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The algal flora of an extremely acidic, metal-rich drainage pond of São Domingos pyrite mine (Portugal)

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Abstract – We examined the algal composition of an extremely acidic, metal-rich drainage pond of a pyrite mine located in southeastern Portugal. Two acidophilic diatom taxa, *Eunotia exigua* ("Sippenkomplex") and *Pinnularia acoricola* var. *acoricola* were found to be dominant at the site. The well known acidophilic taxon *Euglena mutabilis* was also abundant. *Ulothrix tenerrima* and *Chlamydomonas* spp. were also identified. Green jelly balls containing multiple diatoms and chlorococcal forms inside their assemblages were also present. These diatoms displayed greater vitality than their free-living counterparts, exhibiting more vivid colours. Rare specimens of an unidentified colourless flagellate, as well as *Achromatium volutans*, a large sulphur oxidizing bacterium, were also present. The algal species diversity of the S. Domingos mine acid drainage is very low and most of the taxa found can also grow heterotrophically on a variety of organic compounds, a clearly advantageous feature in this hostile environment.

Acid mine drainage / acidophilic algae / diatoms / *Euglena mutabilis* / metal contamination

Résumé – **Flore algale d'un étang extrêmement acide et riche en métaux de la mine de pyrite de São Domingos (Portugal)**. Nous avons examiné la composition algale d'un étang de drainage extrêmement acide et riche en métaux, situé dans le sud-est du Portugal. Deux taxons de diatomées acidophiles, *Eunotia exigua* ("Sippenkomplex") et *Pinnularia acoricola* var. *acoricola* sont dominants dans ce site. *Euglena mutabilis*, taxon acidophile bien connu, était aussi abondant. En outre, *Ulothrix tenerrima* et une espèce de *Chlamydomonas* ont été identifiés. Des boules vertes gélatineuses étaient présentes; elles contenaient de nombreuses diatomées et des formes semblables à certaines Chlorococcales. Les diatomées vivant dans ces boules montraient une plus grande vitalité et une couleur plus vive que celles qui vivaient librement. De rares individus d'un flagellé incolore non identifié, ainsi qu'une grosse bactérie sulfureuse oxydante, *Achromatium volutans*, ont aussi été observés. La diversité spécifique algale de l'étang acide de la mine de São Domingos est très basse et la plupart des taxons présents peuvent également croître d'une manière hétérotrophe sur une variété de composés organiques, ce qui leur confère un avantage certain dans ce milieu hostile.

Drainage acide d'une mine / algues acidophiles / diatomées / *Euglena mutabilis* / contamination par le métal

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INTRODUCTION

São Domingos mine is an old, abandoned mine located on the portuguese side of the massive pyritic belt that runs through the southern part of the Iberian Peninsula. The mine has not been active since the late 1960's, but still generates a considerable volume of acidic effluents, also referred to as Acid Mine Drainage (AMD). These effluents are originated from the spontaneous oxidative dissolution of sulphide-containing minerals, which leads ultimately to the formation of sulphuric acid and solubilization of various metals, thus resulting in highly acidic and metal-enriched solutions (Johnson, 2006; Nordstrom, 2000). In the past 50 years, the scientific community has become aware of the central role played by acidophilic microorganisms in accelerating this oxidative dissolution of sulphide minerals, and this prompted the study of the microbiology of these waters. Two reviews on the prokaryotic communities of pyrite AMDs have appeared recently (Baker & Banfield, 2003; Johnson & Hallberg, 2003), showing a limited diversity of species with predominance of bacteria and archaea involved in the iron and sulphur cycles. The algal communities of AMDs from various sites have also been documented (Albertano, 1995; DeNicola, 2000; Hargreaves *et al.*, 1975; López-Archilla *et al.*, 2001; Round, 1981; Whitton & Diaz, 1981; Whitton & Satake, 1996) and it was shown that acid-tolerant as well as acidophilic representatives of the Chlorophyceae, Chrysophyceae and Bacillariophyceae frequently inhabit these environments, though at a decreased level of diversity with increasing medium acidity.

