Eukaryotic Community Structure from Río Tinto (SW, Spain), a Highly Acidic River

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EUKARYOTIC COMMUNITY STRUCTURE FROM RÍO TINTO (SW, SPAIN), A HIGHLY ACIDIC RIVER

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1. Introduction

A major question in microbial ecology is to identify the limits of life for growth and survival, and to understand the molecular mechanisms that define these limits. Our ongoing exploration of the Earth has led to continued discoveries of life in environments that have been previously considered uninhabitable. Thus, interest in the biodiversity and ecology of extreme environments has grown in recent years for several reasons: some of these are scientific and related to the idea that extreme environments are believed to reflect early Earth conditions; conditions that persisted for most of the time that life has been on the Earth and to which prokaryotes originally evolved and adapted (Schopf and Walter, 1982). Other reasons are more commercial, such as the use of the metabolic properties of some microorganisms for metal extraction.

The discovery and study of extremophiles also contribute to the search for life beyond Earth. Thus, according to the NASA Astrobiology Roadmap (http://astrobiology.arc.nasa.gov), one of the main goals of astrobiological research is to understand life that survives or thrives under the most extreme conditions on Earth in order to characterize the biochemistry that defines the limits for cellular life. In this regard, the study of extremely acidic environments (pH <3) is becoming increasingly important since the environmental acidity is often caused by microbial activity (Hallberg and Johnson, 2001).

Highly acidic environments are relatively scarce worldwide and are generally associated with volcanic activity and mining operation (Baffico et al., 2004). The natural oxidation and dissolution of the sulfidic minerals exposed to oxygen and water results in acid production, and the process can be greatly enhanced by microbial metabolism (Nordstrom and Southam, 1997; González-Toril et al., 2001). At the same time, low pH facilitates metal solubility in water, particularly cationic metals (such as aluminum and many heavy metals), and therefore acidic water tends to have high concentrations of heavy metals (Johnson, 1998).

Río Tinto, a 92-km river located in south-west Spain, is one of the most unusual acidic ecosystems due to its size, rather constant low pH, high concentrations of heavy metals, and a high level of mainly eukaryotic microbial diversity (López-Archilla et al., 2001; Amaral-Zettler et al., 2002).

2. The River and Its Setting

The river raises in Peña del Hierro, in the core of the Iberian Pyritic Belt, flows through it and reaches the Atlantic Ocean at Huelva (Fig. 1). The Iberian Pyritic Belt is one of the richest metal sulfide ore deposits on Earth formed by massive bodies of iron and copper sulfides, as well as minor quantities of lead and zinc (Fernández-Remolar et al., 2003). Consequently, the area has been mined for millenia since the Phoenician and Roman era (Davis et al., 2000).

The river basin can be divided into three main zones on the basis of topological, geological, and geochemical characteristics: the northern, the transitional

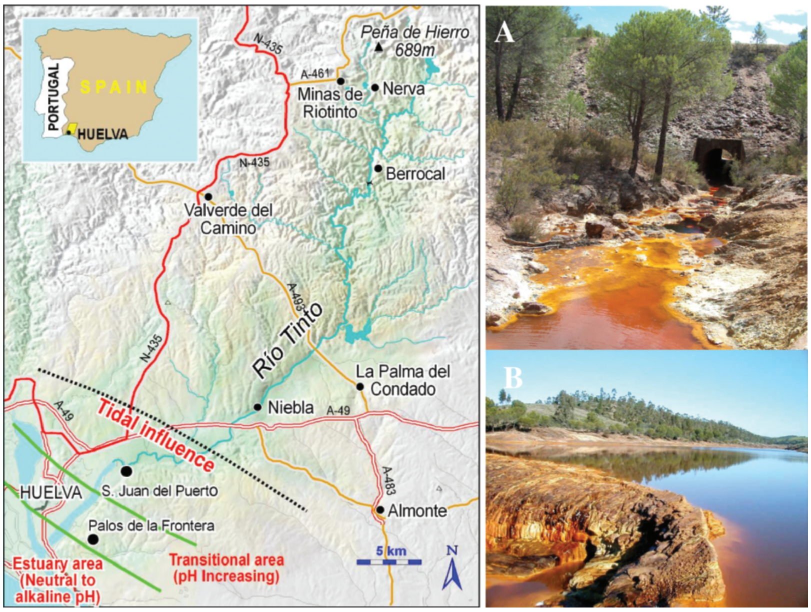

Figure 1. Geographical map of the Tinto River showing the three domains, the north area, the transitional area and the estuary. (A) The river at the origin in Peña del Hierro. (B) The river at La Palma del Condado.

and the estuarine, which have been differentiated by a Pliocene half-graven activity with NE-SW direction (Flores, 1996). The northern zone includes the towns Riotinto, Nerva, Berrocal La Palma del Condado and Niebla and their surroundings (Fig. 1). The area is characterized by highlands (100–660 m above sea level), stable of the hydrochemical parameters such as pH, which remains between 0.9 and 3 (mean value of 2.3) and a high concentration of iron in solution (between 1.5 and 20 g L^{-1}). The transitional area is located between Niebla and San Juan del Puerto, although its position varies depending on the tidal regime and seasonal changes of the river flow.

Thus, the hydrochemical parameters experience periodic variations due to the seawater neutralization including a pH increase (higher than 3) and a decrease in the ferric iron concentration (lower than 0.2 g L^{-1}). The third zone is the estuary near Palos de la Frontera, where the seawater causes ferric iron precipitation as sulfides (Fernández-Remolar et al., 2003).

