pattern in a subsequent comprehensive meta-analysis based on molecular inventory studies from globally distributed AMD and associated environments regardless of the long-distance isolation and the distinct substrate types, implying the conservation of ecological niches exhibited by these highly specialized organisms (Fahy et al., 2015). Indeed, such pH-specific niche partitioning was clearly evident for the dominant lineages such as *Acidithiobacillus*, *Ferroplasma*, and *Leptospirillum* (Kuang et al., 2013). Consistent with this, previous studies have revealed that the lesser known “*Ferroplasma*” spp. are more acid-susceptible than the well-studied AMD species *At. ferrooxidans* and *Leptospirillum ferrooxidans* and prefer to grow under relatively high ferrous iron concentrations and moderate pH conditions (Rowe and Johnson, 2008; Hallberg, 2010).

Spatial analyses of specific sites using high-throughput molecular profiling approaches may also unveil fine-scale variations in microbial composition and the possible mechanisms of adaptation to local environmental stresses. By extensive sampling (and thus obtaining sufficient coverage of) a massive and heterogeneous acidic environments, Liu et al. attempted to resolve the local diversity of these organisms and how communities are shaped by the prevailing

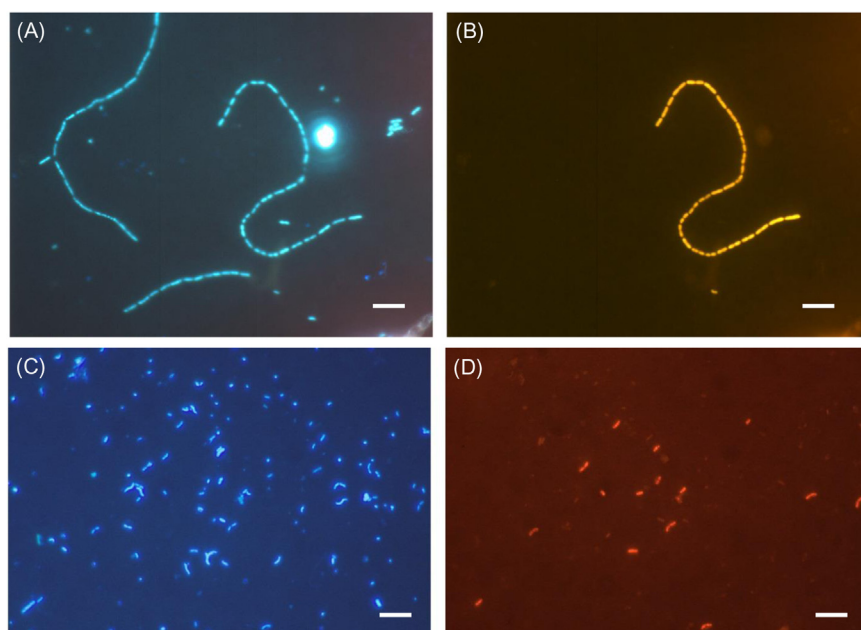


FIGURE 14.2 Acidic prokaryotic cells. (A) Epifluorescence micrographs of water column bacteria, 4',6-diamidino-2-phenylindole (DAPI)-stained cells. Bar = 5 μ m, (B) cells hybridized with ACT4675a probe (Cy3-labeled) specific for *Acidithiobacillus ferrooxidans* group *a*. Bar = 5 μ m, (C) epifluorescence micrographs of water column bacteria, DAPI-stained cells. Bar = 5 μ m, (D) cells hybridized with ACD638 probe (Cy3-labeled) specific for *Acidiphilium* spp. Bar = 5 μ m.