The aim of the present study is to identify and document the algal flora of a drainage water body of the São Domingos mine, in the vicinity of which thrives a strong, monospecific stand of *Erica andevalensis* Cabezudo-Ribera growing on sediments highly contaminated with various metals recently characterized (Turnau *et al.*, 2007). The drainage water has an extremely acidic pH (2.25) and is highly enriched in Fe and Al, as well as Cu, Zn and Pb. We report here the algal species found at the site and show that a very limited number of species are present, with a predominance of the diatoms *Eunotia exigua* and *Pinnularia acoricola* and of *Euglena mutabilis.* Possible reasons for the dominance of these algal species are briefly discussed.

MATERIALS AND METHODS

Site characterization and sample collection

Samples were collected twice, in July 2003 and July 2005, at a permanent water body of a pyrite mine situated at São Domingos, near Mértola (37º 45' N; 7º 33' W), in southeastern Portugal. The Mértola region is part of the Iberian Pyrite Belt (IPB) that also runs through the southwestern part of Spain. This belt consists mainly of a cupriferous pyrite associated with zinc and lead sulphides, but mining activity has essentially stopped throughout the belt. São Domingos mine has been inactive for half a century, but still generates large volumes of AMD that pose an important environmental hazard. The water body where samples were collected was about 3 ×2 meters wide and ca. 1 m deep. Samples were collected using glass flasks at a depth of 30 to 50 cm in the euphotic zone and stored in

polypropylene bottles before taken to the laboratory. Samples were thoroughly homogenised, filtered through a 0.5 μm net and used for study as described below.

Chemical analysis

Water pH determination was carried out using a Radiometer M61 potentiometer. Total metal concentrations were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, TJA, Iris Advantage, Franklin, MA).

Algal identification

Taxonomic studies were based on live and preserved material in 4% solution of formaldehyde. Observations were made using a light microscope Nikon ECLIPS E 600 with Nomarski phase contrast. Samples of the Bacillariophyceae were prepared according to the procedures of Pickett-Heaps (1998) and examined with a PHILIPS XL30 and Hitachi S-4700 SEM.