The hydrological characteristics of Río Tinto are typical of a semi-arid, Mediterranean-type climate. The mean water discharge is ca. 3 m^3 sec^{-1}, although important variations have been observed throughout the year (Elbaz-Poulichet et al., 2000). Rainfall discharge reaches up to 10–70 mm day^{-1} in winter and approaches 0 mm day^{-1} in summer. Evapotranspiration is ca. <1 mm day^{-1} in winter and up to 10 mm day^{-1} during the summer (Sanchez-España et al., 2005). Under these hydrological regime, the river shows a clear bimodality in the annual water availability with a humid and temperate season in winter, followed by a dry and warm summer. This seasonality strongly affects the eukaryotic microbial communities by changing important hydrochemical characteristic such us water flow, water temperature, and element composition.

The northern part of Río Tinto maintains a constant low pH (ranging from 0.9 to 3, mean 2.3), and high concentrations of heavy metals (López-Archilla et al., 2001; Fernández-Remolar et al., 2004). Since pyrite (FeS_2) is the dominant sulfide in the Iberian Pyritic Belt, ferric ion is the dominant oxidant in the river and mainly responsible for the constant pH due to its buffering capacity (Gómez et al., 2004). These extreme conditions are the product of the metabolic activity of microorganisms, including iron- and sulfur-oxidizing bacteria, which can be found in high numbers in its waters. Most of these prokaryotes are autotrophic. Thus, in addition to promoting the extreme conditions of the habitat, they are also primary producers (González-Toril et al., 2001). Recent microbial studies have shown that ca. 80% of the prokaryotic diversity in the water column is explained by three chemolithotrophic bacteria, *Acidithiobacillus ferrooxidans*, *Leptospirillum* spp. and *Acidiphilium* spp., all of them involved in the iron cycle (González-Toril et al., 2003). Although some other species related to the iron cycle have also been identified (i.e., *Ferroplasma acidophilum* or *Thermoplasma acidophilum*), their low number in the water column suggests that they could play a minor role in the function of this cycle (Amils et al., 2004, 2006).

Ferric iron is produced by the metabolism of these iron-oxidizing microorganisms, which are very active in the aerobic part of the river. Sulfuric acid originates

Table 1. Physicochemical parameters at some of the most extreme sampling sites (Mean ± SD).

Location	pH	Conductivity (mS cm^{-1})	Redox potential (mV)	Fe (g L^{-1})	Cu (mg L^{-1})	As (mg L^{-1})	Cd (mg L^{-1})	Zn (mg L^{-1})
Iz-Iz	1.8 ± 0.2	25.7 ± 2.3	569 ± 22	17 ± 4	12 ± 3	16 ± 4	43 ± 16	14 ± 3
Angeles	1.7 ± 0.2	30.8 ± 3.4	471 ± 16	16 ± 3	132 ± 43	24 ± 3	30 ± 12	162 ± 5
UMA	1.7 ± 0.3	40.2 ± 8.3	473 ± 10	18 ± 7	85 ± 36	32 ± 5	40 ± 18	118 ± 4
Richi	1.2 ± 0.3	38.9 ± 16	460 ± 30	22 ± 5	100 ± 36	48 ± 7	34 ± 11	94 ± 31
La Palma	2.5 ± 0.3	3.70 ± 1.1	548 ± 70	0.2 ± 0.1	19 ± 7	0.2 ± 0.1	0.7 ± 0.1	50 ± 10

from sulfides by chemical oxidation or the activity of the sulfur-oxidizing microorganisms, depending on the sulfide mineral substrate (González-Toril et al., 2003). The result is a strongly acidic solution of ferric iron which brings into solution other heavy metals, increasing their concentrations relative to neighboring rivers with higher pH. Thus, Rio Tinto heavy metal concentrations, of Fe at ca. 22 g L^{-1}, Zn at ca. 0.5 g L^{-1}or Cd at ca. 70 mg L^{-1}, can be found (Table 1) (López-Archilla and Amils, 1999).

Comparison of physical conditions and concentrations of heavy metals ions in winter, summer, and late summer denote a clear bimodality in their annual distribution. Oxygen and pH generally show higher values in winter, while heavy metals reach their peaks in summer and late summer.

This fact is coincident with the bimodal pattern of annual water availability reported in previous work on Río Tinto area (Fernández-Remolar et al., 2003).

The climograms of this area showed a clear bimodality in the pluviosity and water availability consisting of a humid and temperate season alternating with a warm and dry season. The high phreatic level maintains the river flow during the summer, although a high rate of evaporation induces high concentrations of heavy metals due to concentration processes.

However, what makes the Río Tinto a unique acidic extreme environment is that eukaryotic organisms are the principal contributors of biomass in the river as well as the unexpected degree of eukaryotic diversity found in its waters (López-Archilla et al., 2001, Amaral-Zettler et al., 2002, 2003).

3. Eukaryotic Biodiversity

It is usually assumed that high metal concentrations in acidic habitats limit eukaryotic growth and diversity due to their toxicity (Gross, 2000). It has been also proposed that metal hydroxide deposition could change the physicochemical conditions of surfaces resulting in a reduction of epilithic growth on rocks. However, colorful biofilms covering large surfaces of the river basin as well as filamentous microbial communities and macroscopic algae are common features of the acidic streams at the Río Tinto. In fact, eukaryotic algae contribute at least 60% of the river biomass (López-Archilla et al., 2001).