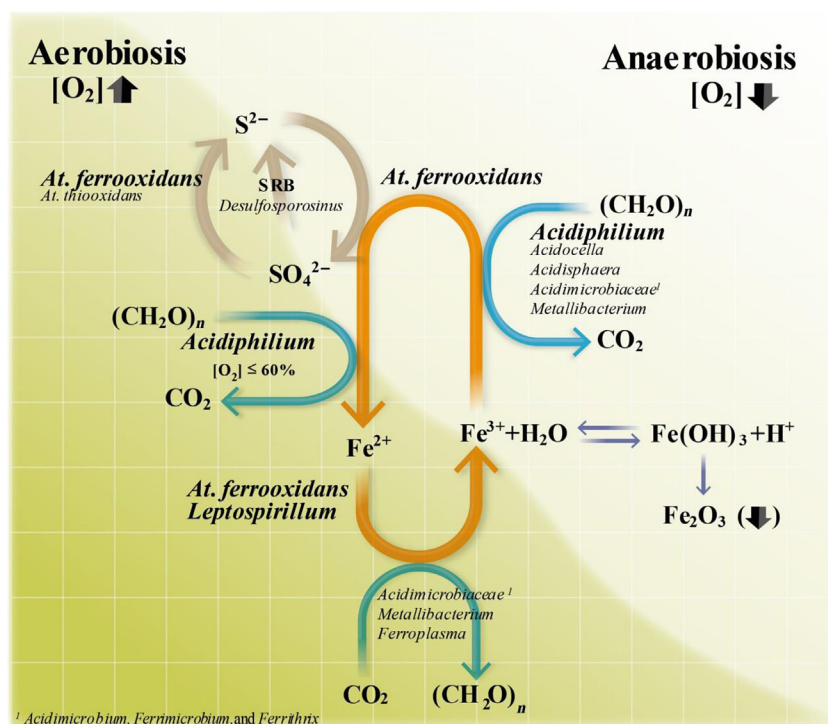


FIGURE 14.3 Geomicrobiological model showing the roles of the different prokaryotic microorganisms identified in acidic water column. Microorganisms are shown associated with their roles in the iron and sulfur cycles. The type size of the organism designation is proportional to the respective cell density.

environmental conditions. 16S amplicon pyrosequencing-based exploration of the phylogenetic differentiation among 90 microbial assemblages showed that overall microbial diversity estimates and relative abundances of most of the dominant lineages were significantly correlated with pH. Furthermore, the effect of pH in shaping phylogenetic structure within specific lineages was also clearly evident, except for *Alphaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes*, which were largely influenced by variations in ferric and ferrous iron concentrations. This is reasonable since energy generation in many species from these classes is associated with ferrous iron oxidation or ferric iron reduction (Bridge and Johnson, 2000; Holmes et al., 2004).

14.3.2 Eukaryotic Microbial Diversity

It is usually assumed that high metal concentrations limit eukaryotic growth and diversity due to their toxicity. However, what makes these acidic ecosystems unique extreme environments is the unexpectedly high degree of eukaryotic diversity found in their waters and that these eukaryotic organisms are the principal contributors to the biomass in most of them (López-Archilla et al., 2001; Amaral et al., 2002; Aguilera et al., 2006a). Extreme acidic ecosystems usually include as well different abiotic extremes than low pH (Rothschild and Mancinelli, 2001). Thus, eukaryotes thriving at these habitats are often also to low nutrient levels (Brake and Hasiotis, 2010), high concentrations of toxic metals (Aguilera et al., 2007), and/or extreme temperatures (González-Toril et al., 2015). Additionally, several studies have revealed representatives from multiple evolutionary eukaryotic lineages, suggesting that the ability to adapt to pH extremes may be widespread (Amaral et al., 2002). This raises the question of whether there are cosmopolitan eukaryotic taxa that have adapted to a wide range of pH extremes (Amaral-Zettler, 2013). Which environmental parameters are most influential in shaping eukaryotic microbial diversity patterns at pH extremes also remains underexplored.