RESULTS

Water physico-chemical features

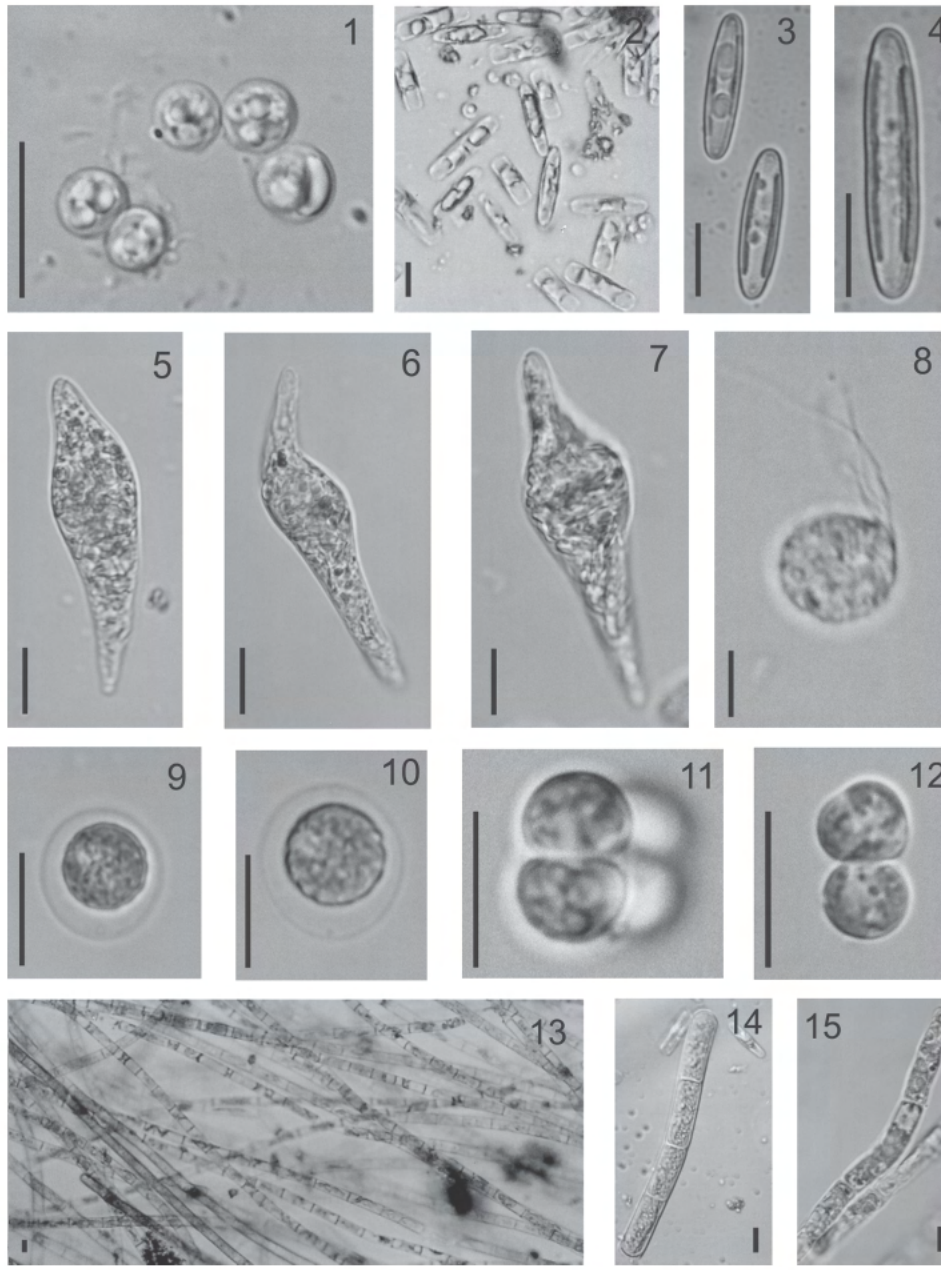
The São Domingos mine is located in the south-east Portugal, a region with a mediterranean climate characterized by rainy winter and dry, hot summer seasons. This marked annual change in precipitation and temperature causes large changes in the pond's water level and accounts for a high fluctuation of metal concentrations found year around as well as of variations in the water pH. On summer months, the São Domingos mine's drainage water showed an average pH value of 2.25 and a Fe concentration of 77 x 10^3 mg l^{-1}. The solution also contained extremely elevated concentrations of Cu (1.9×10^3 mg l^{-1}), Zn (0.7×10^3 mg l^{-1}), Pb (0.8 mg l^{-1}) and As (230 mg l^{-1}).

Algal identification and analysis

We list below the major algal taxa identified in the water samples collected from the mine's water body. The list was prepared according to taxonomical system after Hoek *et al.* (1995). Each species is accompanied by a short description of its typical form and distribution, followed by a note on unusual aspects found in this study, when warranted. We also report the presence of an unidentified colourless flagellate as well as of *Achromatium volutans*, a large sulphur-oxidizing bacterium that was readily detected by light microscopy.