The eukaryotic biodiversity in this ecosystem includes species of most of the major eukaryotic lineages (Fig. 2) (López-Archilla et al., 2001; Amaral-Zettler et al., 2002, 2003; Sabater et al., 2003). Most of the eukaryotic species thriving in

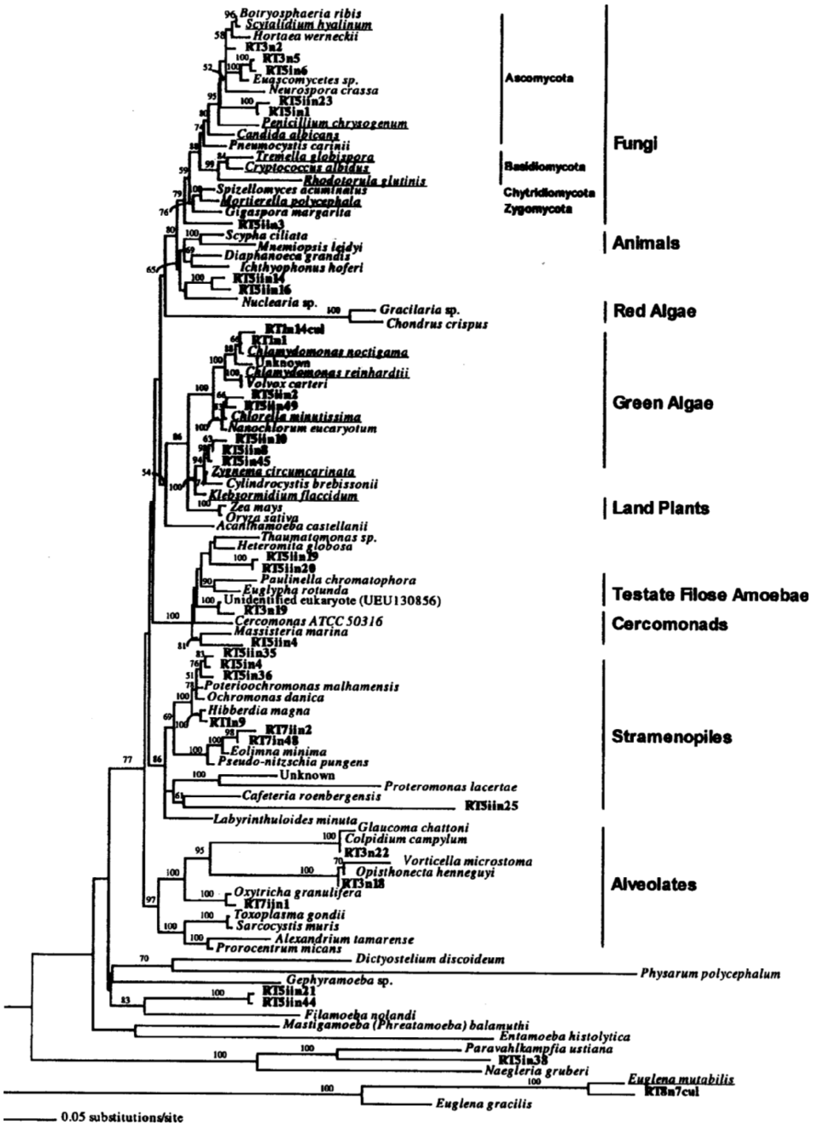

Figure 2. Phylogeny based on minimum evolution analyses of ribosomal RNAs using a likelihood method. Published by Amaral et al. Biological Bulletin (2003) 204: 205–209.

Río Tinto are photosynthetic species. Within them, chlorophytes related to different genera such as *Chlamydomonas*, *Dunaliella*, *Chlorella*, as well as *Euglena mutabilis* are the dominant eukaryotic microorganisms present in the river, forming large green patches along the river bed. These species are known for their high metal and acid tolerance from several investigations in extremely acidic lakes (Olaveson and Nalewajko, 1994; Fisher et al., 1998) and showed the same patchy distribution as in other acidic environments. Filamentous algae, represented by the genera *Zygnemopsis* and *Klebsormidium* have been also found. The occurrence of both filamentous species is highest during the dry summer months, when ion concentrations were highest and most of the physicochemical parameters are most extreme. These species are usually attached to the sediments forming long filamentous biofilms on the water surface that could exceed 1 m in length. During the winter, they almost disappear, probably due to an increase in the water current velocity which makes maintaining these structures difficult. Other chlorophytes, such as species belonging to the genera *Mesotaenium* and *Stichococcus,* have also been found, although at low numbers.

The most acidic part of the river is inhabited by an eukaryotic community dominated by two species related to the genera *Dunaliella* (*Chlorophyta*) and *Cyanidium (Rhodophyta)*. The genus *Dunaliella* includes some of the most extreme acidophiles reported until now, surviving at a restricted pH between 0 and 3 (Gimmler and Weis, 1992). The Cyanidiaceae group is also exceptionally well adapted to warm (ca. 45°C) and acidic (ca. pH 2) habitats (Seckbach, 1999).