Recent studies based on molecular ecology have demonstrated that eukaryotic organisms are exceedingly adaptable, much like prokaryotes, although most habitats have not been sufficiently well explored for sound generalizations to be made. Molecular analysis has also revealed novel protist genetic diversity in different extreme environments (Caron et al., 2004). In this regard, acidophilic environments are not an exception. In general, acidophilic eukaryotic diversity is more extensively studied in environments related to mining activities, probably due to a larger accessibility, relevance to biotechnological applications, and contamination issues. Examples include ARD (Amaral et al., 2002; Aguilera et al., 2007; González-Toril et al., 2015) and AMD environments (Baker and Banfield, 2003; Baker et al., 2009; Nancucheo and Johnson, 2012), and acidic lakes (Kamjunke et al., 2004; Santofimia et al., 2013; González-Toril et al., 2014; Hao et al., 2017).

Members of the phylum Chlorophyta, such as *Chlamydomonas*, *Chlorella*, and *Euglena*, are the most frequent species found in these acidic environments, followed by two filamentous algae belonging to the genera *Klebsormidium* and *Zygnemopsis* (Fig. 14.4). The most acidic areas are usually inhabited by a eukaryotic community dominated by two species related to the genera *Dunaliella* and *Cyanidium* (Rhodophyta), well known for their metal and acid tolerance (Visviki and Santikul, 2000). Molecular ecology techniques have identified algae closely related to those characterized phenotypically, emphasizing the high degree of eukaryotic diversity existing in the extreme acidic conditions (Amaral et al., 2002; Aguilera et al., 2006a,b).

The mixotrophic community is dominated by cercoconads and stramenopiles related to the genera *Bodo*, *Ochromonas*, *Labyrinthula*, and *Cercomonas*. The protistan consumer community is characterized by two different species of ciliates tentatively assigned to the genera *Oxytricha* and *Euplotes*. Amebas related to the genera *Vahlkampfia* and *Naegleria* can be found frequently even in the most acidic parts of the river (pH < 1) and one species of heliozoan belonging to the genus *Actinophrys* seems to be the characteristic top predator of the benthic food chain (Aguilera et al., 2006a,b).

Eukaryotic biodiversity in acidic geothermal springs have also been explored (Ferris et al., 2005; Luo et al., 2005; Brown and Wolfe, 2006; Toplin et al., 2008; Aguilera et al., 2010). In these environments, the unicellular rhodophyte *Galdieria sulphuraria* (formerly *C. caldarium*) is the dominant eukaryote worldwide. This moderate thermophile (35–56°C) (Reeb and Bhattacharya, 2010) may grow as a heterotroph in the absence of light (as may *Euglena*) and has been reported to grow at pH values around zero (Schleper et al., 1995). Related *Galdieria* species can be found also in acidic AMD and ARDs since they are extremely tolerant to heavy metals and absorb high concentrations of them from the acidic water (Nagasaka et al., 2004; Misumi et al., 2008). Acidophilic algae and protozoans have received more attention than fungi and yeasts, although fungi have long been recognized as active participants in acidification of sulfide-rich environments (Armstrong, 1921; Gross and Robbins, 2000). Additionally, most studies related to these

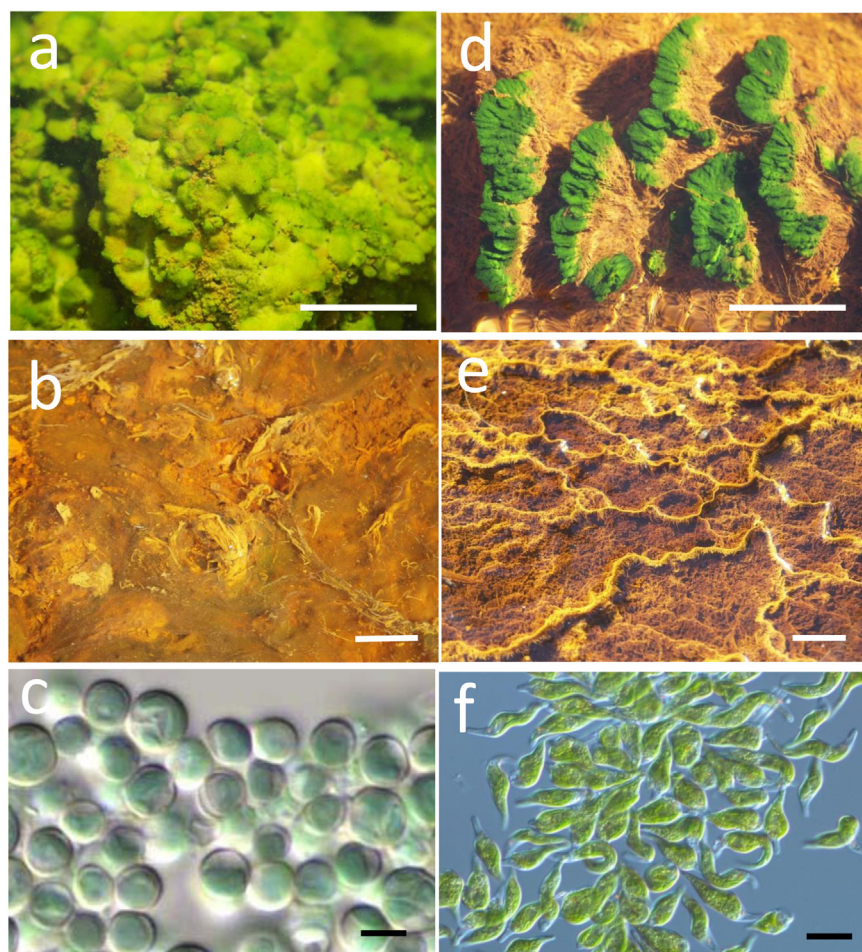


FIGURE 14.4 Microbial biofilms and eukaryotes founded in Río Tinto. (A) Green biofilms formed mainly by *Chlorella* and *Chlamydomonas* located at the origin. Scale bar = 5 cm, (B) prokaryotic streamers. Scale bar = 5 cm, (C) light microscopy micrographs of *Cyanidium*. Scale bar = 5 μ m, (D) biofilms of *Euglena*. Scale bar = 5 cm, (E) terraces formed by iron precipitates located at the origin of the river. Scale bar = 10 cm, (F) light microscopy micrographs of *Euglena*. Scale bar = 30 μ m.

organisms have been focused on biodiversity, and little is known about their physiology or their interactions with other species.

Among the filamentous fungi which have been isolated from acidic sites are some of the most acidophilic of all microorganisms; *Acontium cylatum*, *Trichosporon cerebriae*, and a *Cephalosporium* sp. have all been reported to grow at ca. pH 0 (Schleper et al., 1995). However, most fungi living in acidic habitats should be regarded as acid-tolerant rather than strictly acidophilic because they are also able to grow under neutral or even alkaline pH (Gross and Robbins, 2000). Most field studies on acidophilic fungi have been carried out only using soil samples, and up to 81 fungal species have been described (Gross and Robbins, 2000). Molecular analysis of the small subunit rRNA genes (rDNA) have shown that acidophilic basidiomycetes phylotypes had more than 97% sequence identity to known taxa, whereas the phylotypes of the acidophilic *Zygomycota/Chytridiomycota* had less than 93% sequence identity to sequences available in the GenBank database (Gadanhó and Sampaio, 2006). Some of those phylotypes branched deeply within the fungi (Gadanhó and Sampaio, 2006). Fungi seem to play an important role in these environments because, together with other microorganisms, they form biofilms on the surfaces of rocks. These biofilms are the site of metal and mineral precipitation and provide physical support for other microbial populations. Fungi can display metal resistance and can sequester specific metals allowing less tolerant species to exist (Fournier et al., 1998). Moreover, it has been reported that ferrous iron oxidation by *At. ferrooxidans* was stimulated by the basidiomycetous *Rhodotorula mucilaginosa* (Oggerin et al., 2013). This may act as heterogeneous crystallization nuclei for a passive method of jarosite precipitation, possibly promoting local ion oversaturation for mineral crystallization when suitable physicochemical conditions are present and helping to shape and control the geochemical properties of the environment (Oggerin et al., 2013).

Studies on yeasts in acidic environments are even rarer and are usually concerned with descriptions of yeast biodiversity in situ, rather than how they adapt to acid stress. *Rhodotorula* spp., which form pink colonies on solid media, are frequently encountered in AMD waters, and arthroconidia (*Trichosporon* sp.) are the most commonly encountered fungal isolates, though other genera (e.g., *Candida*, *Cryptococcus*, and *Purpureocillium* spp.) have also been found in extremely acidic environments (Gadanhó and Sampaio, 2006, 2009; Russo et al., 2008).

Among the ascomycetes, species belonging to the Hemiascomycetes and Euascomycetes have been described in the Iberian Pyrite Belt (IPB; *Glomerella* sp. and *Lecythophora* sp.), as well as basidiomycetous yeasts distributed in the classes Hymenomycetes and Urediniomycetes (Gadanhó and Sampaio, 2006). These species have also been described in acidic geothermal areas (Russo et al., 2008). Three novel asexual basidiomycetous yeast species have been recently identified in the IPB (*Cryptococcus aciditolerans* sp. nov., *Cryptococcus ibericus* sp. nov., and *Cryptococcus metallitolerans* sp. nov.) belonging to the order Filobasidiales and form a well-separated clade (Gadanhó and Sampaio, 2009). These *Cryptococcus* species are apparently specialists of acidic aquatic environments since they require low pH for growth, a property that has not been observed before in yeasts.

14.4 EUKARYOTIC ACIDOPHILIC GENOMIC STUDIES

Until very recently, culturability was a prerequisite for genome sequencing and for full access to the genetic complement of individual organisms. To some extent, this limitation has been resolved by the development of the field of metagenomics (DeMaayer et al., 2014) and single cell genomics. Metagenome sequencing, coupled with other “omic” technologies, such as transcriptomics (measuring of mRNA transcript levels), proteomics (study of the protein complement), and metabolomics (study of cellular metabolites), has led to the development of sophisticated systems biology approaches (Zhang et al., 2010) which facilitate the combinatorial study of microbial community functions and their interactions within, and with, the environment (Cowan et al., 2015).

14.4.1 Genomes

Thus far, the genomes of three related thermoacidophilic red algae, *Cyanidioschyzon merolae* (Matsuzaki et al., 2004), *G. sulphuraria* (Schönknecht et al., 2013), and *Galdieria phlegrea* (Qiu et al., 2013), have been sequenced (all belong to the cyanidialean red algae, which inhabit sulfuric hot springs). These genomic studies showed that horizontal gene transfer (HGT) from environmental prokaryotes, the expansion of gene families, and the loss of genes have probably played important roles in the adaptation of Cyanidiales to acidic and high-temperature environments (Qiu et al., 2013). Through HGT, cyanidialean red algae acquired arsenical-resistance efflux pumps that biotransform arsenic and archaeal ATPases, which probably contribute to the algal heat tolerance (Schönknecht et al., 2013). In fact, at least 5% of protein-coding genes of *G. sulphuraria* were probably acquired horizontally (Qiu et al., 2013). In addition, the reduction

in the number of genes encoding voltage-gated ion channels and the expansion of chloride channel and chloride carrier/channel families in the genome has probably contributed to the algal acid tolerance (Schönknecht et al., 2013). Likewise, a study in the acidophilic green alga *Chlamydomonas acidophila* showed that phytochelatin synthase genes of bacterial HGT origin played an important role in the tolerance to cadmium (Olsson et al., 2017).

The draft genome sequence of the acidophilic green alga *Chlamydomonas eustigma* has also been recently published (Hirookaa et al., 2017). Genes encoding heat-shock proteins and plasma membrane H⁺-ATPase are highly expressed. This species has also lost fermentation pathways that acidify the cytosol and has acquired an energy shuttle and buffering system and arsenic detoxification genes through HGT. Moreover, the arsenic detoxification genes have been multiplied in the genome. These features have also been found in other acidophilic green and red algae, suggesting the existence of common mechanisms in the adaptation to acidic environments.

On the other hand, comparative analysis of the chloroplast genome of *Euglena mutabilis* shows also that this species differ from other *Euglena* species, making it very likely that the emergence at the base of the phylogenetic tree of the genus *Euglena* (Dabbagh and Preisfeld, 2017). This was corroborated by many similarities in gene arrangement and orientation with *Strombomonas* and *Monomorphina*, rendering the genome organization of *E. mutabilis* in certain clusters as plesiomorphic feature (Dabbagh and Preisfeld, 2017).

14.4.2 Transcriptomes

For species without a reference genome, mRNA sequencing technology can detect transcripts corresponding to the existing genomic sequences and provide abundant information for a wide range of biological studies (Surget-Groba and Montoya-Burgos, 2010; Liu et al., 2014b; Hong et al., 2017). Transcriptomic studies related to acidophilic eukaryotes are still scarce and mainly related to heavy metal resistance. Thus, the genus *Dunaliella* is particularly attractive for studies on adaptive mechanisms to extreme environmental conditions since two of the most extremophilic eukaryotic species described until now, *Dunaliella salina* and *Dunaliella acidophila*, belong to it. Besides, *Dunaliella* species are well known for their extraordinarily high tolerance to salinity, temperature, nutrient limitation, and irradiance (Ben-Amotz et al., 2009; García-Gomez et al., 2012; Hong et al., 2017). These facts strongly suggest that this microalga has acquired efficient adaptive mechanisms to cope with the stresses associated to these ecosystems.

Comparative transcriptomic approaches, using massive Illumina sequencing for a de novo transcriptome assembly, identified changes in response to high cadmium concentrations and natural metal-rich water *D. acidophila* (Puentes-Sánchez et al., 2014). The results strongly suggest a constitutive biotic response produced in *D. acidophila*, even when the stress metal conditions and possible natural chelators are not present in the growth media. Thus, a higher variety of transcripts related to general and oxidative stress within the nondifferentially expressed transcripts than in the differentially expressed ones is observed. Besides, the abundances of these nondifferentially expressed transcripts were also significantly higher, reaching up to five times the levels found in the differentially expressed ones. It is worth noting that these high levels of transcripts related to general and oxidative stress were also observed in the control library suggesting a constitutive high expression of these defense mechanisms in *D. acidophila*, even after several years growing under absence of metal stress. This is particularly relevant in the case of phytochelatin synthase, metallothioneins, and glutathione *S*-transferase related transcripts, since all of them are directly related to heavy metal response and detoxification (Hirata et al., 2005; Nishikawa et al., 2006; Rea, 2012), showing up to ten times higher transcripts abundances than the average for the nondifferentially expressed transcripts found in this study. Similar results have been reported for *C. acidophila*, in which constitutive high levels of glutathione *S*-transferase have been found, suggesting that this microalgae has developed detoxification system to prevent cell destruction due to Cd toxicity (Nishikawa et al., 2006).

Furthermore, a significantly higher basal metabolic and energetic activity under natural metal-rich water growth conditions has been observed. It is tempting to speculate that this higher metabolism could be linked to a higher protein turnover. This assertion was further supported by the fact that, in the presence of natural metal-rich water, *D. acidophila* transcriptome was significantly enriched in proteasomal catabolic process transcripts, revealing a more active protein degradation in comparison to the other conditions. In addition, the higher nitrogen metabolism observed in this transcriptomic library involving amino acids metabolism and nitrogen compounds transport may also be linked, at least in part, to a higher protein turnover implying proteasome-mediated degradation. As heavy metals are known to lead to an alteration of the functionality of the proteins, such behaviors could help this acidophile to maintain the functional integrity of its proteome under environmental conditions (Casiot et al., 2004; Jacobson et al., 2012; Halter et al., 2015).

The transcriptomic response to copper has also been assayed in *C. acidophila* (Olsson et al., 2015). Although copper is a trace metal that aerobic organisms need in small amounts, at high concentrations it is toxic. Local concentrations of copper can reach up to 6 mM in some acidic environments, such as Río Tinto (SW, Spain). To thrive under these

environmental conditions, *C. acidophila* have developed different molecular mechanisms of tolerance and resistance. For example, there were no alterations in transcripts that code for antioxidant defense in spite of the extremely high copper levels assayed (0.5 mM). Also surprisingly, even very high amounts of copper that are lethal to other species seem not to significantly reduce photosynthetic activity in *C. acidophila*.

The differentially expressed genes in this study included transcripts involved in sugar metabolism, signaling proteins, transporters and membrane proteins, and there were also both up- and downregulated genes with unknown function. A large portion of copper responsive genes in *Chlamydomonas reinhardtii* were uncharacterized prior to the study performed by [Castruita et al. \(2011\)](#), demonstrating that copper homeostasis in green algae is not yet well understood. Thus, it was not surprising that also many genes in *C. acidophila* did only get annotated as predicted proteins. The fact that many transcripts did not get any blast hit at all tells us that there are big differences between *C. reinhardtii* and *C. acidophila*, some of them being key factors that allow this species to live in an extreme environment.

A recent transcriptomic study regarding the daily photosynthetic performance of a natural *E. mutabilis* biofilm reveals that this photosynthetic protist undergo large-scale transcriptomic reprogramming during midday due to a dynamic photoinhibition and solar radiation stress ([Puente-Sánchez et al., 2016b](#)). In this particular case, photoinhibition is due to UV radiation and not to light intensity, as revealed by pulse amplitude modulated (PAM) fluorometry analysis. In order to minimize the negative effects of solar radiation, the transcriptomic results support the presence of a circadian rhythm in this euglenophyte that increases their opportunity to survive, with photosynthetic related processes enhanced in the morning, DNA synthesis occurring in the evening, and cell division taking place at night. The transcription pattern during the day is mainly altered in genes involved in Photosystem II stability and repair, UV damaged DNA repair, nonphotochemical quenching and oxidative stress, supporting the photoinhibition detected by PAM fluorometry at midday. This low light acclimation has also been reported in *Galdieria* and *Cyanidium*, which often grow endolithically, and, therefore, autotrophic cell growth is restricted to upper rock layers and/or to periods of high photosynthetically active irradiation. However, although *Galdieria* maintains a high photosynthetic rate, even at high light irradiation (2000 $\mu\text{E}/\text{m}^2/\text{s}$), photoinhibition occurs abruptly at about 250 $\mu\text{E}/\text{m}^2/\text{s}$ which means a pronounced sensitivity to high levels of light ([Puente-Sánchez et al., 2016b](#)).

14.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The systematic use of meta “omics” methodologies will contribute to a better understanding of the complex microbial ecology involved in extreme acidic environments, and it is clear that we are facing a revolution in this field, in which the confluence of basic sciences and biotechnology should reap great benefits in the near future. Further functional and structural analyses are needed to understand in detail the role of new genes both in heavy metal detoxification and other possible functions. The democratization of “omics” technologies, in terms of pricing, analytical tools, as well as the integration of “omic” technologies into all-encompassing systems biology approaches, will almost certainly continue to challenge scientific paradigms on the ecology of extremophiles.

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