Achromatium volutans (Hinze) Van Niel — Fig. 1

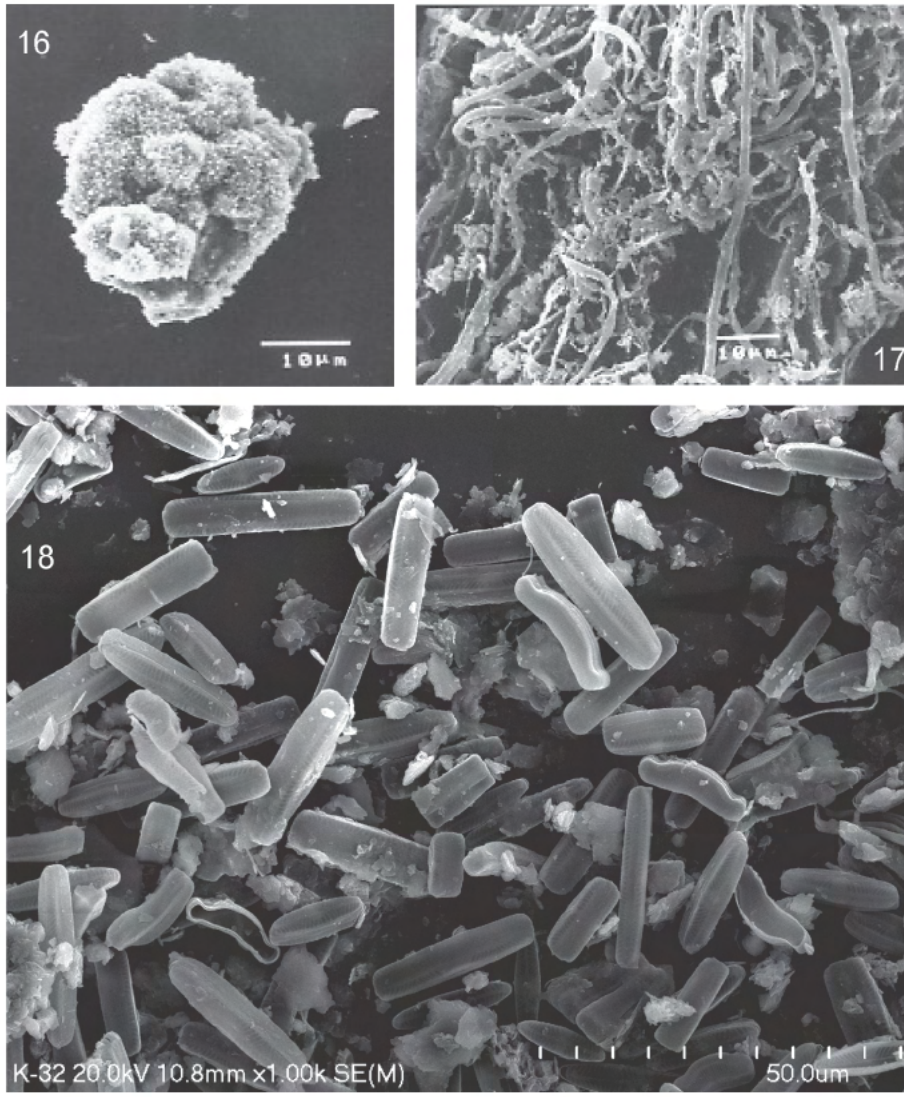
Cells 3-6 μm in diameter with clearly visible small particles of sulphur inside. Cosmopolitan, reported from polluted waters (Hausler, 1982).



Figs 1-15. Light micrographs. **1.** *Achromatium volutans.* **2.** Living cells of *Pinnularia acoricola* var. *acoricola* and *Eunotia exiqua.* **2-4.** Living cells of *Pinnularia acoricola* var. *acoricola.* **5-7.** *Euglena mutabilis.* **8.** Unidentified colourless flagellate. **9, 10.** *Chlamydomonas* ssp. **11, 12.** Unidentified chlorococcal forms. **13-15.** *Ulothrix tenerrima.* Scale bar = 10 µm.

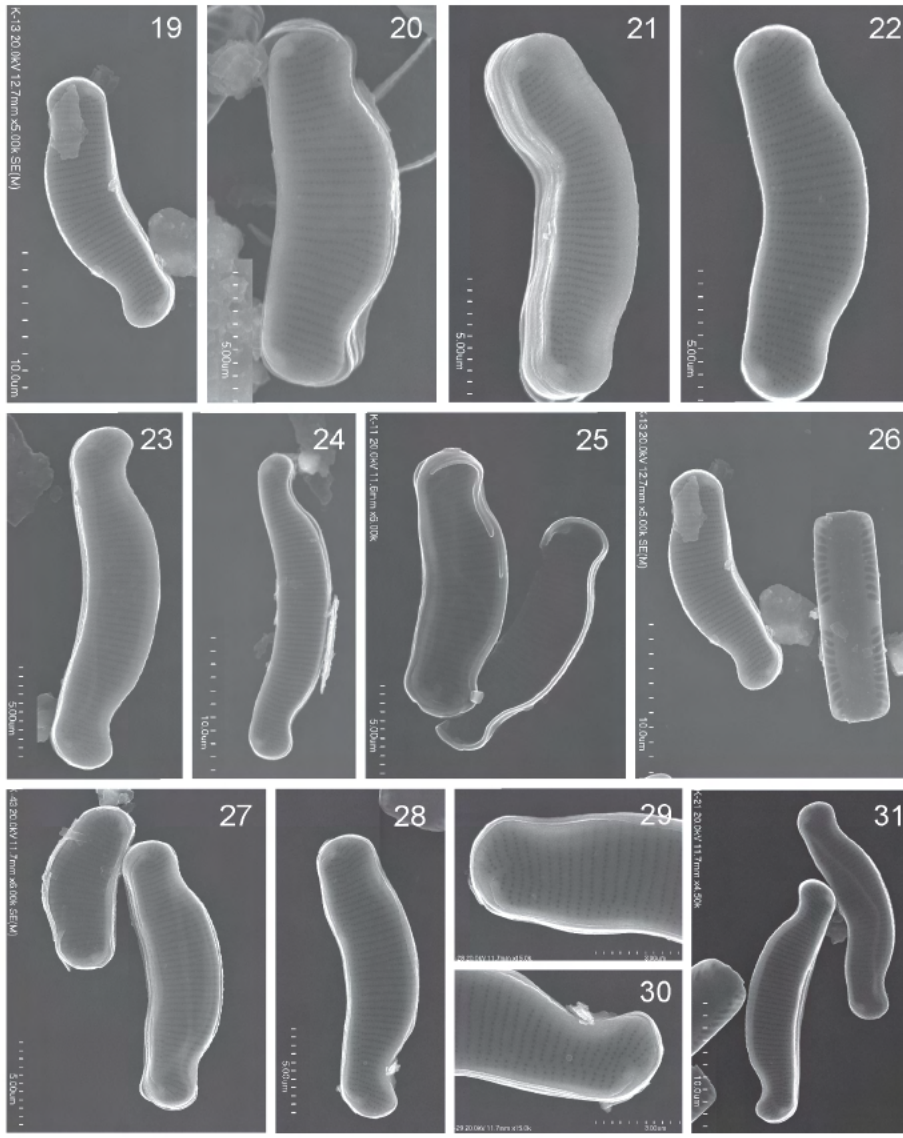
Eunotia exigua (Brébisson *ex* Kützing) Rabenhorst **Figs 2, 18, 19-31**

Valves (9.5) 10.5 – 19.5 (25.0) µm long, 3.7-5.0 µm wide; dorsi-ventral with a slightly concave ventral margin, convex and smooth dorsal margin and capitate apices that are deflexed; valve slightly narrowing towards the ends; striae parallel and slightly denser near the apices, with a density of 21-26 / 10 µm. Species found both growing freely in water or within spherical bodies formed on plant parts present in the water. **Note:** Some of the sampled specimens were



Figs 16-18. Scanning electron micrographs. **16.** Macroscopically visible green jelly ball reminding *Ophridium versatille* inside which numerous diatoms were found. **17.** Filaments of *Ulothrix tenerrima* inside the green ball. **18.** *Eunotia exigua* "Sippenkomplex", and *Pinnularia acoricola* var. *acoricola* inside the green ball.

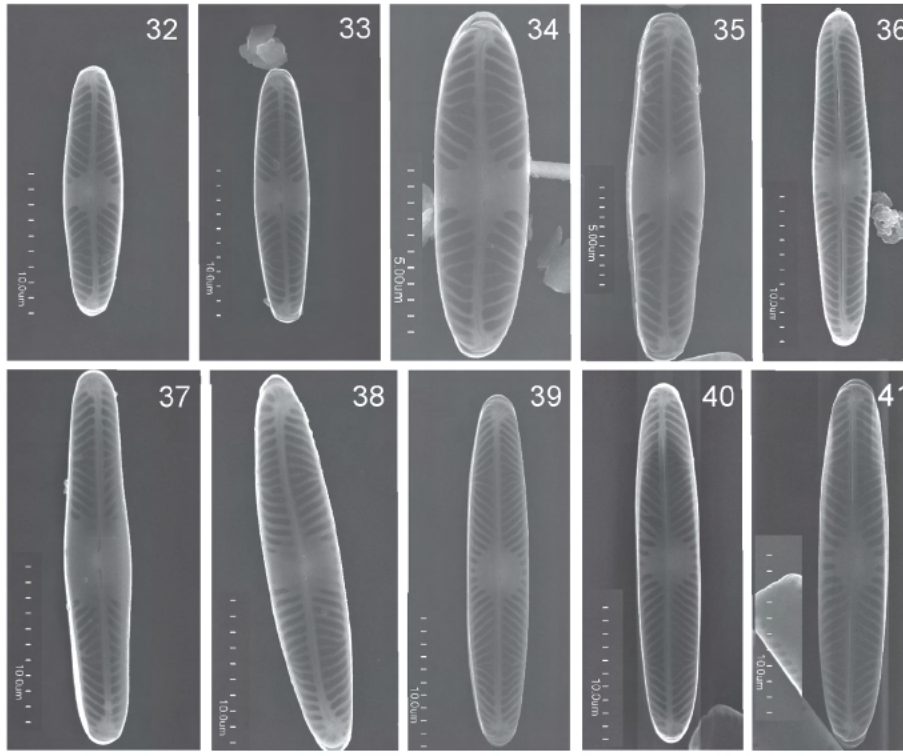
asymmetric at the ends of the valves (Figs 26-28). The morphological variability of the specimens found can be treated as the *E. exigua* "Sippenkomplex" described by Krammer & Lange-Bertalot (1991). The diatoms found within the spherical bodies of green algae showed much higher chloroplast pigment concentration than their free-living counterparts that were somewhat bleached. Cosmopolitan, acid-tolerant and widespread; reported from North America and Europe in lakes and streams receiving acid mine drainage (DeNicola, 2000), most often associated with oligotrophic ponds (Siver *et al.*, 2005).



Figs 19-31. Scanning electron micrographs, *Eunotia exigua* "Sippenkomplex", showing frustule variability and structural details.

Pinnularia acoricola var. ***acoricola*** Hustedt **Figs 2-4, 18, 32-41**

Valves elliptic-lanceolate, elliptic in small specimens with convex sides, ends obtusely rounded cuneiform, 13.5-19.6, 21.2 -27.5 µm long, 3-4-5.5 µm wide; raphe branches straight, central pores small, curved a little to one side, terminal fissures relatively large; axial area very narrow, linear; central area with moderately broad fascia of varying breadth and shape; striae 14 to 16 per 10 µm, divergent with a sudden change in direction half-way to the ends, strongly radiate towards the valve centre, strongly convergent towards the ends, an acute angle being formed at the meeting point of the two striae groups, alveoli open. Found growing freely in water or within spherical bodies formed on plant parts present in the water. **Note:** This species shows high morphological variability (Krammer & Lange Bertalot, 1986). According to DeNicola (2000), it closely resembles *Pinnularia obscura* Kraske in size and divergence of striae but differs primarily in the more lanceolate shaped valves of the latter. That author highlighted the similarity of *P. chapmaniana* Foged to *P. acoricola*. Our specimens are very similar to *Pinnularia divergentissima* (Grunov) Cleve var. *divergentissima*, which has larger valves (25-46 µm long, 5.8-6.3 µm wide), and to *Pinnularia acidophila* Hofmann & Krammer, which has linear-lanceolate valves that are smaller than those of *P. acoricola*. Cosmopolitan, reported from both natural and artificial acidic habitats (DeNicola, 2000; Hargreaves *et al.*, 1975; Negoro, 1985; Watanabe & Asai, 1995; Whitton & Diaz, 1981).



Figs 32-41. Scanning electron micrographs, *Pinnularia acoricola* var. *acoricola*, showing variability of frustules.

Euglena mutabilis F.Schmitz (Synonym: *Euglena acus* f. *mutabilis* G.A. Klebs) **Figs 5-7**

Cells 4-9(-11) µm wide, 60-95(-122) µm long, narrowly cylindrical, slender or elongated at the posterior end. **Note:** The chloroplasts of the specimens found here were smaller and dispersed throughout the whole cell and not adpressed to the wall, unlike the original description (Schmitz, 1884 after Starmach, 1983; Wołowski & Hindak, 2005). Common in small acidic water bodies and frequently abundant in waters contaminated by heavy metals, with low pH; known in unpolluted to moderately polluted water, acidobiont (Wołowski, 2002).

Unidentified colourless flagellate taxon **Fig. 8**

Cells 7.8-10.5 µm in diameter, colourless, swimming actively, with four flagella. Only a few specimens were observed.

Chlamydomonas spp. **Figs 9, 10**

Cells 7.5-8.0 µm in diameter enshrouded by a thin colourless layer about 3 µm thick (Pedrozo *et al.*, 2001).

Unidentified chlorococcal forms **Figs 11, 12**

Cells spherical, 3-5 µm in diameter, chloroplast cup-shaped; pyrenoid not visible; usually as 2 to 4 packets of cells. Probably cosmopolitan. In a diverse range of aquatic habitats, sometimes on sub-aerial surfaces or dominating at depth (Kapfer *et al.*, 1997; Nix dorf *et al.*, 1998).

Ulothrix tenerrima Kützing **Figs 13-15, 17**

Filamentous, thin, 4-5 µm wide cells, 1.5 to 2 times longer than wide, thin-walled; chloroplasts small, plate-like or irregular. **Note:** In the strongly acidic habitat the filaments were modified, lacking chloroplasts with folded walls. Examination of the algae by SEM revealed some ornamentations on the surfaces of *Ulothrix* of unknown origin and resembling adsorbed inorganic particles. Cosmopolitan, frequently reported from sites of acid pollution (Warner, 1971).

DISCUSSION

The metal concentrations found in the drainage water of the São Domingos mine show annual fluctuations, but overall the Fe and other metal levels are more than one order of magnitude higher than those found in the well-known Rio Tinto (Stained River, from its high ferric ion concentration) that runs through the same Iberian Pyritic Belt (Lopez-Archilla., 2001), as well as in many other acidic water bodies worldwide (Johnson, 2003; Johnson & Hallberg, 2003). The pH value of S. Domingos AMD is also extremely low and these two factors,

the extreme acidity and high metal concentration, concur to greatly reduce the biological diversity of these waters. Recently, Bryan *et al*. (2006) reported a number of prokaryotic species, both archaea and bacteria, inhabiting this environment; our investigation represents the first study of the algal community.

We found the diatoms *Eunotia exigua* (Figs 18, 19-31) and *Pinnularia acoricola* var. *acoricola* (Figs 2-4, 18, 32-41) to be dominant in the drainage water of São Domingos mine. The two species are classified as acidophilic and are known to occur in both natural and artificial environments (DeNicola, 2000). Several of the *Eunotia* taxa usually found in highly acidic environments belong to *E. exigua* like-complex, which includes species such as *E. exigua*, *E. tenella* (Grunow) Cleve, *E. levistriata* