Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ugmb20

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Version of record first published: 13 Sep 2010

To cite this article: Sandra S. Brake & Stephen T. Hasiotis (2010): Eukaryote-Dominated Biofilms and Their Significance in Acidic Environments, Geomicrobiology Journal, 27:6-7, 534-558

To link to this article: http://dx.doi.org/10.1080/01490451003702966

Geomicrobiology Journal, 27:534–558, 2010

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490451003702966

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Eukaryote-Dominated Biofilms and Their Significance in Acidic Environments

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Biodiversity of benthic eukaryotic microorganisms in highly acidic (pH ≤ 3.5) aquatic environments is limited to species that have developed strategies to tolerate elevated concentrations of H^+ and dissolved metals and low nutrients levels that commonly characterize these environments. To survive adverse conditions, some algae, protozoa, and fungi have developed mechanisms to make their cell membranes impermeable to protons and maintain cytosolic pH at near neutral levels; others have developed a cell boundary mechanism that blocks H^+ ions from entering the cell. High concentrations of heavy metals are also toxic, adversely impacting growth by disrupting physiological, biochemical, or metabolic processes. Some algae, fungi, and protozoans are able to tolerate high metal concentrations via metal complexation outside the cell, extracellular binding and precipitation of metals, reduced metal uptake, increased metal efflux, and detoxification or compartmentalization of metals within the cell. In acidic environments, benthic eukaryotic microorganisms form biofilm communities in which they are the dominant members numerically and ecologically. Eukaryote-dominated benthic communities produce heterogeneous microenvironments that vary spatially and temporally in their physicochemical character. The eukaryotes in these biofilms can be considered ecosystem engineers as they directly or indirectly modulate the availability of resources to other species within the biofilm. These eukaryote-dominated communities may play a significant role in mediating their environment by actively and passively contributing to metal attenuation through various processes of biosorption and via formation of laminated organosedimentary structures, which may be used as analogs for similar structures in the rock record.

Keywords algae, AMD, euglena, microorganisms, protozoan, stromatolites

INTRODUCTION

The purpose of this article is to provide a brief overview of research on the diversity of eukaryotic microorganisms in acidic systems and to discuss the potential role they may play in mediating their environment. There is a wealth of information on the subject of eukaryote diversity in acidic environments, and this article makes no claim to be comprehensive or complete. We focus our attention, instead, on those acid-tolerant and acidophilic species commonly reported in acidic aqueous environments, specifically with regard to benthic eukaryotes as opposed to planktonic species. We postulate, based on research to date, that benthic biofilm communities show the greatest potential for mediating their environment via bioaccumulation of heavy metal and the formation of such iron-rich organosedimentary structures as stromatolites.

Highly acidic (pH ≤ 3.5) aqueous environments are common on Earth and originate either naturally as in volcanic springs, peat bogs, and natural acid rock drainage (ARD) or through anthropogenic activities as in acid mine drainage (AMD) and acidification of lakes and ponds (Dixit and Smol 1989; Albertano 1995; Gross 2000; Gross and Robbins 2000). The acidity of many of these environments is attributed to the production of sulfuric acid. In the case of volcanic hot springs, sulfuric acid is generated when volcanic gases of hydrogen sulfide and sulfur dioxide react chemically and dissolve in the hydrothermal water (Iwasaki and Ozawa 1960).

In ARD and AMD environments, sulfuric acid is derived from the oxidation of sulfide minerals exposed at the Earth's surface during erosion or by mining processes (Drever 1997; Langmuir 1997). Surface water bodies also undergo acidification in areas where sulfur dioxide emissions from smelting produce acid rain (Gunn et al. 1995). These acidification processes promote ionization and dissolution of contained metals in minerals that result in increased concentrations of dissolved elements (Stumm and Morgan 1996), with iron and aluminum

Received 15 September 2009; accepted 3 November 2009.

We thank G. M. Gadd and J.A. Raven for the invitation to contribute this review article. We thank Diane Winter for confirming our taxonomic assessment of diatoms in AMD at Green Valley. We also thank Springer for providing copyright permission to publish Figures 1a, b, and c from Aguilera et al. (2007), and the Society for Sedimentary Geology for providing copyright permission to publish Figures 4a and d from Brake and Hasiotis (2008).

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being highly concentrated due to their abundance in the Earth's crust. Other such acidic water bodies as soft water lakes, which are lime deficient and low in nutrients, and organic-rich bogs are not considered in this review article because their water chemistry is generally significantly different (e.g., Wieder 1985; Murphy 2002).

High acidity along with elevated concentration of dissolved metals is toxic to most aquatic organisms (Swift 1982). Additionally, these environments tend to be deficient in such nutrients as inorganic carbon and phosphorus, which limits primary production (Kapfer 1998; Nixdorf and Kapfer 1998). These factors also limit diversity and richness of biota to a few species of microorganisms (Mulholland et al. 1986; Verb and Vis 2000) that include prokaryotic bacteria and Archaea (Hallberg and Johnson 2003; Coupland and Johnson 2004; Druschel et al. 2004; Bruneel et al. 2006) and eukaryotic algae, protozoa, and fungi (Cooke 1966, 1976; Bennett 1969; Hargreaves et al. 1975; Albertino 1995; DeNicola 2000). Most microorganisms living in acidic environments are acid tolerant (i.e., acidotolerant), having a wide pH tolerance with optimum growth at or near normal pH, as opposed to being exclusively acidophilic with optimum growth and competition under acid conditions (e.g., Gross 2000; Gross and Robbins 2000; Konhauser 2007).

Acidic environments, thus, provide excellent natural laboratories to investigate the ecology of microorganisms under conditions of limited diversity and in the absence of grazers, bioeroders, and metazoans that would otherwise prey on microbial communities. The majority of studies on microorganisms in acidic environments focus on chemolithotrophic bacteria because of their ability to mediate their environment by dissolving minerals (e.g., Hallmann et al. 1993; Fowler et al. 1999; Pogliani and Donati 2000) and catalyzing redox reactions (e.g., Colmer et al. 1950; Kleinmann and Crerar 1979; Suzuki et al. 1990) that may lead to biomineralization (e.g., Lazaroff et al. 1982; Ferris et al. 1989; Bigham et al. 1990; Kim et al. 2002). It is well documented, for example, that iron-oxidizing chemolithotrophic bacteria of the genera *Acidithiobacillu*s and *Leptospirillum* generate acidity by catalyzing the oxidation of ferrous to ferric iron (Colmer et al. 1950; Kleinmann and Crerar 1979; Suzuki et al. 1990; Nordstrom and Southam 1997; Bond et al. 2000).

The influence of eukaryotic microorganisms on the physicochemical conditions in acidic environments, on the other hand, is not as well documented (Mann et al. 1989; Casiot et al. 2004; Brake and Hasiotis 2008). Although research has shown that some algal species in freshwater environments have the capacity to attenuate contaminants (e.g., Ferguson and Bubela 1974; Nakajima et al. 1981; Darnall et al. 1986; Kaplan et al. 1987), this activity may not be as viable in acidic environments for reasons that will be discussed later in the text. Recent research is beginning to establish a more important role for eukaryotic microorganisms in acidic environment. Brake et al. (2004) and Brake and Hasiotis (2008), for example, have documented the potential role of eukaryote-dominated biofilms in trapping and binding sediment to form iron-rich organosedimentary structures—that is stromatolites—in acidic environments. In this article we explore the potential effect of such activities on acidic ecosystems.

SURVIVAL IN ACIDIC CONDITIONS

Such eukaryotes as algae, protozoans, and fungi are found in most highly acidic environments where there is a sufficient energy source to sustain life, with the exception of high temperature environments exceeding 60°C (e.g., Roberts 1999; Rothschild and Mancinelli 2001). For life to exist, species have developed genetic adaptations and mechanisms to tolerate the adverse conditions (e.g., Gadd 1993, 2007; Pick 1999; Gross 2000). In highly acidic environments, cells must overcome the high H^+ concentration that may lead to rapid acidification of the cytosol (Gross 2000), as well as develop strategies to protect themselves from adverse effects commonly associated with acidity, such as decreased nutrients, increased dissolved metal concentrations (Olaveson and Stokes 1989), and limited supply of CO_2 for photosynthesis because of the absence of a bicarbonate pool (Olaveson and Stokes 1989; Gross 2000).

Major adaptations by eukaryotes to low pH conditions, therefore, are likely through the (1) modification of the plasma cell membrane to decrease permeability to protons and (2) overexpression or modification of the plasma membrane proton and ion transporters, as the ion channels and transporters are in contact with a low pH medium (Pick 1999).

For eukaryotes to maintain near-neutral cytosolic pH (see Table 1 for near neutral intracellular pH values for several acidophilic eukaryotic microorganisms), they must be able to actively pump H^+ out of the cytosol and back into the extracellular medium, develop an intracellular regulatory system to control pH level, or possess a plasma membrane that can maintain a high proton gradient that would be relatively impermeable (Pick 1999; Gross 2000; Messerli et al. 2005). Several studies reviewed here have proposed different adaptive molecular structures and physiological controls used by organisms to survive in acidic environments.

Pick (1999) studied *Dunaliella acidophilia*, a halotolerant unicellular green algae, in an attempt to understand how these and other eukaryotes survive in pH conditions as low as 1 or 0. In particular, the focus of the study was to determine what modifications or structural adaptations of basic transport mechanisms are necessary to (1) maintain a neutral intracellular pH, (2) accumulate essential organic and inorganic elements, and (3) make the extracellular plasma membrane surface structurally capable of limiting the influx of protons without hampering ionic transport. *Dunaliella acidophilia* maintains a neutral intracellular pH, resisting cytoplasmic acidification by maintaining a positive inside transmembrane potential, possessing a positive extracellular surface charge, and having a potent plasma membrane (PM) H^+ATPase.

The positive extracellular surface charge is not an adaptive feature, but a result of low external pH and serves to reduce

TABLE 1
Intracellular pH values for some acidophilic eukaryotes living in acidic (pH $\leq$ 3.5) water

Microorganisms	Phylum	Internal pH	External pH	Habitat	Locality
Chlamydomonas sp.	Chlorophyta	6.6	1.7–2.5	AMD	Rio Tinto, Spain
Dunaliella acidophila	Chlorophyta	6.2–7.2	0.5–3.0	Acidic water and soils	Italy and Czechoslovakia
Cyanidium caldarium	Rhodophyta	6.6	2.1	Hot springs	Europe, Asia, and America
Euglena mutabilis Schmitz	Euglenozoa	5.0–6.4	2.8	AMD	Pennsylvania, USA

Data from Lane and Burris (1981), Pick (1999), Messerli et al. (2005), and Seckbach and Oren (2007).

proton flux into the cytosol. The positive-inside electrical potential ($\Delta\Psi$) helps maintain a large ΔpH gradient across the plasma membrane that is generated by a strong PM H^+ATPase, which is an adaptive feature and provides for high capacity H^+ extrusion. A result of having a positive-inside $\Delta\Psi$ is a lower plasma membrane force that likely helps the alga avoid kinetic limitations on H^+ extrusion, as well as to decrease the driving force for H^+ influx. Furthermore, such extremophiles as *D. acidophilia* have a much higher internal buffering capacity than the neutrophilic species of *Dunaliella*. This provides added protection from rapid cytoplasmic acidification under stressful conditions (Gimmler and Weis 1992).

Finally, the uptake of K^+ ions is modulated mostly through K^+/H^+ cotransporter rather than through K^+ pumps, as K^+ uptake cannot be driven by the membrane potential. This allows *D. acidophilia* to maintain a 100x greater concentration of K^+ against the large electrochemical gradient. Overall, the adaptations and envirophysiological consequences associated with *D. acidophilia* that allow it to survive in highly acidic environments may also be present in other acidophilic protists, which live under similar conditions.

Gross (2000) reviewed the prerequisites for acidophilic and acidotolerant algae to live in highly acidic environments. Algae must have fairly impermeable plasma membranes with proton permeability coefficients (ppc) that range from $\sim$1 to 100 nm s^{-1} to control the proton concentration within the cytosol; the ppc of a typical plant, on the other hand, ranges from 10 to 50,000 nm s^{-1}.

Under conditions of complete darkness and insufficient energy supply, however, the cytosol of acidophilic and acidotolerant algae will rapidly acidify unless these algae can make its cell membrane impermeable temporarily to protons through the incorporation of sterols, proteins, or saturated fatty acids, or by having a highly positive membrane potential and positive surface charge (e.g., *Dunaliella acidophila*; see Gimmler et al. 1989). The acidity of the water also limits the availability of dissolved inorganic carbon (DIC). The pool of CO_2—the source of virtually all DIC in acidic water with pH < 4—can be quickly depleted during photosynthesis; however, this is counterbalanced in acidic waters by the high exchange rate between dissolved CO_2 and atmospheric CO_2, compared to neutral pH waters. Algae can also counter this issue by living in media at the sediment-water-air interface (i.e., terrestic or endolithic), which allows the algae to achieve greater and more rapid exchange rates with atmospheric CO_2 (e.g., Moreira et al. 1994; Gross and Oesterhelt 1999). Some algae, such as the acidotolerant *Chlorella saccharophila*, have an active carbon concentrating mechanism (CCM).

In *D. acidophilia*, CO_2 that enters the cell is converted to HCO_3^- in the cytosol and will accumulate until equilibrium between extra- and intracellular CO_2 concentrations is achieved. The intracellular DIC will be increased by 10 to15 times in this manner; however, this form of passive CCM is not very efficient. Overall, Gross (2000) concluded that acidophiles and acidotolerant organisms deal with incredible proton gradients across the plasma membrane without expending all their energy to maintain neutral cystolic pH, the mechanisms of which still need to be understood in detail as algae physiology varies widely. As a whole, these and other eukaryotes also have a remarkable tolerance to trace (i.e., heavy) metals and some toxic anions of which the exclusionary and complexation mechanisms are still understood poorly.

More recently, Messerli et al. (2005) studied the transmembrane electrochemical H^+ gradient in *Chlamydomonas* sp., an acidophilic protist, isolated from the Rio Tinto, Spain, to determine the energetic cost for eukaryotic acidophiles living in acidic pH. They hypothesized that some eukaryotic acidophiles maintain their transmembrane H^+ gradient by actively pumping out protons rather than imposing H^+ surface barriers to the extracellular portion of the cell membrane. Messerli et al. (2005) were able to determine through experimental analyses that *Chlamydomonas* sp. can tolerate a wide range of extracellular pH and maintain a near-neutral cytosolic pH in acidic conditions by actively pumping H^+ out of the cell. This is indicated by the burning of ATP at a faster rate compared to the same organism reared in neutral conditions.

Messerli et al. (2005) concluded that the primary structure of H^+ transporters in some acidophiles—for example, *Chlamydomonas* sp.—are no different than those found in neutral-growing protists, as their contractile vacuoles may help maintain the near neutral cystolic pH without exposing H^+ transporters or exchangers to highly acidic extracellular environment. They

also surmised that acidophiles likely evolved two classes of enzymes—plasma membrane channels and cell wall lysins—to function under acidic pH that allows them to live in such extreme environments.

Adverse Effects of Heavy Metal Toxicity

High concentrations of heavy metals associated with acidic environments are also toxic to most aquatic microorganisms and adversely impact growth by disrupting physiological, biochemical, or metabolic processes (Rai et al. 1981). Metals affect metabolic processes by causing the denaturation of proteins, including blockage of functional groups, decomposition of essential metabolites, displacement of essential metals, and disruption of the cell membrane (Gadd 1986; Ford et al. 1995; Das et al. 2009). Rai et al. (1981) provided a comprehensive table summarizing reported effects of heavy metals on various algal species. In spite of these adverse effects, some algae, fungi, and protozoans are able to tolerate high metal concentrations (Whitton 1970; Albertano et al. 1980a, 1980b) by genetic adaptations that prohibit entry of metals into the cytoplasm via metal complexation outside the cell, extracellular binding and precipitation of metals, reduced metal uptake, increased efflux, and intracellular detoxification or compartmentalization of metals (Gadd and Griffiths 1978; Gadd 1986, 2007; Reed and Gadd 1989; Novis and Harding 2007).

Another potential strategy for protecting cells against adverse environmental conditions is through the production of extracellular polymeric substances (EPS), also referred to as mucilage or exopolymers. Various organisms, including bacteria, algae, protozoa, and fungi, produce EPS to hold cells together, particularly in biofilm communities (Neu et al. 2003; Baffico et al. 2004). The EPS consists mainly of polysaccharides (Edgar and Pickett-Heaps 1984; Hoagland et al. 1993), although specific composition is dependent on the microorganism (Neu et al. 2003). The EPS forms a three-dimensional, highly hydrated, matrix responsible for the structural and functional integrity and physiochemical and biological properties of the biofilm (Flemming and Wingender 2001).

The EPS is generally secreted as stalks, tubes, pads, filaments, and sheaths (Patrick and Reimer 1966; Hoagland et al. 1993; Winsborough 2000) that not only aid in adhesion but may protect cells from rapid physicochemical changes by chelating and adsorbing metals and acting as a diffusion barrier that protects cells against toxins (Gadd 1990, 2009; Hoagland et al. 1993; Decho 1994; McClean et al. 1996; Steward 1998). Exopolymers often contain such functional groups as carboxyl, hydroxyl, and amine groups that can potentially sequester ions and molecules, reducing toxic exposure of cells (Decho 2000). Additionally, Decho (2000) suggested that biofilms are highly structured, and microorganisms may be able to position themselves in the EPS to optimize nutrient gathering and to avoid toxic compounds that are sequestered within parts of the biofilm. Because biofilms are generally composed of spatially and temporally mixed-species communities, the EPS may allow them to coexist in a variety of heterogeneous chemical zones (e.g., Costerton et al. 1995).

The binding of metals by EPS is thought to inactivate some enzymes by blocking essential functional groups on the enzyme, displacing essential metal ions already present in the enzyme, and modifying the structural formation of the enzyme (Babich et al. 1985; Decho 2000). Decho (2000) suggested that the biofilm functions most efficiently when metal-sequestering exopolymers are localized in separate areas of the biofilm that are further from the cells, allowing necessary extracellular enzymes to occupy positions in closer proximity to the cells. This process allows enzymes in the biofilms to function in the presence of elevated metals, while keeping toxic metals away from the cells. Microbial exopolymers may also retard or limit diffusion of material to the cell surface or removal of metabolic products by the same process (Costerton et al. 1994; Lawrence et al. 1994; Stewart 1998; Lewis 2001). The rate of diffusion within the biofilm is dependent on the diffusion coefficient of the biofilm and of the solute of interest in the water (Stewart 1998).

Species of the green algae *Chlorella*, the cyanobacterium *Chroococcus*, and the dinoflagelate *Gymnodinium*, for example, secrete thick gelatinous sheaths as a partial barrier to acidity (Findlay and Kasian 1986). García-Meza et al. (2005) showed that biofilms composed mostly of the chlorophyte *Chlorococcum* sp. and the cyanobacterium *Phormidium* sp. in mine-tailing sediment produced increased amounts of EPS with increasing metal exposure. Similarly, Pistocchi et al. (2000) found that cultures of diatoms and dinoflagellates also increased EPS production in response to increasing toxic metal concentrations. These processes, in general, appear to not only provide protection against adverse environmental conditions, but may also create favorable physicochemical conditions within the biofilm that allow less adaptive species to exist under conditions that would otherwise be too toxic (e.g., Costerton et al. 1995).

EUKARYOTIC DIVERISTY AND BIOFILM COMMUNITIES

A significant volume of work addresses the diversity of eukaryotic species in acidic environments, particularly with regard to algae (e.g., Bennett 1969; Hargreaves et al. 1975; Sheath et al. 1982; Dixit and Smol 1989; Albertino 1995; Gross 2000; Sabater et al. 2003). One of the problems plaguing research and review of the literature is the misidentification of some microorganisms within a genus. For example, *Eunotia tenella* reported by Bennett (1969) may be Warner's (1971) *E. exigua* (see De Nicola, 2000 for other examples). Additionally, species names have changed through time (e.g., *Hormidium rivulare* renamed to *Klebsormidium rivulare*).

The following discussion focuses on benthic eukaryotes because of their potential impact on acidic environments and because increasing acidity tends to decrease planktonic production and increase benthic biomass (Müller 1980; Dixit and Smol 1989), which maybe why a significant volume of

literature examines benthic species (e.g., Kapfer 1998; Verb and Vis 2000; López-Archilla et al. 2001; Brake et al. 2001b, 2002, 2004; Sabater et al. 2003; Baffico et al. 2004; Aguilera et al. 2006, 2007a, b, 2008; Brake and Hasiotis 2008). Table 2 lists frequently reported benthic eukaryotic species in aquatic environments measuring pH $\leq$ 3.5. Species are grouped according to green, red, and golden algae, diatoms, euglena, ciliates, amoeba, flagellates, heliozoa, and fungi. Also included in Table 2 is the environment and location where species were identified. Additionally, we have noted species documented as being acidophilic.

Some of the early hallmark studies on eukaryotic microorganisms in acidic environments were conducted on streams impacted by AMD in the United States and Great Britain. These include studies by Lackey (1938), Joseph (1953), Bennett (1969), and Hargreaves et al. (1975). Lackey (1938) identified 67 species of algae and protozoa in AMD measuring pH $<$ 4.0 at sites in Indiana and West Virginia, USA. Species tolerant of highly acidic conditions included the green alga *Chlamydomonas* sp., the golden alga *Chromulina* sp., euglenid *Euglena mutabilis*, heliozoa *Actinophrys sol*, cilitates *Oxytricha* sp. and *Urotricha farcta*, and diatoms *Navicula* sp. and *Urothrix zonata,* with *E. mutabilis*, *Oxytricha* sp. and *Navicula* sp. being the most abundant. Joseph (1953) found an abundance of diatoms in AMD-impacted streams in West Virginia and Pennsylvania, USA, with *Navicula viridis* being the dominant species. Also present in abundance were *E. mutabilis* and *E. viridis*, with the latter being as abundant as *N. viridis*. Bennett (1969) noted 107 different species of microorganisms in AMD-impacted environments in West Virginia with 25 of these species occurring exclusively in the most contaminated and acidic water. Of the 25 species, *E. mutabilis* and the diatoms *Eunotia tenella* and *Pinnularia braunii* were most abundant. Hargreaves et al. (1975) investigated AMD sites in Great Britain and identified 24 algal species at 14 sites with pH $<$ 3.0. Species that were widely distributed at the sampling sites included diatoms *Nitzschia* spp. and *Pinnularia acoricola* and green algae *Hormidium rivulare* (i.e., *Klebsormidium rivulare*) and *Zygogonium ericetorum*. Hargreaves et al. (1975) also provided detailed information on seasonal variations in algal distribution and extent of stream bottom coverage. All of these studies, along with numerous other studies (e.g., Pentecost 1982; Sheath et al. 1982; Nakatsu and Hutchinson 1988; Kapfer 1998; Brake et al. 2001b; Sabater et al. 2003; Casiot et al. 2004) cite *E. mutabilis* as one of the more widely distributed eukaryotic species in AMD environments. In addition, *E. mutabilis* and *Chlamydomonas acidophila* are reported to occur in environments with high concentrations of heavy metals (Fott 1956; Brake et al. 2001a).

Several other studies of eukaryotes in acidic environments are noteworthy. Sheath et al. (1982) examined benthic microbial communities in tundra ponds acidified by sulfur dioxide and sulfuric acid aerosols from burning bituminous shales. Primary productivity and biomass of the biofilms was dominated by *Chlamydomonas acidophila*, *Euglena mutabilis*, and diatom species of *Nitzschia communis*, *Eunotia arcus*, and *E. glacialis*. In geothermal systems, several unique thermo-acidophilic species of unicellular red algae (Rhodophyte) were identified in acidic springs, streams, and fumeroles (DeLuca and Taddei 1970; Brock 1978; Albertano et al. 2000; Cozzolino et al. 2000). These consisted of *Cyanidium caldarium*, *Cyanidioschyzon merolae*, and *Galdieria sulphuraria*, which have adapted to temperatures in the range of 45–56°C and very low pH (pH $<$ 2–4) conditions (Doemel and Brock 1970; Seckbach et al. 2007). Aguilera et al. (2006, 2007a, b) examined benthic biofilms in Spain's highly contaminated and acidic Río Tinto River that flows through the Iberian Pyrite Belt and identified several diverse communities dominated by eukaryotic microorganisms. The biomass was composed of eukaryotic species similar to those reported in other AMD systems, including diatoms (*Pinnularia* sp.), *E. mutabilis*, and several green algae (*Chlamydomonas* sp., *Chlorella* sp., *Dunaliella* sp., *Zygnemopsis* sp., *Klebsormidium* sp.). They also identified a species of red algae (*Cyanidium* sp.) and protozoa, consisting of two amoebae (*Vahlkampfia* sp. and *Naegleria* sp.), a species of heliozoa (*Actinophrys* sp.), four flagellates (*Bodo* sp., *Cercomonas* sp., *Ochromonas* sp., and *Labirynthula* sp.), and two ciliates (*Oxytrichia* sp. and *Colpidium* sp.). Similar protozoa (i.e., *Bodo* sp., *Actinophrys sol*, *Vahlkampfia* sp. and *Oxtricha* sp.) were also identified by Lackey (1938). Although such protozoa as these have been identified in benthic communities, they have not been reported as forming the bulk of the biomass. Packroff and Woelfl (2000) provided a review of heterotrophic protists in extremely acidic environments and indicated that the ciliates *Urotricha*, *Vorticella*, and *Oxytricha* and the heliozoan *Actinophrys* sp. are the most commonly reported species.

Several studies focus exclusively on diatoms in acidic environments, possibly due to their widespread abundance. The acidophilic diatom *Eunotia exigua* is the most commonly reported species, with widespread occurrences in lakes and streams receiving AMD in North American and Europe (Dixit and Smol 1989; Kwandrans 1993; Nixdorf and Kapfer 1998; Verb and Vis 2000; Koschorreck and Tittel 2002). *Eunotia exigua* is also reported to occur with *E. paludosa*, *E. tenella*, and *Pinnularia subcapitata* in highly acidic streams in Poland (Kwandrans 1993). In acidic mining lakes in Germany, *E. exigua* and *P. obsecura* form dense layers that cover the sediment (Koschorreck and Tittel 2002), and *E. exigua* and *Frustulia rhomboids* are the dominant flora in AMD-impacted streams in Ohio, USA (Verb and Vis 2000). DeNicola (2000) provides a comprehensive review of mainly benthic diatoms in highly acidic environments measuring pH $\leq$ 3.5.

Fewer articles address fungi in highly acidic aquatic environments (e.g., Cooke 1966, 1976; Gross and Robbins 2000; López-Archilla et al. 2004; Das et al. 2009), particularly with regard to their occurrence in biofilm communities (e.g., Baker et al. 2004). Fungi are reported to have a wide range of tolerance for varying levels of pH (Johnson 1998). Species of *Asperillus*, *Penicillium*, and *Fusarium* can survive at pH $\sim$2

TABLE 2
Summary of literature dealing with benthic eukaryotes commonly reported in aquatic environments of pH ≤ 3.5

Microorganism	Acidophilic (Y or N)	Optimum pH for growth	Location	Habitat	Reference
Diatoms (Bacillariophyta)					
Eunotia sp.	N	—	USA	sulfide ore AMD	Krishnaswamy and Hanger 1998; Niyogi et al. 2002
Eunotia arcus	N	—	Canada	acidic tundra ponds	Sheath et al. 1982
Eunotia cf. *denticulata*	N	—	Germany	acid mine lakes	Kapfer 1998
Eunotia exigua	Y	5.2–5.4	England, Germany, Portugal, USA	coal mine AMD, sulfide ore AMD, acid mine lakes	Cholnoky 1968; Warner 1971; Patrick 1974; Hargreaves et al. 1975; Kwandrans 1993; Kapfer 1998; DeNicola 2000; Verb & Vis 2000; Koschorreck & Tuttel 2002; Luís et al. 2009
Eunotia glacialis	N	—	Canada	acidic tundra ponds	Sheath et al. 1982
Eunotia pectinalis	N	—	USA	coal mine AMD	Pattrick 1974; DeNicola 2000
Eunotia steineckei	N	—	Germany	acid mine lakes	Koschorreck & Tittel 2002
Eunotia tenella	N	—	Germany, Poland, USA	acid mine lakes, coal mine AMD, sufide ore AMD	Bennett 1969; Lampkin & Sommerfeld 1982; Kwandrans 1993; Koschorreck & Tittel 2002
Frustulia rhomoides	N	—	USA	coal mine AMD	Bennett 1969; Warner 1971; Patrick 1974; DeNicola 2000; Verb & Vis 2000
Navicula sp.	N	—	USA	sulfide ore AMD, coal mine AMD	Lackey 1938; Krishnaswamy & Hanger 1998
Navicula viridis	N	—	USA	coal mine AMD	Joseph 1953
Nitzschia communis	N	—	Canada	acidic tundra ponds	Sheath et al. 1982
Nitzschia elliptica	N	—	England	coal mine AMD	Hargreaves et al. 1975
Nitzschia capitellata/ subcapitellata	N	—	England, USA	coal mine AMD, sulfide ore AMD	Hargreaves et al. 1975; Lampkin & Sommerfeld 1982
Nitzschia palea	N	—	Canada, England, USA	acidic tundra ponds, coal mine AMD	Patrick 1974; Hargreaves et al. 1975; Sheath et al. 1982
Pinnularia sp.	N	—	Spain, USA	sulfide ore AMD, coal mine AMD	Krishnaswamy & Hanger 1998; Aguilera et al. 2006, 2007a
Pinnularia acoricola	N	—	England, Portugal, Spain, USA	coal mine AMD, sulfide ore AMD, acidic hot springs	Warner 1971; Hargreaves et al. 1975; Whitton & Diaz 1981; Sabater et al. 2003; Luís et al. 2009
Pinnularia braunii	Y	1.5	Italy, Japan, USA	sulfuric acid volcanic habitats, coal mine AMD	Bennett 1969; Satake & Saijo 1974; Albertino 1995
Pinnularia ferroindulgentissima	N	—	USA	coal mine AMD	Czarnecki & Cawley 1997
Pinnularia obscura	N	—	Germany, USA	acid mine lakes; coal mine AMD	Patrick 1974; Koschorreck & Tittel 2002

(Continued on next page)

TABLE 2
Summary of literature dealing with benthic eukaryotes commonly reported in aquatic environments of pH ≤ 3.5

Microorganism	Acidophilic (Y or N)	Optimum pH for growth	Location	Habitat	Reference
Pinnularia subcapitata	N	—	Poland, USA	sulfide ore AMD; coal mine AMD	Kwandrans 1993; DeNicola 2000; Verb & Vis 2000
Pinnularia termitina	N	—	USA	coal mine AMD	Warner 1971
Green Algae (Chlorophyta)					
Chlamydomonas sp.	N	—	England, Germany, Spain, USA	coal mine AMD, sulfide ore AMD, acid mine lakes	Bennett 1969; Hargreaves et al. 1975; Kapfer 1998; Brake et al. 2001; Aguilera et al. 2006
Chlamydomonas adicophila	Y	3.0–5.0	Canada, Spain	coal mine AMD, sulfide ore AMD, acidic tundra ponds	Lackey 1938; Sheath et al. 1982; Lopez-Archilla et al. 2001; Gerloff-Elias et al. 2005;
Chlorella sp.	N	—	Spain	sulfide ore AMD	Lopez-Archilla et al. 2001; Aguilera et al. 2007a
Cylindrocapsa sp.	N	—	USA	sulfide ore AMD	Krishnaswamy & Hanger 1998
Dunaliella sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006, 2007a
Dunaliella acidophilia	Y	1.0	Italy	hot springs	Gimmler & Weis 1992;
Klebsormidium sp.	N	—	England, Portugal, Spain	coal mine AMD, sufide ore AMD	Lopez-Archilla et al. 2001; Aguilera et al. 2007a; Valente & Gomes 2007
Klebsormidium rivulare (formerly *Hormidium rivulare*)	Y	3.5–4.0	England, USA	coal mine AMD	Hargreaves et al. 1975; Stevens et al. 2001
Klebsormidium flaccidum	N	—	Spain	sulfide ore AMD	Sabater et al. 2003
Penium jenneri	N	—	USA	coal mine AMD	Bennett 1969
Stigeoclonium sp.	N	—	USA	coal mine AMD	Lackey 1938; Stevens et al. 2001
Ulothrix sp.	N	—	USA	coal mine AMD, sulfide ore AMD	Bennett 1969; Krishnaswamy & Hanger 1998
Ulothrix subtilis	N	—	USA	coal mine AMD	Bennett 1969
Ulothrix tenerrima	N	—	USA	coal mine AMD	Warner 1971
Ulothrix zonata	N	—	USA	coal mine AMD	Lackey 1938
Zygnema sp.	N	—	Spain	sulfide ore AMD	Lopez-Archilla et al. 2001
Zygnemopsis sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006, 2007a
Zygogonium ericetorum	Y	3.0	England, Germany, Spain, USA	acid mine lake, coal mine AMD, sulfide ore AMD, acid hot springs	Lynn & Brock 1969; Hargreaves et al. 1975; Aguilera et al. 2006; Kleeberg et al. 2006

Golden Algae (Chrysophyta)					
Chromulina sp.	N	—	USA	coal mine AMD	Lackey 1938
Gloeochrysis sp.	N	—	Argentina	volcanic acid water	Baffico et al. 2004
Gloeochrysis tufosa	N	—	England	coal mine AMD	Hargreaves et al. 1975
Red Algae (Rhodophyta)					
Gladieria sulphuraria	Y	2.0	Italy, Spain	sulfide ore AMD, acidic hot springs	Albertano et al. 2000; Lopez-Archilla et al. 2000; Pinto et al. 2007
Cyanidium sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006, 2007a
Cyanidium caldarium	Y	1.5	Italy, USA	acidic hot springs, AMD	Doemel & Brock 1971; Belly et al. 1973; Albertano et al. 2000; Pinto et al. 2007
Euglena (Euglenaphyta)					
Euglena mutabilis	Y	3.0–4.0	Canada, England, France, Germany, Portugal, Spain, USA	coal mine AMD, sulfide ore AMD, acid mine lakes, acidic tundra ponds	Lackey 1938; Bennett 1969; Warner 1971; Hargreaves et al. 1975; Sheath et al. 1982; Olaveson & Stokes 1989; Kapfer 1998; Krishnaswamy & Hanger 1998; Brake et al. 2001; Sabater et al. 2003; Casiot et al. 2004; Aguilera et al. 2006, 2007a; Valente & Gomes 2007
Ciliates					
Colpidium sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006
Oxytricha sp.	N	—	Germany, Spain, USA	coal mine AMD, sulfide ore AMD, acid mine lakes	Lackey 1938; Packroff & Woelfl 2000; Aguilera et al. 2006
Urotricha armata	N	—	Germany	acid mine lakes	Packroff & Woelfl 2000
Urotricha farcta	N	—	USA	coal mine AMD	Lackey 1938
Vorticella sp.	N	—	Germany	acid mine lakes	Packroff & Woelfl 2000
Amoeba					
Naegleria sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006
Vahlkampfia sp.	N	—	England, Spain, USA	sulfide ore AMD, coal mine AMD	Lackey 1938; Johnson 1998; Baker et al. 2004; Aguilera et al. 2006

(Continued on next page)

TABLE 2
Summary of literature dealing with benthic eukaryotes commonly reported in aquatic environments of pH ≤ 3.5

Microorganism	Acidophilic (Y or N)	Optimum pH for growth	Location	Habitat	Reference
Flagellates					
Bodo sp.	N	—	Spain, USA	coal mine AMD, sulfide ore AMD	Lackey 1938; Aguilera et al. 2006
Cercomonas sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006
Labirynthula sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006
Ochroomonas sp.	N	—	Spain	sulfide ore AMD	Aguilera et al. 2006
Helizoa					
Actinophrys sp.	N	—	Germany, Spain	sulfide ore AMD, acid mine lakes	Packroff & Woelfl 2000; Aguilera et al. 2006
Actinophyrs cf. *sol*	N	—	Germany, USA	acid mine lakes, coal mine AMD	Lackey 1938; Packroff & Woelfl 2000
Fungi					Aguilera et al. 2006
Acidomyces richmondensis	N	—	USA	sulfide ore AMD	Baker et al. 2004
Alternaria sp.	N	—	USA	coal mine AMD	Joseph 1953
Aspergillus sp.	N	—	USA	coal mine AMD	Joseph 1953
Bispora sp.	Y	1.0–1.5	China	uranium mine AMD	Luo et al. 2009
Cladosporium sp.	N	—	USA	coal mine AMD	Joseph 1953
Cryptococcus sp.	N	—	Canada	acidic tundra ponds	Hutchinson et al. 1981; Havas & Hutchinson. 1983
Ochroconis gallopavum (formerly *Dactylaria gallopava*)	N	—	USA	hot springs	Belly et al. 1973
Penicillium sp.	N	—	USA	coal mine AMD	Joseph 1953
Trichoderma sp.	N	—	USA	coal mine AMD	Joseph 1953
Trichothecium sp.	N	—	USA	coal mine AMD	Joseph 1953

with an upper growth limit near pH 10 (Langworthy 1978). As an example of this range of tolerance, such species as *Phycomyces blakesleeanus* and *Marasmius foetidus* are acidophilic, having optimum growth near pH 3, but can survive to pH 7 (Langworthy 1978). The most acidophilic fungi reported in the literature are *Acontium velatum*, *Trichosporon cerebriforme*, and *Cephalosporium* sp., growing at pH values near zero. *Acontium velatum* and *Cephalosporium* sp. were isolated from acid solutions used at an industrial plant (Starkey and Waksman 1943), and *T. cerebriforme* was obtained from bottles of sulfuric acid reagent (Sletten and Skinner 1948).

Several studies on fungi in acidic habitats report culturing species from water, stream sediment, and soil samples. Cooke (1966, 1976) identified a large number of fungi residing in water, soil, bank soil, and upland soil impacted by AMD in Ohio and West Virginia, USA. López-Archilla et al. (2004) studied fungal populations in the acidic Tinto River, Spain, and identified 90 strains of yeast, 349 strains of hyphomycetes, and a few strains of ascomycetes and zygomycetes from 144 samples collected at 5 cm depth of water close to the riverbank. Luo et al. (2009) isolated an acidophilic fungus *Bispora* sp. from wastewater at a uranium mine in China and were able to grow the fungus at pH 2.5–3.0. Gross and Robbins (2000) compiled a detailed list of acidophilic and acidotolerant fungi and yeast. The majority of the species were isolated from soil, bogs, and swamps, with only a few isolated from acidic water: *Cryptococcus* sp. from acidic tundra ponds; *Ochroconis gallopavum* from hot spring effluent in Yellowstone National Park, USA; and *Scytalidium acidophilum* from uranium AMD. Whether the fungi reported in the studies here are directly associated with identifiable biofilms is unknown.

Only a few studies document the occurrence of fungi in biofilms (Brake et al. 2002; Baker et al. 2004). Baker et al. (2004) found that, although fungal diversity was low, fungal hyphae formed a significant portion of total biofilm biomass in extremely acidic water at the Richmond Mine in California, USA. They genetically identify phylotopes of the Eurotiomycetes and Dothideomycetes families belonging to the phylum Ascomycota. Brake et al. (2002) also noted the occurrence of fungal hyphae in autotrophic eukaryote-dominated biofilms growing in highly acidic mine drainage at the abandoned Green Valley coal mine site in Indiana, USA; however, identification of fungal species was not determined. Table 2 lists those fungal species commonly reported in such acidic aquatic environments as AMD, acidified ponds, and hot springs. For a more detailed list of fungi found in other acidic environments, refer to Gross and Robbins (2000).

An interesting aspect of microbial biofilms is the population structure within a community. Although we have a fairly good understanding of microbial diversity in acidic environments, little is known about microbial distribution within biofilms. In marine environments, benthic microbial mats have been extensively studied because of the role they play in building organosedimentary structures (i.e., stromatolites). Marine microbial mats are composed of primary producers, consumers, and decomposers (Stolz et al. 1989) that are often vertically stratified downwards from aerobic to anaerobic and from phototrophic to chemotrophic (Horodyski et al. 1977; Bauld 1981; Stolz 1990; Stal 1994; Briggs 2003) along steep physicochemical gradients of such factors as dissolved oxygen, sulfide, and light intensities (Bauld 1981; Revsbech et al. 1983; Jørgensen et al. 1987, 1988; Kühl and Jørgensen 1992; Visscher and Stolz 2005). Stratification is often visibly defined by colors, representing the type of microorganisms present within each layer. The top layer is green and generally comprised of oxygenic phototrophic cyanobacteria along with eukaryotic diatoms and euglenid flagellates (Golubic 1976; Awramik and Riding 1988; Paterson et al. 2003) that secrete EPS during locomotion and for anchorage (Edgar and Pickett-Heaps 1984). Beneath the phototrophs is a purple-pink layer of anoxygenic photosynthetic bacteria (e.g., purple sulfur bacteria), followed by a black layer consisting of anaerobic heterotrophs and sulfate-reducing bacteria (Knoll and Awramik 1983; Stal 1994).

Similar microbial stratification has not been well documented in biofilms from acidic environments. Studies on these biofilms tend to focus on species diversity rather than on microbial stratification (e.g., Brake et al. 2002; Baker et al. 2004; López-Archilla et al. 2004; Aguilera et al. 2006, 2007b; Souza-Egipsy et al. 2008). Here we highlight a few studies that have begun to look more closely at the population structures within biofilms in acidic systems. At the Green Valley mine site, Brake et al. (2002, 2004) and Brake and Hasiotis (2008) identified several distinctive eukaryotic biofilms dominated by *Euglena mutabilis* and diatoms. In the *E. mutabilis*-dominated biofilm, they reported crude stratification consisting of an upper layer of entwined *E. mutabilis* cells with very minor fungal hyphae, *Chlamydomonas* sp., diatoms, and bacteria overlying a thin undermat ($\sim$ 1 mm) composed of several bacterial species (i.e., primarily gram-negative bacilli) with lesser fungal hyphae, diatoms, and algal filaments (Brake et al. 2002). Fang et al. (2007) analyzed samples from the site consisting of biofilm attached to laminated iron-rich stromatolites for which formation will be discussed later in this text. Lipid analyses preformed on both the biofilm and underlying stromatolite to determine microbial biomass and community structure showed a microbial zonation comparable to communities that build marine stromatolites. The top biofilm layer contained the highest concentration of hydrocarbons, which decreased with depth into the stromatolite. Fatty acid compositions indicated that the biofilm layer was comprised mainly of phototrophic microorganisms, making up 83% of the total biomass; whereas, prokaryotic microorganisms (i.e., bacteria and Archaea) and fungi dominated the stromatolite layers, with fungal biomass being highest in the upper part of the stromatolite. Analytical data also suggested that Gram-positive bacteria and acidophiles occurred with fungi in the upper layers of the stromatolite. The lower stromatolite layers, on the other hand, contained biomolecular evidence of anaerobic

sulfate-reducing bacteria (SRB), with SRB and bacterial diversity increasing with depth. The reader is referred to Fang et al. (2007) for more detailed discussion on the lipid analyses.

Aguilera et al. (2008) identified three distinct eukaryote-dominated biofilms in the Río Tinto River: *Euglena mutabilis*-dominated biofilms with minor *Pinnularia*, *Chlorella* sp.-dominated biofilms, and biofilms composed of filamentous algae belonging to the genus *Zygnemopis* with minor *Cyanidium* sp. Preliminary microscopic analysis of some of these biofilms shows a population structure composed of different layers of eukaryotic species separated by a possible layer of EPS (Aguilera et al. 2007b). This included biofilms where EPS separated: (1) an upper layer of *Euglena* from a lower layer *Pinnularia* (Fig. 1a); (2) an upper layer of *Dunaliella* cells from a lower layer of filamentous fungi and fine minerals (Fig. 1b); and (3) a densely packed diatom layer from a lower layer of *Chlorella* (Fig. 1c). Souza-Egipsy et al. (2008) also analyzed similar eukaryotic biofilms from the Río Tinto River to characterize spatial relationships between prokaryotic and eukaryotic microorganisms. They observed *Pinnularia*-dominated biofilms in which bacteria formed layers around *Pinnularia* cells and dense layers of *Cyanidium* biofilms with bacteria in clusters around layers of *Cyanidium*. Prokaryotic diversity was found to be lower in thinner biofilms, with species closely related to those in the water column; whereas, thicker biofilms maintained higher prokaryotic diversity, possibly because the biofilms provided microniches for less adapted species. They also noted that fungi and bacteria were located primarily at the water-sediment interface.

Although some studies have noted a certain level of microbial stratification within eukaryote-dominated biofilms in acidic environments, additional questions remain to be answered. If eukaryote-dominated biofilms in acidic systems are microbially stratified, are they stratified along similar steep geochemical gradients as in marine microbial mats that build biolaminated structures? Are there trophic levels in acidic biofilm communities and how do they compare with those in marine biofilms? What is the relationship between microorganisms in the biofilm and between microorganisms in stratified layers? Answers to these questions will further enhance our understanding of the dynamics and importance of eukaryotic species in acidic environments.

SIGNIFICANCE OF EUKARYOTIC MICROBIAL ACTIVITY

The significance of microorganisms in mediating the environment is commonly viewed from the perspective of their contribution to geochemical cycles. Of particular interest in acidic environments are biological activities that result in chemical changes, especially those causing changes in redox state of iron and sulfur, mineral precipitation, dissolution of minerals, and adsorption of metals, since these factors can either exacerbate or naturally attenuate acidic conditions. Microbiological research has focused primarily on chemolithotrophic bacteria because they possess the ability to catalyze chemical reactions and produce complexing agents that affect speciation and mobility of metals, resulting in the generation of acidity, dissolution and precipitation minerals, and changes in redox states of aqueous iron and sulfur species (Bigham et al. 1992; Ledin and Pedersen 1996; Warren and Ferris 1998; Wood et al. 2001; Ehrlich 2002). The biological activity of eukaryotes, on the other hand, is often overlooked in terms of their impact on environmental conditions. In the following discussion, we suggest that eukaryotic microorganisms also play a significant role in mediating their environment to the extent that they contribute to the attenuation of metals in contaminated water and to the formation of organosedimentary structures that may be used as analogs for similar structures in the ancient rock record.

Metal Attenuation via Biosorption and Intracellular Sequestration

Both prokaryotic and eukaryotic microorganisms are known to accumulate heavy metals at significantly higher concentration than found in the environment (Schulz-Baldes and Lewin 1976; Brierley et al. 1989; Kelly 1999). Metal sequestration by eukaryotic microorganisms, especially algae and fungi, has been extensively studied in contaminated freshwater and marine environments (e.g., Rai et al. 1981; Foster 1982; Gadd 1986, 1993, 2007, 2009; Kaplan et al. 1987, 1988; Brierley et al. 1989; Lawrence et al. 1998). One of the main mechanisms for metal accumulation in biomass is via biosorption. Biosorption is the removal of organic or inorganic substances from solution by biological material—living or dead biomass (Gadd 2009). This may be achieved by a number of mechanisms that include sorption, ion exchange, surface complexation, precipitation, and intracellular accumulation (Brierly et al. 1989; Gadd 2009). Metal mobility, however, is dependent on the microorganisms involved, the environment, and the physicochemical conditions in which they live (e.g., Gadd 2004, 2007, 2009).

In eukaryotes, metals are often bound to the cell walls or membranes or to secreted EPS (Nakajima et al. 1981; Rai et al. 1981; Bistricki and Munawar 1982; Kelly 1999). Cell surfaces contain such reactive chemical groups as carboxylates and phosphates that are available for ionic interactions with solutes (Fortin et al. 1997). In algae, for example, there are a number of functional groups (e.g., carboxyls) that deprotonate under normal growth conditions, allowing algae to sequester a wide range of metals in excess of 100 mg of metal per g biomass dry weight (Volesky and Holan 1995; Konhauser 2007). The capacity for fungi and yeast to sequester metals has also been extensively studied (Gadd 1993). Cellular interactions in fungi can result in extracellular precipitation and complexation of metals, metal binding to cell walls, transport of toxic metals through the plasma and vacuolar membranes, and intracellular sequestration, which includes binding of toxic metals to proteins and peptides and vacuolar compartmentalization (Gadd 1993). The polymer of chitin in fungal cell walls, for example, contains protonated amino groups that are important in sorption

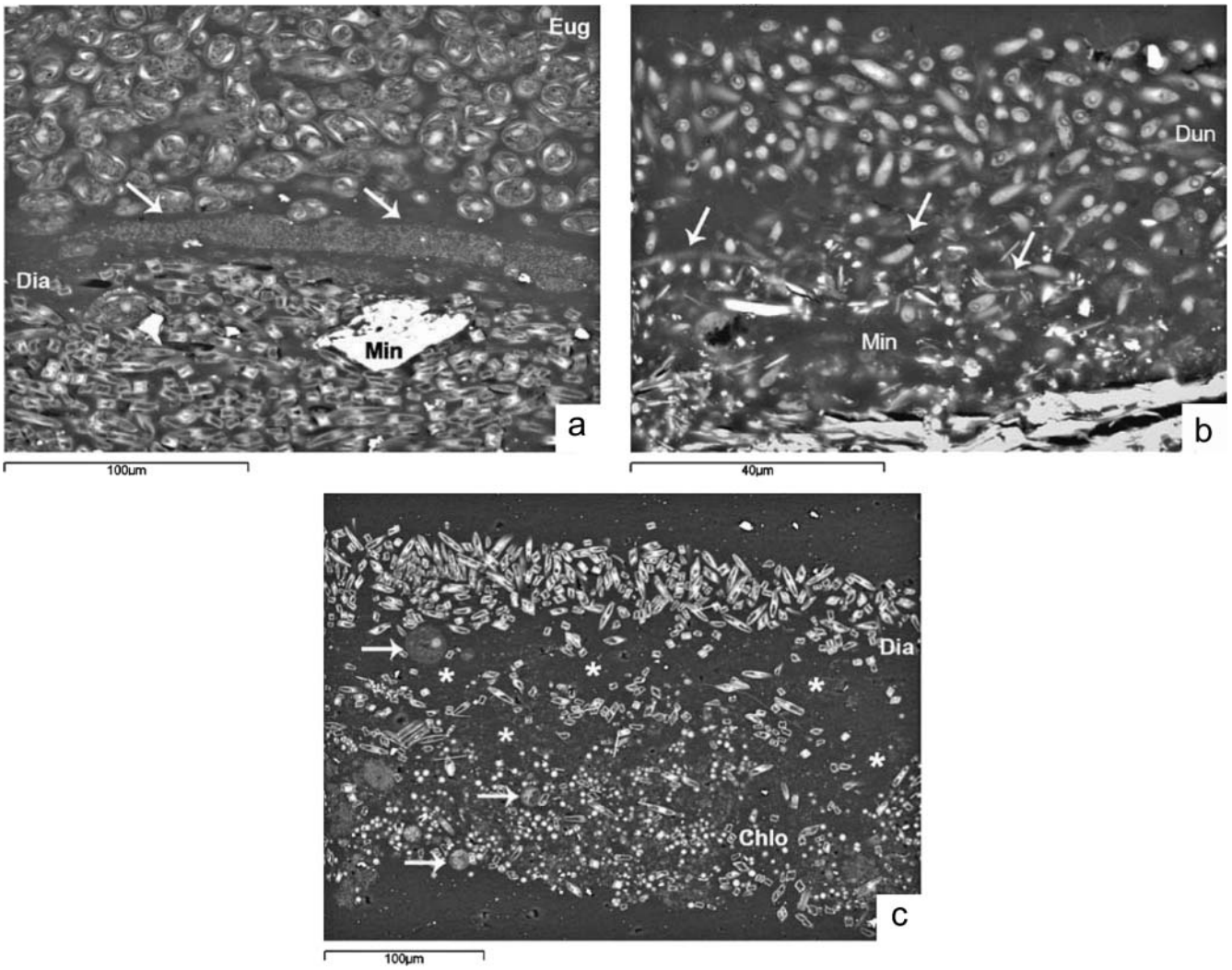

FIG. 1. SEM photomicrographs of eukaryote-dominated biofilms from the Río Tinto River showing community structures (from Aguilera et al. 2007). (a) Biofilm consisting of an upper layer of *Euglena* (Eug) cells separated by EPS (arrows) from an underlying layer of *Pinnularia* diatoms (Dia). (b) *Dunaliella*-dominated (Dun) biofilm overlying a layer of fungi (arrows) and mineral particles (Min). (c) Biofilm composed of a dense layer of near vertical diatoms (Dia) separated by EPS (asterisks) from a lower layer of *Chlorella* (Chlo). Arrows indicate the presence of some protists.

of anionic species (e.g., Tsezos 1986) and carboxyl and phosphate groups that are linked to adsorption of cationic species (e.g., Tobin et al. 1990; Konhauser 2007). Phenolic polymers and melanins in fungal cells possess oxygen-containing groups (e.g., carboxyl, carbonyl, phenolic and alcoholic hydroxyl, and methoxyl groups) that are also effective in metal sorption (e.g., Caesar-Tonthat et al. 1995; Konhauser 2007; Gadd 2009).

EPS is also capable of sequestering metals, particularly in association with biofilms (e.g., White and Gadd 1998; Brake and Hasiotis 2008; Gadd 2009). The affinity of EPS anionic ligands for such multivalent cations as iron, calcium, magnesium, and copper is strong and may facilitate mineral precipitation, particularly in metal-laden water (e.g., McClean and Beveridge 1990; Schultze-Lam et al. 1996). The exopolymer matrix may also contribute to metal immobilization by providing ion exchange sites, which may contribute to mineral nucleation (Lawrence et al. 1998). This strategy of cell protection via EPS production may have developed to limit access of toxic elements to the cell interior (Gaur and Rai 2001; Gadd 2009). From an environmental perspective, the processes discussed here contributes to natural attenuation of metals by removing soluble metals from the water column, with the metals being either sequestered in the sediments or introduced into the food chain (Beveridge 1984). Brierley et al. (1989) and Gadd (2009) provide detailed reviews of microbial processes involved in the removal and recovery of metals.

Biosorption of metals, however, appears to be less effective in acidic environments because solution pH controls the amount of sequestration of heavy metals by cells (Gross 2000). As metal concentrations increase with increasing acidity, the rate of

surface binding and uptake is significantly reduced (Peterson et al. 1984; Gadd 1986; Ferris et al. 1989). The availability of negatively charged anionic sites on cell surfaces and in EPS in acidic environments is greatly reduced, thereby lowering the amount of heavy metal sorption under lower pH conditions (James and Healey 1972; Peterson et al. 1984; Gadd 1986; Ferris et al. 1989). If, however, an anionic group on the cell wall material has a low pK_a value (logarithmic measure of the acid dissolution constant) than other ionizable functional groups, it could maintain cation exchange capacity at low pH (e.g., Crist et al. 1992; Konhauser 2007). For example, sulfate ester groups within extracellular polysaccharides have low pK_a values. Sulfate is common to extremely abundant in volcanic acidic, ARD, and AMD environments (Konhauser 2007). Green, brown, and red algae are known to contain such sulfate esters as sulfated heteropolysaccharides (fucoidan) and sulfated galactans (Hunt 1986; Konhauser 2007); these esters are also present in the EPS of acidophilic protozoa. Many green algae contain sulfate esters in their cellulose that facilitate metal sorption in acidic conditions (Crist et al. 1981, 1992; Konhauser 2007). Relating the degree of sorption via pK_a values based on titration curves to specific functional groups, however, may be inaccurate due to the considerable variation in these values for the same functional group as these values are controlled by the structure of the molecule to which the functional group is attached (Martell and Smith 1977; Konhauser 2007).

Ferris et al. (1989) observed the reduction in biosorption in acidic habitats in a study on metal uptake by bacterial biofilms. They found that metal sequestration by bacterial biofilms under neutral conditions was enhanced up to 12 orders of magnitude over uptake under acidic conditions. A study on metal uptake by the green algae *Chlorella* also indicated that pH impacted metal-binding capacity (Darnall et al. 1986), with Cd^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}, Be^{2+}, Al^{3+}, Cu^{2+}, Pb^{2+}, and UO^{2+} being strongly bonded at pH $\geq$ 5, and metal anions of $PtCl_4^{2-}$, CrO_4^{2-}, and SeO_4^{2-} being strongly bonded at pH $\leq$ 2.0 (Brierley et al. 1989). Similarly, Taboada-de la Calzada et al. (1999) tested bioaccumulation capacity for As(III) in *Chlorella vulgaris* in solutions ranging from pH 3–11 and found that As(III) did not significantly accumulated until pH 10.

In spite of the studies mentioned here, there are a number of studies that show metal sequestration by some eukaryotes in acid environments (e.g., Mann et al. 1986; Stevens et al. 2001; Sabater et al. 2003). Mann et al. (1986), for example, detected significant metal uptake in an acidophilic *Euglena* sp. growing in biofilms in highly acidic tailings water at the Elliot Lake Mining District, Ontario, Canada. Samples of *Euglena* sp. contained up to 0.13% U by weight with other metals concentrated up to 10^4 to 10^6 times that of the sampled water. Stevens et al. (2001) noted increased concentrations of Al, Fe, and Mn in *Klebsormidium rivulare*-dominated algal mats compared to concentrations in ambient water and soil samples from an area impacted by AMD in southeastern Ohio. Sabater et al. (2003) analyzed copper accumulation in algal mats growing in an experimental stream that simulated physicochemical conditions of the Río Tinto River. Copper was found to be concentrated up to one order of magnitude greater in the algal mats than in the simulated water. Likewise, algae-dominated microbial mats in AMD-impacted streams in Virginia were shown to bioaccumulate 60–70% more metals than concentrated in the stream water (Krishnaswamy and Hanger 1998).

One major caveat regarding the analysis of biosorption, particularly in AMD environments, is that metal-rich precipitates adhering to biofilms may inflate the amount of metals actually bound to the cell surface or EPS. Acidic mine solutions are generally high ionic strength and, thus, in disequilibrium, resulting in the continuous precipitation of iron oxyhydroxide and oxyhydroxysulfates. These precipitates have low crystallinity with high specific surface area (Bingham et al. 1996; Marina et al. 2005), making them efficient scavengers of other metals from AMD solutions (Dzombak and Morel 1990; Smith 1999). The precipitates are easily trapped within EPS and may stick to the cell membrane. At the Green Valley mine, we have observed *Euglena mutabilis* cells entwined around precipitates, precipitates trapped within EPS secreted by diatoms, and precipitates adhering to linked chains of diatoms (Brake et al. 2004). Precipitates have also been documented in eukaryote-dominated biofilms occurring in a number of AMD environments (e.g., Boult et al. 1997; Lawrence et al. 1998; Sabater et al. 2003; Valente and Gomes 2007). Studies that evaluate metal uptake should carefully document removal of these precipitates to guarantee that analyses measure the fraction of metal uptake due to sorption.

In addition to surface bioaccumulation, metals may be metabolically sequestered from the surrounding environment via intracellular influx. Documentation of this process is more extensive for fungal species compared to other eukaryotic species. Fungi and yeast are known to intracellularly sequester such divalent cations as Cu^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Ca^{2+}, and Sr^{2+}, with cations being compartmentalized within vacuoles, localized into cytoplasmic granules, or precipitated as minerals on cell walls or within vacuoles (Gadd 1986 and references therein). Intracellular influx in fungi, however, proceeds at a slower rate than surface adsorption, and as with surface binding, the amount of influx decreases with increasing acidity (Gadd 1986). For a more detailed discussion on intracellular influx of metals in fungi, see Gadd (1986, 1993).

Intracellular uptake has also been documented in some euglena species living in acidic habitats (Mann et al. 1987; Brake et al. 2001a, 2002; Casiot et al. 2004). Mann et al. (1987) noted bioaccumulation of metals in a *Euglena* sp. thriving in highly acidic mine tailings water at the Elliot Lake District. They found that pure isolates of *Euglena* sp. contained up to 40% Fe by dry wt. and had accumulated Co, Ni, Zn, Mn, Th, U, Ti, Ag, and V at concentrations $\geq 10^8$ over concentration in the AMD. Using transmission electron microscopy (TEM) and electron diffraction imagery, they identified intracellular microcrystalline lepidocrocite [γ-FeO(OH)] aggregates within the cell. Brake et al.

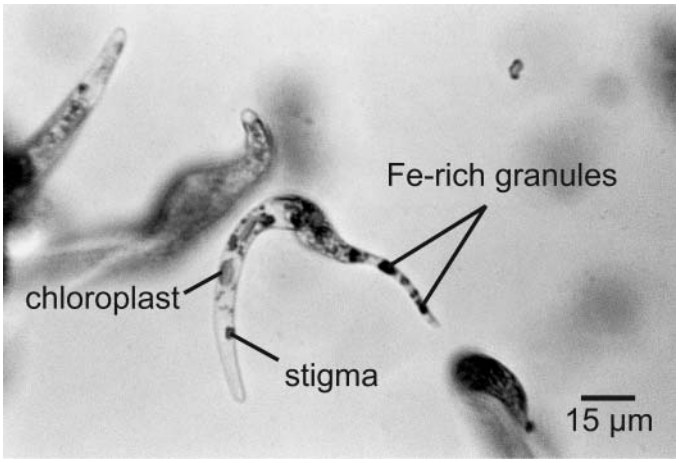

FIG. 2. Photomicrograph of *Euglena mutabilis* using phase contrast microscopy. Iron-rich intracellular granules appear a dark spots.

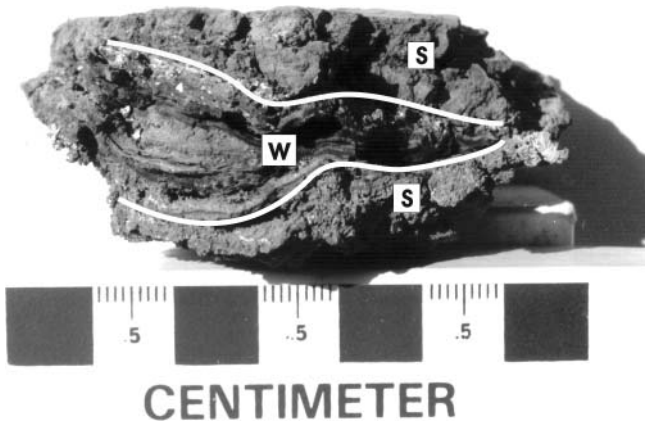

FIG. 3. Iron-rich stromatolite sample showing thinly laminated wavy (w) layers surrounded by porous sponge-like (s) layers.

(2001a, 2002) noted intracellular sequestration in *E. mutabilis* collected from AMD at the Green Valley mine site. Microscopic analysis of the cells showed several small irregularly shaped, orange to dark-red amorphous granules within the cytoplasm (Fig. 2). Analysis of a concentrate of the granules by environmental scanning electron microscopy (ESEM), scanning transmission electron microscopy (SEM), and inductively coupled plasma-mass spectrometry (ICP-MS) indicated that they were Fe-based with minor concentrations of K, S, O, P, Si, Al, Cl, Ca, Se, Zn, Co, As, Cu, Cr, V, Zr, Th, Nb, Mo, Ag, Pb, and Tl (Brake et al. 2002). Casiot et al. (2004) analyzed arsenic concentrations in *E. mutabilis* collected from AMD at the Carnoulès Mine, southern France, and in *E. mutabilis* cells reared in the lab in AMD and synthetic media. In addition to finding increased adsorption of arsenic on the cell surface, they identified intracellular accumulation of arsenic at a concentration factor between 1.5 and 3, compared to arsenic concentrations in solution. Arsenic speciation was also noted to change dramatically within 5 days from As(III) to As(V) in the presence of *E. mutabilis*, indicating increased oxidation rate of As(III) in the presence of *E. mutabilis*. The influx of metals into the cell and the formation of internal precipitates in euglenids and fungi likely represent compartmentalization of elements converted to inert forms as a means of detoxification to prevent the internal build-up of toxic elements that diffuse across the cell boundary (e.g., Gadd 1990).

Formation of Biolaminated Deposits

The presence of iron-rich biolaminated structures has been reported in several acidic environments associated with AMD (Brake et al. 2002; Gónzalez-Toril et al. 2003; Brake and Hasiotis 2008). At the Green Valley site, we have studied such structures since 2000. These features are referred to as stromatolites because they meet the criteria established by Awramik et al. (1976) as being accretionary organosedimentary structures biologically derived by microbial mat-building communities. The Green Valley stromatolites vary from the definition of stromatolites in that they are derived primarily from the biological activity of eukaryotes rather than prokaryotes (Brake et al. 2002). Eukaryotic microorganisms, including diatoms and fungi, have been implicated in the partial or complete formation of stromatolites in freshwater environments (Winsborough and Golubíc 1987), acidic hot spring waters (Jones et al. 2000), alkaline lakes (Braithwaite and Zedef 1996), and marine subtidal environments (Winsborough 2000), as well as in freshwater environments dating from the Cretaceous (Beraldi-Campesi et al. 2004). The deposits in the AMD environment are significant because they represent the attenuation of contaminants and serve as a record of microbial activity that may be useful as a modern analog for ancient eukaryote-dominated stromatolites, including such iron-rich biolaminates as banded iron formations (BIF). The following discussion on the formation of these structures is based on our work at Green Valley.

Iron-rich stromatolites (Fig. 3) at Green Valley consist of a succession of finely laminated, wavy layers that alternate with thicker porous, sponge-like layers (Brake et al. 2002). The wavy layers are composed of very fine-grained amorphous Fe precipitates with interbedded remnants of decaying *E. mutabilis*-dominated biofilm. Analysis of this layer by SEM shows interbedded layers of pennate diatom frustules (Fig. 4a) and layers of mineralized casts of bacterial cocci (Fig. 4b). The sponge-like layers contain iron-coated diatom frustules (Fig. 4c) with remnants of fungal and algal filaments (Brake et al. 2004). Some of the spongy layers also exhibit radiating morphologies (Fig. 4d) with linked diatom frustules within radiating segments, suggesting that the layer formed when precipitates were trapped or deposited on diatom communities in growth position (Brake et al. 2004).

We have identified several biological processes that we postulate contribute to the formation of the iron-rich stromatolites at Green Valley. These include: 1) active or passive nucleation of iron-rich minerals on cell surfaces; 2) generation of oxygen

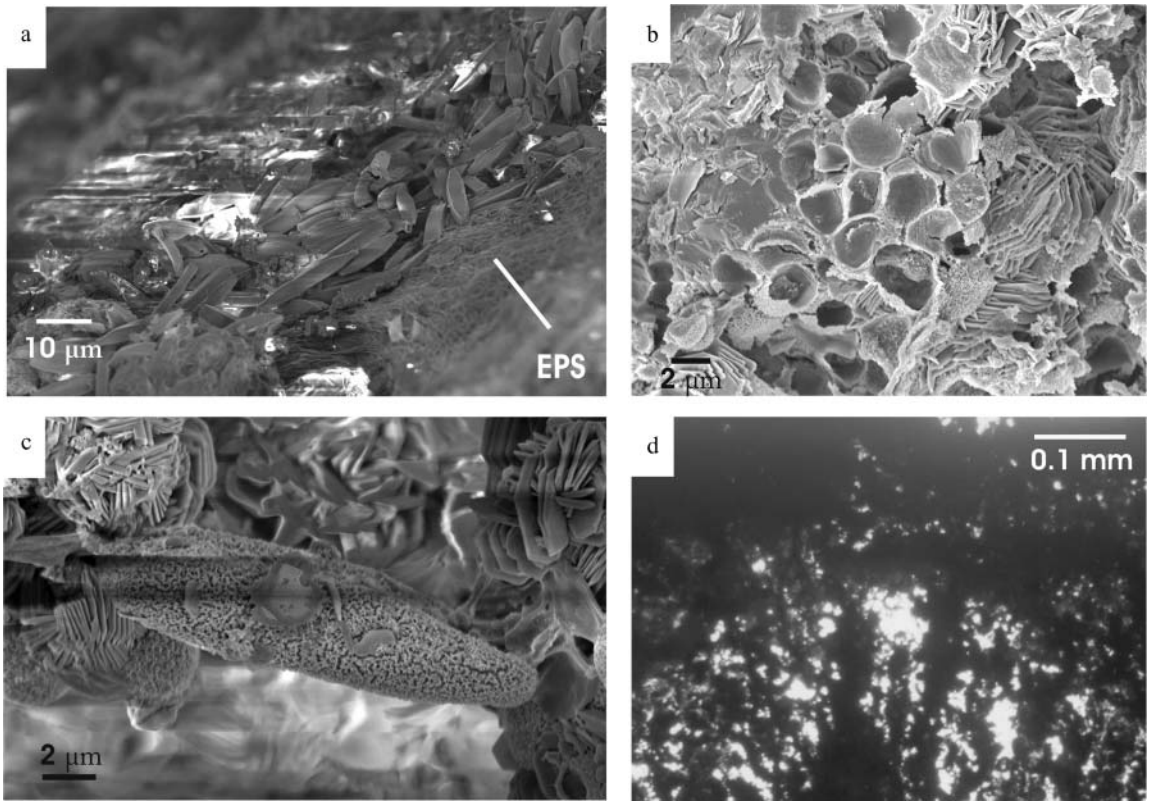

FIG. 4. SEM photomicrographs showing evidence of microbial life preserved in an iron-rich stromatolite sample. (a) Interbedded layer of pennate diatoms within a finely laminated stromatolite layer. EPS layer adjacent to diatoms is draped over iron-rich chemical sediments (from Brake and Hasiotis 2008). (b) Mineralized casts of bacterial cocci within a finely laminated wavy layer. Casts are composed of iron oxyhydroxysulfates. (c) Iron-encrusted diatom frustule within a spongy layer. Encrustation suggests iron minerals were precipitated directly on the frustule. (d) Transmitted-light photomicrograph of spongy layer showing radiating upward texture (from Brake and Hasiotis 2008). At higher magnification, radiating segments contained linked diatom frustules, suggesting that morphology resulted from deposition or trapping of iron precipitates that covered diatoms in growth position.

via photosynthesis within the eukaryotic biofilms that further facilitates precipitation of reduced iron species; 3) formation of intracellular iron compounds in *Euglena mutabilis*, which, after death, contributes to the solid fraction of the biolaminates; and 4) trapping and binding of chemical sediments via EPS and microbial motility (Brake and Hasiotis 2008).

One of the important questions regarding nucleation of minerals on cell surfaces is how much is enzymatic induced and how much is via passive accumulation. It is well known that enzymatic processes associated with bacteria can lead to biomineralization on and within cell walls (e.g., Lowenstam 1981; Pentecost and Riding 1986; Westall et al. 2003; Ferris et al. 2004; Inkseep et al. 2004). Likewise, biomineralization is also associated with the biological activity of fungi (Gadd 1993; 2007). Fungi produce secondary minerals by direct nucleation on such cellular macromolecules as melanin and chitin in fungal cell walls or by indirect precipitation of minerals through microbially mediated changes in solution chemistry (e.g., extracellular secretion of acids) (Gadd 1993; 2007 and references therein). Metabolically induced precipitation of minerals by algae and euglenoids, however, is not well documented, except for the rare cases of mineral-solid formation within the cells of acidophilic euglena as discussed earlier (see Mann et al. 1987; Brake et al. 2002).

At Green Valley, bacteria, diatoms, and other algae show evidence of mineral accumulation on cell surfaces. For bacteria, we observed traces of their presence as finely crystalline, mineralized casts (see Fig. 4b) composed of iron oxyhydroxysulfates. For diatoms, we noted iron minerals encrusted on both living diatoms (Fig. 5) and diatoms preserved in stromatolites (see Fig. 4c). In living diatoms, iron minerals were attached to linked chains of diatoms, and in some cases, chains were completely encrusted. Algae strands were also encrusted in iron precipitates (Fig. 6). Iron encrustation on *E. mutabilis* cells was not observed, possibly because its flexible cell membrane precludes mineral accumulation. We surmise that a significant portion of the iron accumulated on cell surfaces is due to passive accumulation from continuous precipitation of iron minerals in this system, rather than from biomineralization associated with enzymatic processes. Any object (e.g., spiders, leaves, worms, etc.) that falls into the AMD is covered in iron precipitates within a few days.

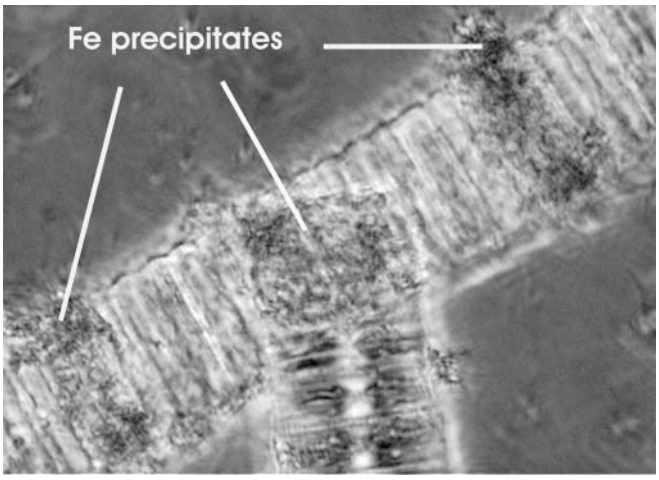

FIG. 5. Transmitted light photomicrograph of linked pennate diatoms assigned to *Nitzschia* sp. Some segments are encrusted with iron particles. Field of view is 0.07 mm.

Precipitation of minerals that form stromatolites may be further enhanced by oxygenic photosynthesis within eukaryotic biofilms, which supersaturates the water with oxygen above the biofilms (e.g., Brake et al. 2001, 2002). This process may promote ferrous iron oxidation and oxidation of reduced metal species by creating conditions favorable for autooxidation by increasing pH and raising oxygen concentrations in the water surrounding the biofilms (Ehrlich 2002). This process likely explains the observed oxidation of arsenic from As(III) to As(V) in the presence of *E. mutabilis* mentioned earlier (see Casiot et al. 2004).

Also contributing to the material of the stromatolites are the intracellular iron-rich granules that form within *E. mutabilis*. We hypothesize that upon death the granules are released to the developing stromatolites and may serve as nucleation sites for further precipitation of authigenic iron phases (Brake et al. 2002). Rounded, amorphous iron-rich granules, similar in size to the intracellular granules in *E. mutabilis*, occur within the stromatolitic layers. Whether these granules are produced biologically or derived abiotically from precipitation of iron-rich phases has not been determined.

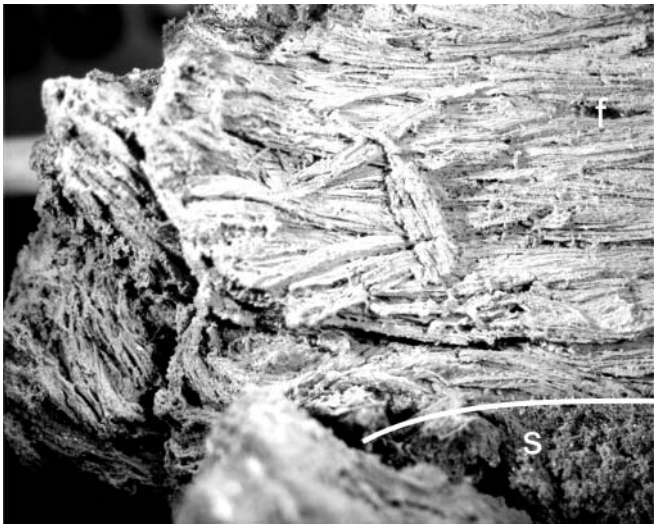

FIG. 6. Iron encrusted algal filaments (f) collected from a stromatolite layer. Preserved filaments overly a porous, spongy (s) layer. Field of view is 4 cm.

Although the processes discussed here contribute to the formation of stromatolites at this site, we surmise that the bulk of the deposits are constructed by the trapping and binding of chemical precipitates. One of the challenges facing biofilm communities is to keep pace with the continuous sedimentation of iron-rich precipitates. We hypothesize that the communities survive by trapping precipitates within the biofilm and then binding them to the channel bottom via locomotion to form the layered fabric of the stromatolite, similar to biostabilization of sediments and formation of stromatolites in marine environments (e.g., Holland et al. 1974; Paterson 1989; Dade et al. 1990; Noffke and Paterson 2008). In modern marine environments, stromatolitic fabric is generally constructed by cyanobacteria and diatom communities that keep pace with sedimentation by trapping and binding sediments to the stromatolite using EPS during locomotion to place the cells higher in the mat for photosynthesis and nutrient availability—phototaxis and chemotaxis, respectively (Edgar and Pickett-Heaps 1984; Paterson et al. 2003). Grant et al. (1986) reported that sediments in an intertidal environment were bound when mucus strands secreted by diatoms, protozoa, fungi, and other organisms were attached to sand grains. EPS was also observed to enhance sediment stability in riverine environments by filling void spaces in the sediment that strengthen interparticle forces (Gerbersdorf et al. 2008). In AMD environments, we suggest that similar processes operate to bind chemical sediments to the channel bottom. We have observed remnants of EPS (Fig. 4a) covering iron-rich precipitates within thinly laminated layers. We have also observed iron particles entrapped in EPS associated with diatom-dominated biofilms and by entwining *E. mutabilis* cells and algal filaments (Brake et al. 2002, 2004). Valente and Gomes (2007) observed similar accumulations of iron-rich particles on the outside of algal cells and in the EPS associated with *E. mutabilis* in AMD in northern Portugal.

Eukaryotic biofilm communities likely keep pace with chemical sedimentation via phototactic and chemotactic behavior. Both *E. mutabilis* and diatom cells exhibited phototactic and chemotactic behavior in the field and laboratory by moving through chemical sediment to reestablish the biofilm community at the sediment-water interface within a few days after being covered by precipitates (Brake et al. 2004). We propose that the mucilage secreted by the biofilm community during locomotion assists in binding sediment to the channel bottom topography (previously bound stromatolitic layers) to form the biolaminated structures observed on the channel bottom. In some cases, decaying layers of *E. mutabilis*-dominated biofilms and radiating upward linked frustules of diatoms indicative of growth position are interbedded in the biolaminates, suggesting that at least

some of the biofilm communities were unable to keep pace with high rates of chemical sedimentation. The highest rates of iron and aluminum precipitation and burial of biolaminates generally occur when pH increases due to increased discharge from rainfall events.

The succession of laminae types within the AMD stromatolites can serve as a record of the spatial and temporal changes in the AMD system with regard to microbial activity and physicochemical conditions through time. Each lamina marks not only the position of the sediment-water interface, but also the type of microbial community and associated biostructures that formed under given physicochemical conditions (e.g., Hofmann 2000; Seong-Joo et al. 2000). Succession of layers also indicates the duration and variability of particular physicochemical conditions within the channel. We have observed that thick (up to 3 cm) layers of chemical sediments can form within a few hours after a rainfall event, while thin (< 3 mm) lamina can take from a few days to several months to form under normal AMD conditions. Thus, an important caveat is that some thicker lamina represents shorter periods of deposition, whereas thinner lamina may represent extended periods of surface stability (Brake et al. 2004).

Due to the lack of clear evidence of enzymatic biomineralization on the part of the eukaryotic microorganisms in the biofilm communities at Green Valley, it may be argued that the observed laminated features do not constitute stromatolitic deposits (e.g., Fernández-Remolar and Knoll 2008). Fernández-Remolar and Knoll (2008) suggest that the laminated deposits in the Río Tinto River are generated from chemical sedimentation and precipitation, rather than from the activity of microbial mats, particularly due to the lack of preserved mat-building organisms. The preservation potential of mat-building biofilm communities in such acidic environments, however, is very low because many of the eukaryotic microorganisms have flexible cells membranes that preclude preservation and the oxidized conditions typically destroy the microbes. This is a common problem that plagues our interpretation of ancient stromatolites (Noffke and Paterson 2008). Also, the lack of evidence of mat-building communities does not signify their nonexistence. Instead, we need to focus on interpreting better those macro- and microstructures identified in modern environments that will help us recognize the biogenic origin of stromatolites in the rock record.

IMPLICATIONS AND FUTURE RESEARCH

As we continue to explore the diversity of microorganisms in acidic systems, we are beginning to recognize their significance in mediating their environment. Future work will continue to establish the importance of eukaryotes as potential bioremediators of contaminated acidic environments, as bioengineers that modulate their habitat for other organisms, and as builders of iron-rich biolaminated structures that may serve as analogs in the study of microbial processes that operated on early Earth and perhaps on other celestial bodies.

Bioremediation

One of the approaches in treating contaminants in acidic environments is through the use of bioremediation. Bioremediation measures have focused primarily on the use of sulfate-reducing and metal-transforming bacteria to retard metal mobility (Ledin and Pedersen 1996). Metal-accumulating microorganisms, including eukaryotes, may also attenuate metals. Studies have demonstrated the efficiency of biosorption of metals in freshwater systems, but have been less forthcoming regarding the effectiveness of this process in acidic environments. The conundrum that exists is the degree to which eukaryotes can attenuate contaminants in acidic environments given that their ability to attract cations to their surface decreases with increasing acidity. One would expect limited success in bioremediation given this factor, yet several studies (i.e., Mann et al. 1986; Krishnaswamy and Hanger 1998; Stevens et al. 2001; Sabater et al. 2003) discussed earlier suggest otherwise. How valid are the results of these studies? How much of the reported metal removal in acidic environments is due to biosorption, and what fraction, if any, represents the inadvertent analysis of metal-bearing precipitates that are difficult to remove from cell surfaces and associated mucilage?

Our work on *Euglena mutabilis* and eukaryote-dominated biofilms at Green Valley offers additional insight on the potential for metal attenuation via such activities as: (1) *E. mutabilis* internally sequestering iron and other contaminants from the effluent to form granules that after death may serve as nuclei for further precipitation of authigenic mineral phases; (2) actively or passively accumulating iron-rich precipitates on cell surfaces; and (3) driving precipitation of iron and other reduced metals from acid water via highly oxygenic photosynthetic activity. Although we have documented that contaminants are sequestered by these methods, we have not observed a significant change in the chemistry of the acidic effluent from the point of discharge above the eukaryotic mats to the point of discharge below the mats (Brake et al. 2001), suggesting that the biofilms in this system are not significantly changing water chemistry.

This brings us to the question of where we stand in the use of eukaryotes as effective bioremediation tools in highly contaminated acidic systems. Research suggests that some eukaryotes are effective in attenuating metals, but can attenuation be achieved to a degree that improves water quality in these highly polluted environments? Future studies will need to identify and target those species that show the most potential for metal attenuation. Studies should also: (1) address interactions between microorganisms that might lead to unexpected effects; (2) determine the degree to which eukaryotic microorganisms are capable of attenuating contaminants to levels that result in improved water quality; and (3) consider the amount of time it takes to accomplish this process. Longer studies on bioaccumulation are probably warranted as many of the eukaryotes in acidic environment have a seasonal life cycle, and should be combined with the geochemical and hydrologic aspects of

the system to maximize success in building a self-sustaining bioremediation system that is cost effective.

Ecosystem Engineers

Our review has highlighted some of the significant roles that eukaryotes play in mediating acidic systems. We have not looked at these biological activities, however, from the perspective of their potential for engineering or modulating habitats for other organisms. Eukaryotes can modify acidic environments by such biological activities as oxygenic photosynthesis that adds oxygen to the water column, sequestration of metals via bioadsorption and intracellular uptake to remove contaminants, and by secreting mucilage that aids in trapping and binding chemical sediments. Can these same biological processes create or modify the availability (quality, quantity, distribution) of resources to other species and create suitable microenvironments for other, less acidotolerant microbes to live in acidic environments?

Jones et al. (1994) define ecosystem engineers as those organisms that create, modify, and maintain habitats by directly or indirectly modulating the availability of resources to other species. They identify two types of ecosystem engineers: autogenic engineers that change the habitat via their own physical structures, and allogenic engineers that change the environment by shifting living or nonliving material from one physical situation to another. We propose that benthic eukaryotic microorganisms in biofilm communities in acidic environments are both allogenic and authogenic engineers. An example of allogenic engineering is the photosynthetic activity of some eukaryotes that results in supersaturation of oxygen in such highly acidic environments as AMD, which is often undersaturated in oxygen (e.g., see Brake et al. 2001). The production of oxygen has a twofold effect. First, it may be responsible for slightly increasing the pH immediately surrounding the biofilm community due to carbon dioxide consumption and the production of oxygen during photosynthesis (Ghiorse and Ehrlich 1992) to create a microhabitat more hospitable for other less adaptive microorganisms. Second, the production of oxygen may contribute to the oxidation and precipitation of reduced metal species in the vicinity of the biofilm, thereby reducing the amount of toxic elements impacting other microbial species. Likewise, metal attenuation by biosorption or intracellular uptake may also modulate the supply of metals in the vicinity of biofilm communities.

Further evidence of potential allogenic engineering is in the formation of iron terraces that commonly occur in AMD environments (e.g., Brake et al. 2001b; Sánchez España et al. 2007). Both Brake et al. (2001b) and Sánchez España et al. (2007) noted the occurrence of benthic biofilms on iron terraces at Green Valley and in the Tintillo River, Spain, respectively. These terraces show macro- and micromorphologies indicative of being derived, in part, by microbial activity. At Green Valley, the microbial communities occupying terrace deposits were dominated by eukaryotes (Fig. 7); whereas, terraces in

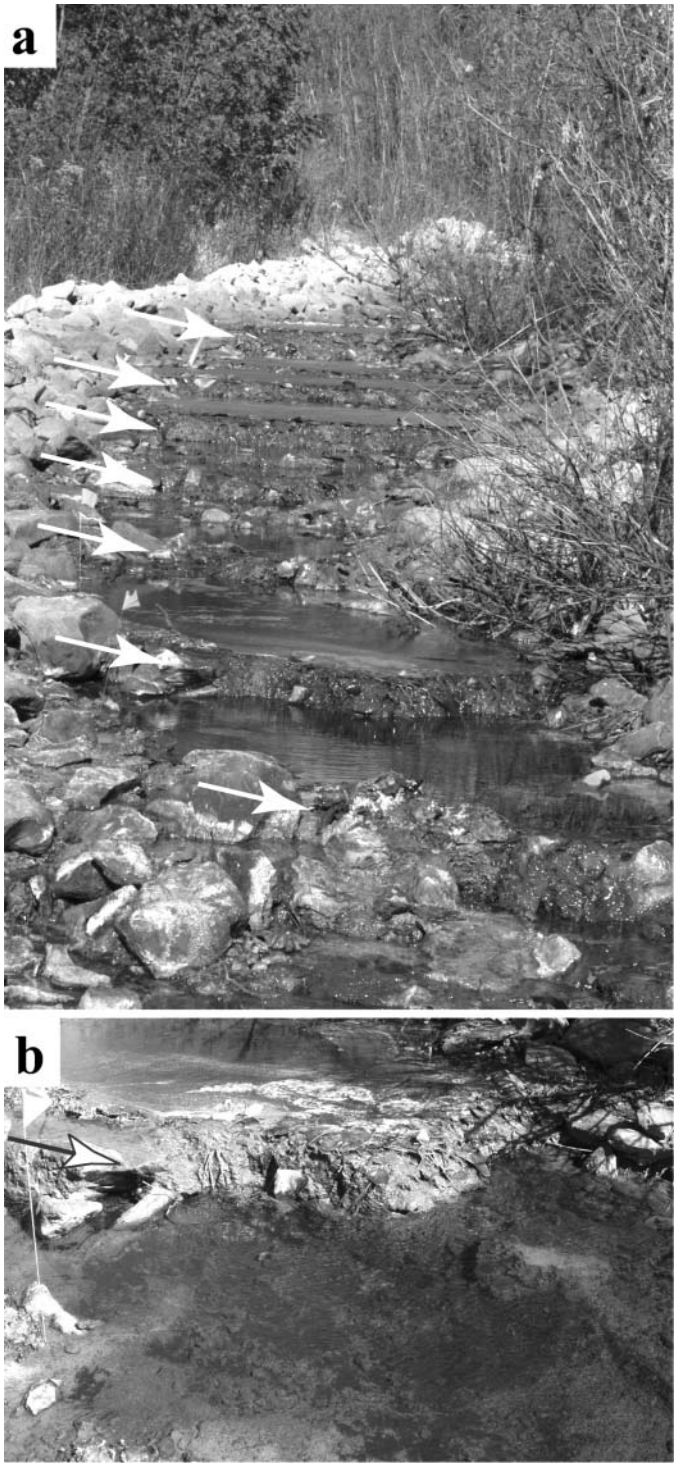

FIG. 7. Iron rich terraces in a constructed AMD channel at Green Valley. (a) Overview of constructed channel with terraces. All terraces are covered by *Euglena mutabilis*-dominated biofilms. Arrows mark position of terraces. (b) Close-up of iron-rich terrace. Both the terrace and the bottom of the pool below the terrace are covered by *E. mutabilis*-dominated biofilm. Arrow marks terrace location. Field of view is 4.5 meters.

the Tintillo River commonly contained white bacterial streamers (Sánchez España et al. 2007). The formation of these terraces reduces channel roughness and alters hydrology, analogous to such structures as beaver dams (e.g., Jones et al., 1994). The terraces change flow dynamics to create calm pools behind the terrace where riffles were once present. These pools may trap chemical sediments and allow the accumulation of organic matter resulting from the death of members from biofilm consortia within the AMD channel (Brake et al. 2001b). It is within this organic matter that essential nutrients can accumulate in concentrations to make them available to the microbial communities.

Evidence of autogenic engineering that modifies habitats for other organisms is the secretion of EPS (or mucilage) by some eukaryotes (e.g., see also Jones et al. 1994). Mucilage can provide a barrier to toxic elements, allowing less adaptive microorganisms to proliferate within the biofilms community. Mucilage also glues chemical sediments together to create a solid media for future benthic communities, which is also, in part, the formation process of the layering of stromatolites. Trapping and binding of sediments also reduces channel roughness, thereby changing the dynamics of hydrologic stream flow, which may in turn affect the type of benthic microorganism adhering to the surface bottom.

Modern Iron-rich Stromatolites as Geologic Proxies

Stromatolites represent the mineralized counterpart of microbial mats. They are Earth's earliest record of life (e.g., Schopf et al. 2007) and are one of the biostructures used in our search for life on extraterrestrial bodies (e.g., Westall et al. 2003). Much paleoenvironmental and paleobiological information on the early biosphere, atmosphere, and geosphere on Earth is preserved in their macrostructures and microstructures. Life on early Earth existed under what we today consider as extreme conditions. Modern highly acidic environments are also extreme environments that similarly restrict life to single-celled microorganisms. We propose that modern eukaryote-dominated biofilms in acidic environments and AMD stromatolites serve as a valuable source of environmental and biological information that may provide important insights on the role of ancient and extant microbial communities in the formation of stromatolitic deposits. Each layer of AMD stromatolites, for example, serves as a proxy record of the diversity, biological activities, and environmental conditions of formation. The biogeochemical processes involved in modern eukaryotic biofilms and in the formation of iron-rich stromatolites may be analogous to some of the processes responsible for the development of such iron-rich structures as late Archean–early Proterozoic BIF and oxygenation of the early atmosphere (e.g., Brake et al. 2002).

Advanced techniques in paleobiology and biogeochemistry over the past decade have led to the discovery of: 1) fossil eukaryotes in the 2.1-Ga-old Negaunee Iron Formation from North America (Han and Runnegar 1992); 2) hydrocarbon biomarkers indicative of eukaryotes in 2.45 Ga oil-bearing fluid inclusions in fluvial metaconglomerates in Canada (Dutkiewicz et al. 2006); and 3) the much debated hydrocarbon biomarker evidence of eukaryotes in 2.7-Ga-old rocks from Australia (Brocks et al. 1999; Rasmussen et al. 2008). These occurrences overlap with the main phase of BIF deposition from 2.6–1.8 Ga (Klein and Beukes 1992) and the small but significant rise in oceanic and atmospheric oxygen ~2.5 billion years ago (Anbar et al. 2007; Kump and Barley 2007). If early primitive photosynthetic eukaryotes were present during the late Archean–early Proterozoic and functioning as eukaryotes do today, then they may have contributed to the oxygen budget and to the oxidation of reduced iron in the ocean, complementing the contribution of ancient prokaryotes in producing oxygen and stripping iron from seawater (e.g., Brake et al. 2002).

One of the problems in studying ancient stromatolites is that microfossil and microstructural evidence is rarely preserved within deposits or such macrostructures as stromatolites and BIF (Grotzinger and Knoll 1999; Schopf et al. 2007). Added to this complexity is the lack of preservation of many eukaryotes because their flexible cell membranes preclude preservation. If we are to find evidence of eukaryotes in the ancient rock record, we will need to continue to identify microstructures and molecular biosignatures and biomarkers indicative of eukaryotic microorganisms and their biological activity that will survive decomposition and alteration associated with burial and diagenesis. Our understanding of the biogeochemical conditions in acidic environments will provide us with the tools to recognize biosignatures of eukaryotic life and activity that can be used as proxies to identify similar activity in the ancient rock record as well as in iron-rich strata in extraterrestrial environments.

CONCLUSION

Acidic aquatic environments are excellent natural laboratories for investigating biological patterns (microbial biofilms and modern stromatolites), adaptive strategies, and biosignatures of life. High acidity and elevated concentration of metals in acidic environments limit diversity to a few species of microorganisms including, bacteria, Archaea, and single-celled eukaryotes that must develop adaptive strategies to protect their cells from the harsh environmental effects. Microbiological research has extended our understanding on the diversity of acidophilic and acid-tolerant eukaryotic species and has provided valuable insight into the type of eukaryotes that commonly populate benthic biofilm communities. Several benthic taxa appear repeatedly in highly acidic aquatic environments. These include: the euglenoid *Euglena mutabilis*; diatoms *Pinnularia* spp., *Eunotia exigua*, *Eunotia* spp., *Nitzschia* spp., and *Navicula* spp.; green algae *Chlamydomonas acidophila*, *Klebsormidium* spp., *Ulothrix* spp., and *Zygogonium ericetorum*; red algae *Gladieria sulphuraria* and *Cyanidium caldarium*; and the amoeba *Vahlkampfia* sp. Although the diversity of benthic eukaryotes in acidic environments is well documented, additional research is needed to characterize relationships between

microorganisms within the biofilm communities and to identify any microbial stratification that may be similar to marine mat-forming communities.

The most intriguing feature of eukaryote-dominated biofilms is the role they play in mediating acidic environments. Unfortunately, much of the research on biological processes in acidic environments focuses on the role of prokaryotic microorganisms because of their ability to catalyze chemical changes. The biological activity of benthic eukaryotic communities in acidic environments, on the other hand, is not as well documented. Recent work has started to highlight the importance of eukaryote-dominated biofilms in attenuating metals and in the formation of iron-rich biolaminated structures. The former has important implications in the natural attenuation of metals. Additional studies are necessary to further assess the viability of using eukaryotic microorganisms to attenuate metals and improve water quality in these highly contaminated systems. The formation of iron-rich biolaminated structures by eukaryote-dominated communities in some AMD environments has broad implications for understanding the role of ancient and extant microbial communities in the formation of stromatolites and the identification of biosignatures of life that indicate mediation of the environment by eukaryotes.

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