Pennate diatoms are also present in the river forming large brown biofilms. These biofilms are usually clearly dominated by only one species related to the genus *Pinnularia*, although some other minoritary genera have been identified, including *Nitzschia* or *Cyclotella* (Sabater et al., 2003). Species belonging to these genera, especially *Pinnularia*, are fairly widespread at environments with pH values around 3.0 (DeNicola, 2000). From all the environmental variables that affect freshwater diatoms, pH seems to be the most important and, most taxa show a preference for a narrow pH range (Battarbee et al., 1986). The low diversity of diatoms present in Río Tinto in comparison with the diversity found in neighboring freshwaters, supports the idea that there is a threshold between pH 4.5 and 3.5 in which many species of diatoms are eliminated (DeNicola, 2000).

In addition to photosynthetic species, heterotrophic protists are also widely distributed throughout the river. The mixotrophic flagellates are dominated by members of the genera *Bodo* and *Ochromonas*. These organisms show the same ecological strategies as the phytoflagellates to overcome limitation in nutrient supply such as mixotrophy and mobility which results in an important advantage in these environments (Lessmann et al., 2000). Phagotrophic species such as ciliates, cercomonads, amoebae, and heliozoans have been also found in Río Tinto.

At least two species of ciliates are members of the community. The dominant ciliate taxa belongs to the order Hypotrichida. Although two different species have been microscopically observed, only clones related to *Oxytrichia granulifera* have been molecularly identified. The other morphotype could be tentatively assigned to the genus *Euplotes*. The reduction of species diversity and ciliate abundance with increasing acidity is well documented (Beaver and Crisman, 1981; Bienert et al., 1991). Hypotrichida species predominantly thrive in soils or benthos usually associated with algal clumps and they can be found in almost all sampling sites in the river. They are relatively large and slow-swimming, and this may contribute to the lack of predatory impact. Similarly, amoebas are found frequent, even in the most acidic parts of the river, eating large diatoms. *Vahlkampfia* species have been identified microscopically as well as other species, including lobosea-like amoebas and acanthamoeba-like amoebas.

One species of heliozoan belonging to the genus *Actinophyris* is also present in the river. Heliozoa seem to be characteristic top predators of the benthic food chain in the river. They are omnivorous and can consume organisms larger than themselves, including rotifers, algae and ciliates that get stuck at their adhesive podiae. In Río Tinto, we have observed them ingesting algae, mainly *Chlorellas*, *Chlamydomonas, and Euglena*. The only animal found in the river is a species of bdelloid rotifer related to the genus *Rotifera* (Amaral-Zettler et al., 2003). This low diversity of rotifers is consistent with prior observations that few species of rotifers have been found in waters of $\mathrm{pH} < 3.0$ (Deneke, 2000). This pioneer rotifer species can persist because of their high physiological tolerance of severe acidic stress and the lack of other more efficient competitors.

Among decomposer fungi are the most abundant, and both unicellular and filamentous forms are present (López-Archilla et al., 2001; López-Archilla et al., 2005). While many species of fungi have been isolated from the river, one fungus (related to *Hobsonia*) has been identified in many parts of the river where it forms thick denditric macrofilaments closely associated with other protists. When the fungus is present, a whole community forms embedded in a mucilaginous substance that could protect the inner microbial community from the external conditions by creating differential physicochemical conditions.

Although all these species have been previously described in other acidic environments, recent studies using molecular techniques showed the presence of novel eukaryotic lineages closely related to the base of the animal-fungal radiation (Amaral-Zettler et al., 2003). Further studies regarding eukaryotic biodiversity are underway in order to further identify additional novel lineages in the river.

4. Dynamics of Eukaryotic Communities in the River

The seasonal bimodality of physical conditions and levels of heavy metals ions (Fig. 3) greatly influences the eukaryotic community biomass (Aguilera et al., 2006). In winter the eukaryotic biomass is usually lower than in summer. The increase in the eukaryote population in the summer and late summer is mainly due to the occurrence of two species of filamentous green algae (*Klebsormidium* sp. and *Zygnemopsis* sp.) as well as to the presence of higher amounts of *E. mutabilis*. In general, green algae are responsible for nearly the total eukaryotic biomass increase

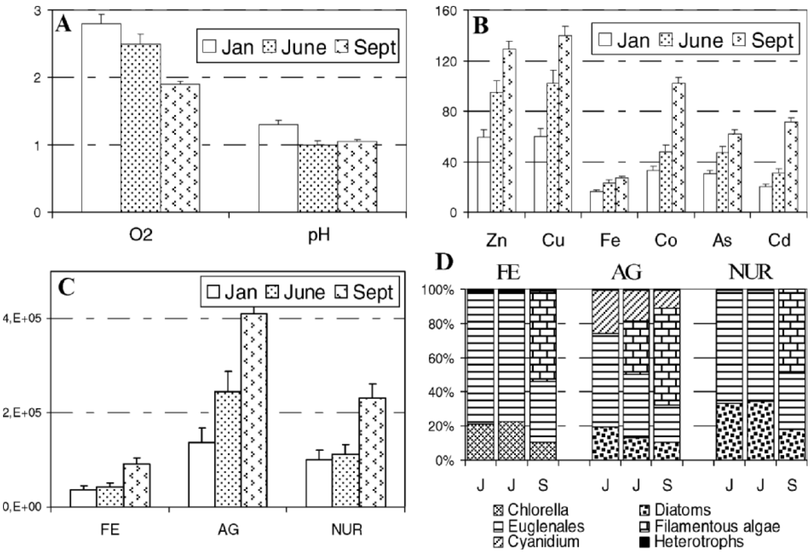

Figure 3. (A) Means and standard errors of water dissolved oxygen (mg L^{-1}) and pH at RI, one of the most extreme sampling stations of Río Tinto. (B) Heavy metal concentrations (mg l^{-1} except for Fe in g L^{-1}) at the same sampling point. (C) Benthic eukaryotic cell densities (cells cm^{-2}) at three different sampling sites (FE, AG and NUR). (D) Percentages of seasonal distribution of dominant taxa at three different sampling sites (FE, AG, and NUR).

during summer, which agrees with other studies in extremely acidic environments (Walton and Johnson, 1992; Nixdorf et al., 1998; Nordstrom and Alpers, 1999).

This fact is closely related to the significant increase in temperature values and daylight, as well as to the decrease in water flow. These factors facilitate cell deposition and biofilm formation.

There is a strong indication that sites with lower pH show lower species diversity. This finding agrees with other studies conducted in acidic lakes in which increases in acidity produces a reduction in species richness (Niederlehner and Cairns, 1990; Locke, 1992; Packroff, 2000). In the same manner, data from sampling stations located along the river show that the highest diversity index appeared at the stations located closer to the mouth of the river where the physicochemical water conditions are less severe.

5. Biofilm Formation as a Mechanism for Surviving Extreme Conditions

In aquatic ecosystems eukaryotic biofilms are diverse species communities. In Río Tinto, these are typically dominated by micro-algal consortia that form thick biofilms all over the riverbed (Fig. 4). Microscopic observations of the biofilms revealed a variety of prokaryotic morphotypes, algae, protozoa, or fungi. The

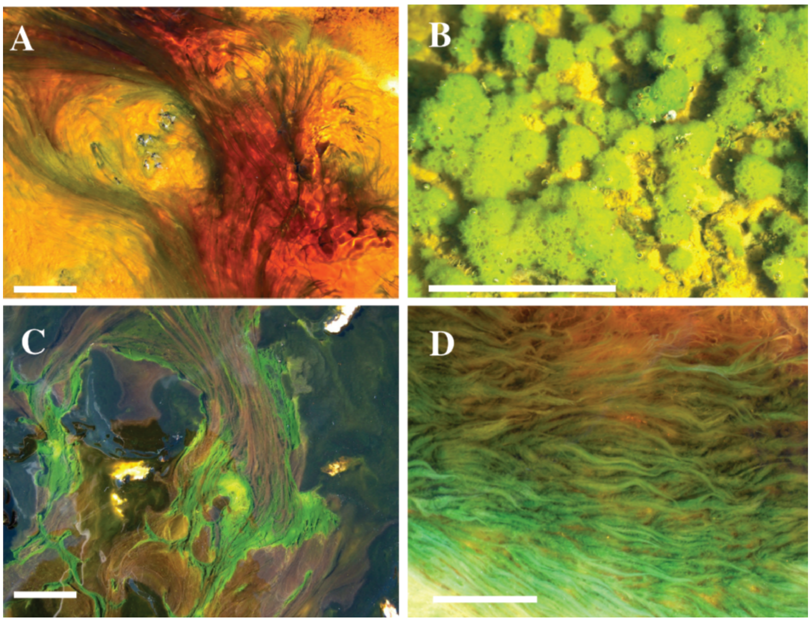

Figure 4. Microbial eukaryotic biofilms founded in Río Tinto. Scale bar = 5 cm. (A) Green filaments formed mainly by *Zygnemopsis* and *Klebsormidium* located at the origin. (B) Biofilms of *Chlamydomonas* and fungi. (C) Euglenas and diatoms are the main components of these biofilms. (D) Biofilms of *Chlorella* and fungi.

whole community is usually embedded in a thick mucilaginous coating that could protect the inner microbial community from the external water conditions.

In situ colonization studies have shown that the microbial colonization sequence starts with the fixation of an organic conditioning biofilm formed mainly by fungal hyphae, and bacterial and detrital mineral particles that will remain permanently attached to the substrate (Aguilera et al., 2006). Pioneer motile eukaryotic species, such as amoeba or heterotrophic flagellates then colonize, and this is followed by the establishment of an increasing number of sessile eukaryotes such *Chlorella* or diatoms. Finally, biofilm formation involves the incorporation of filamentous algae. A similar pattern of biofilm growth has previously been observed in other freshwater aquatic environments, and the nonspecific, permanent adhesion of bacteria and fungi to inert surfaces has been thoroughly described (Tolker-Nielsen and Molin, 2000). Thus, the development of these preconditioned substrata is of major importance to ensure colonization by autotrophs, especially in lotic systems (Korte and Blinn, 1983).

An earlier study of metal-exposed diatom biofilms has shown that the extracellular mucous matrix could be partially responsible for an increase in biofilm tolerance (Barranguet et al., 2000). This matrix is mainly composed of extracellular polymeric substances (EPS) which provide a suitable microenvironment for microbial development. EPSs could act as detoxification agents against heavy metals because they contain high amounts of negatively charged functional groups that could act as metal-binding sites (Sutherland, 1999). In this regard, the capacity of the acidic biofilms to accumulate metals was recently reported by García-Meza et al. (2005). Their results showed that EPS production was a critical feature for the survival of algal biofilms on mining tails. In addition, EPS synthesis increased when the biofilms were exposed to different heavy metals.

Many studies have investigated the physiology and structure of bacterial biofilms in order to understand the underlying processes of their formation. Analysis of the internal structure of biofilms by confocal laser scanning microscopy (Norton et al. 1998) have shown that biofilm communities form highly structured microbial assemblies (Moller et al. 1998). For eukaryotic biofilms, the internal structure has been less studied; however scanning electron microscopy in backscattered mode has permitted the observation of transversal sections of the biofilms still attached to the substrate (Aguilera et al. 2006). Microscale structural differences among naturally grown biofilms have been observed in different localities of Río Tinto. Although some of the biofilms formed a well defined layered structure (Fig. 5A), others showed several layers of cells loosely packed between minerals or decaying organic matter (Fig. 5B). Many biofilms were dominated by a specific photosynthetic microorganism bacteria were conspicuous throughout them (Fig. 5C). Some biofilms such as the ones formed by *Dunaliella* (*Chlorophyta*), only appear in the most acidic localities, and have a clear association with fungal communities (Fig. 5D). In addition to the vertical distribution of the same organism, different biofilms are distributed horizontally under the same environmental conditions. In general the surface is heterogenously colonized, the factors responsible for this distributional pattern are still not well known, but might be related with specific growth of different microorganisms and surface characteristics (Hutchinson et al., 2006). The structural study has shown the importance of metabolic interactions within the microbial communities themselves, and also between different biofilms.

Different factors may be responsible for these structural differences. Several layers of sediment indicate fluctuations of the flow and rapid recolonization of the new surfaces. Several studies have demonstrated that age is a determining factor of biofilm structure with the more loosely structured mats representing a younger state in biofilm development (Horodyski et al., 1977; Heinen and Lauwers, 1985). In our case, most of the biofilms are dramatically reduced every year during the rainy season. Thus age is not the only parameter that increases structure. Differences in water velocity may also play a significant role in determining biofilm structure (Battin et al., 2003) and the amount of material accumulated on the sediments, allowing a higher density of material to accumulate. It has been demonstrated that some biofilm-forming microorganisms can produce sticky excretions that are able to trap allochthonous mineral particles

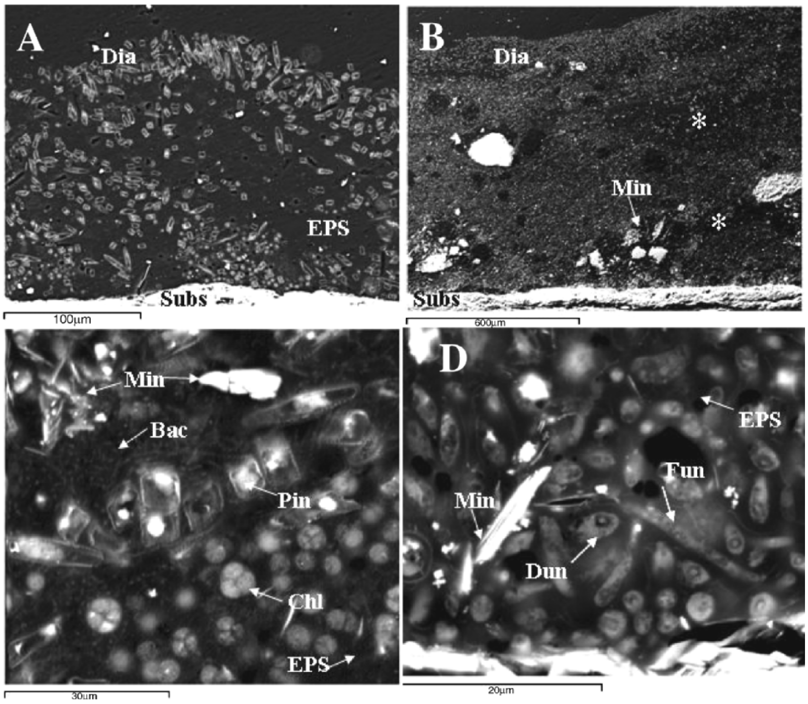

Figure 5. SEM-BSE images of transversal sections of microbial biofilms found in Río Tinto. (A) Diatom biofilm showing distribution of cells. The upper and lower part in contact with the substrate (Subs) are dense areas whereas the inner part is less compact and has more extracellular polymeric substances (EPS) between the cells. (B) Biofilms of diatoms can reach thickness of several mm over the surfaces (Subs). Some areas marked with asterisks are filled with decaying organic matter. (C) The areas where two photosynthetic biofilms contact (*Pinnularia* and *Chlorella*) shows also important bacteria communities (Bac) surrounded by extracellular polymeric substances (EPS). (D) Biofilms of *Dunaliella* within a matrix of extracellular polymeric substances (EPS) are present in the most extreme conditions and show a clear relation with fungi.

within the mats. This may be an adaptation to prevent the mats being swept away by the current (Golubic et al., 2000).

Finally, intrinsic physiological features of the microorganisms forming the biofilm can be responsible for specific internal, lateral structure, and distribution on the riverbed.

In biofilms, the matrix around the cells is a critical feature associated with physiology and environmental conditions. Some studies have stated that diatoms in marine habitats produce variable amounts of extracellular polymeric substances under different environmental conditions (Taylor et al., 1999). In the case of bacteria, the complex regulation of surface attachment, surface binding, biofilm maturation and ultimately, biofilm detachment, is affected by the physiology of the cells and by the physicochemical parameters of solid surfaces and environments (Davies et al., 1998). However, in eukaryotic biofilms, data regarding intercellular regulation in the formation of the biofilms is scarce. Although the development of microorganisms forming biofilms in the presence of a variety of stressful environmental conditions has been well documented (Allan, 1995), specific adaptation that explain the mechanisms why biofilm survives are not yet well understood.

Thus, biofilm formation and structure reflect the adaptation of the microorganisms to different environmental conditions of Río Tinto. Periphytic algae when forming biofilms with other microorganisms might have nutritional advantages or specific microenvironmental conditions that allow them to be exposed to much less severe physical and chemical conditions than those of the external habitat. Further in situ microsensor techniques studies and controlled mesocosmos experiments will be necessary to fully understand the factors controlling biofilm formation and the association among different microorganisms.

6. Eukaryotic Isolates and Their Properties

Culturing of extremophilic organisms has received an increasing attention due to economic importance in industries for agricultural, chemical, and pharmaceutical applications. Thus far, enzymes from extremophiles are the most useful molecules commercialized (i.e., Taq polymerase), although some organisms can be used directly in bioremediation or as nutritional supplement (Hough and Danson, 1999). In the same manner, interest in organisms that thrive in extreme environments has grown because they can provide important insights into molecular biology such as information on protein folding, membrane properties, or cellular repair mechanisms.

Río Tinto has yielded a number of species of eukaryotes that can be grown in culture. Thus, different types of chlorophytes (*Chlorella* sp. and *Dunaliella* sp.), euglenids (*E. mutabilis*), rhodophytes (*Cyanidium* sp.) or amoebae (*Vahlkampfia* sp.) have been isolated. However, most of the physiological studies regarding acidophilic eukaryotes from Río Tinto have been performed with different strains of one species of *Chlamydomonas* (*Chlorophyta*) closely related to *Chlamydomonas pitschmanii*.

Messerli et al. (2005) have characterized the electrochemical H^+ gradient that exists across the plasma membrane in aclonal isolate of *Chlamydomonas* from Río Tinto and compared it with *Chlamydomonas reinhardtii*. The optimal pH for growth of the acidophilic strain occurred between pH 2 and 6, while *C. reinhardtii* showed optimal growth between pH 5.5 and 8.5. The acidophilic isolate maintained an average cytosolic pH of 6.6 in culture media at both pH 2 and pH 7, while *C. reinhardtii* showed an internal pH of 7.1 in pH 7 culture. This indicates that these acidophilic algae are maintaining a slightly acidic cytosol even under more neutral conditions, reversing the electrochemical force on H^+. Furthermore, the transmembrane electric potential difference of the acidophilic *Chlamydomonas* strain was close to 0 mV, a rare value for plants and protists. At the same time, these acidophilic strains consumed about 7% more ATP per second at pH 2 than at pH 7. Due to these facts, the protection mechanism proposed is the active extrusion of H^+ into a cytosolic vacuoles. These vacuoles would help to maintain neutral cytosolic pH without H^+ membrane transporters exposed to the extreme acidic environment (Messerli et al., 2005).

The effect of cadmium on the growth and ultrastructure of three strains of *Chlamydomonas* isolated from Río Tinto has been studied (Aguilera and Amils, 2005). Most research on heavy metals and phytoplankton physiology are related to environments polluted by industrial and domestic wastes. However, little is known about the toxicity of these substances in environments with a natural, nonanthropogenic source of high levels of heavy metals. Acidic environments are the ideal ecosystems in which these studies could be carried out. pH has a considerable effect on the availability and, as a consequence, the toxicity of heavy metals (Mason and Jenkins, 1995). As previously mentioned, acidic environments tend to contain unusually high concentrations of heavy metals because their solubility increases markedly as the pH decreases (Nordstrom and Alpers, 1999).

Acidophilic *Chlamydomonas* strains show an unusual tolerance to cadmium (EC_{50} 0.2 mM of cadmium), being resistant to up to two orders of magnitude more Cd than other phytoplanktonic species analyzed to date grown under similar conditions except for pH. Light microscopy shows that cytological changes induced by Cd are evident even at low concentration (0.1 mM Cd) (Fig. 6). A significant reduction of the chlorophyll content was observed in all the concentrations assayed from the first day. Cultures grown under 0.4 and 0.8 mM Cd remained bleached throughout, and the cells became nonmotile, even in cultures at 0.2 mM Cd. TEM analysis confirmed these results, and Cd impacts include changes to chloroplasts, pyrenoids, starch granules, and vacuoles. The chloroplasts of *Chlamydomonas* sp. exposed to Cd were significantly smaller than controls, indicating a decrease in the photosynthetic activity of this alga. This reduction has been widely reported for other species of microalgae growing under Cd exposure, and it has been suggested that these changes could be due to loss of chlorophyll a and b and/or carotenoids (Rai et al., 1990).

Exposure to 0.1 and 0.2 mM Cd resulted in a significant increase in the number and relative volume of starch granules. The pyrenoid was also affected with a reduction in volume (Fig. 6). These results are consistent with observations on *Dunaliella minuta* and *Chlamydomonas bullosa* (Visviki and Rachlin, 1993). TEM microanalysis highlighted the accumulation of condensed electron-opaque material, within vacuoles. Similar dense granules have been visualized in other microalgae species treated with heavy metals (Nassiri et al. 1997). EDXA analyses have shown that these dense bodies contain Cd, P, and Fe. Metal deposition in vacuoles is a mechanism that may contribute to heavy metal tolerance, indeed,

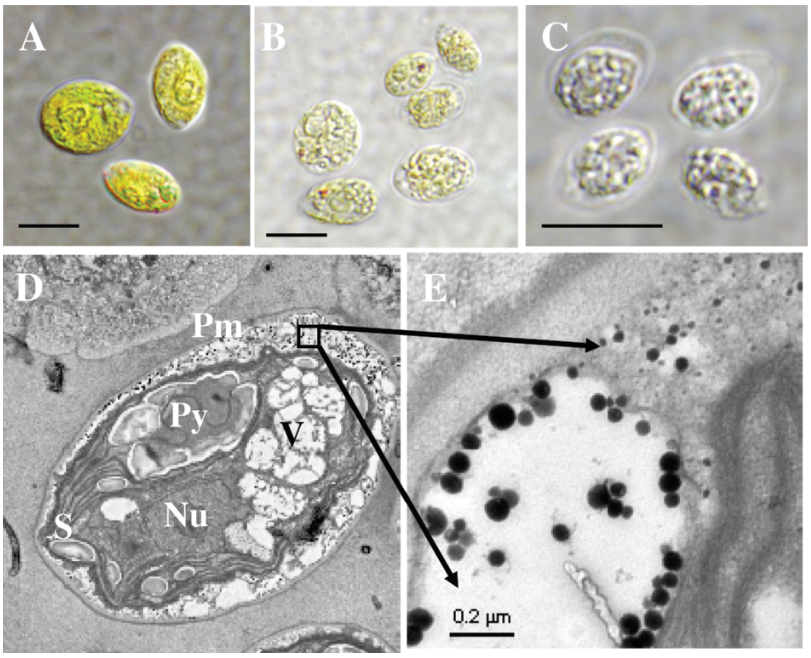

Figure 6. Light microscopy photographs of acidophilic *Chlamydomonas* sp. growing under different amounts of cadmium: (A) controls; (B) 0.2 mM Cd; (C) 0.4 mM Cd. Scale bar 10 µm. Electron micrographs of ultrathin sections: (D) cells grown under 0.4 mM Cd, the periplasmalemma space (Pm) surrounds the cell is increased, as well as the number and volume of cytoplasmic vacuoles (V) and starch granules (S). The nucleolus (Nu) is seen within the nucleus (Nu) as well as the pyrenoid (Py); (E) Dense precipitates are now located in the cytoplasmic vacuoles.

the accumulation of Cd in the vacuoles seems to be the most effective system for maintaining a low cytoplasmic Cd elsewhere in the cell.

In addition to algae, fungi are the major organisms isolated from Río Tinto. These fungi have been used as models for their tolerance to different heavy metals, as well as their capacity to remove metals from the environment. Thus, these organisms may have important applications in bioremediation (Durán et al., 2001).

Canovas et al. (2003) reported the isolation of a filamentous fungus that was *Aspergillus* sp. from Río Tinto, able to grow at 200 mM arsenic. Further, this strain showed a polyresistant phenotype, being able to grow at concentrations of As, Cu, and Cr at least 50-fold higher than other strains of *Aspergillus*. Similar to *Chlamydomonas*, this fungus grows at heavy metals concentrations over an order of magnitude higher than those found in the river. At the same time, the strain sequestered ca. 50%, of these heavy metals. Vacuolar compartmentalization has been suggested to play an important role in the regulation of metal concentrations in the cytosol (Gadd, 1993). In this acidophilic strain, this mechanism might partly explain the resistance to low concentrations, but it cannot account for the whole phenomenon.

Although further studies on the specific mechanisms of heavy metal resistance should be performed, interest in organisms that thrive in extreme environments can provide important data regarding the molecular biology and genetic of metal tolerance and detoxification processes.

7. Río Tinto as a Model of Astrobiological Interest

The discovery by the MER Opportunity Rover of the extensive *Terra Meridiani* iron formation on Mars has led to Río Tinto being recognized as an important mineralogical analog to the Martian site (Fernández-Remolar et al., 2004, 2005). Although thermal oxidation mechanisms have been proposed for its origin on Mars, geomorphology and IR spectroscopy support an aqueous environment as host to its secondary iron-enriched mineralogy. It has also been suggested that such environments are acidic due to sulfur complexation and iron buffering of aqueous solutions (Fairén et al., 2004). Although other terrestrial hydrothermal systems have been proposed as analogs to understand these interesting Martian environments, different studies found hematite as a mineral phase of Mar's iron rocks, not the silica that frequently appears in hydrothermal systems. However, the acidic waters from Rio Tinto produce ferric iron-enriched sediments from which silicates are absent. These features make the river an interesting analog for the Mars hematite site if an aqueous origin of these oxides is considered.

The existence of high numbers of eukaryotic organisms thriving in the extreme conditions of Río Tinto poses interesting questions of astrobiological interest. First, that complex eukaryotic systems, like those described in this chapter, can rather easily develop in the extreme acidic conditions and high concentration of heavy metals present in the Tinto ecosystem. Furthermore, the level of eukaryotic diversity found in this environment is several orders of magnitude higher than the prokaryotic diversity. This contradicts our current notions of biology and ecology. Although we do not understand at present the reasons for this adaptation (probably related to the availability of iron) (Amils et al., 2004; Gómez et al., 2004), it is clear that complex and evolved organisms can develop on other planets in conditions that had been thought to be incapable of sustaining life only a few years ago. Obviously this observation has to have an important impact on the design of future astrobiological exploration missions.

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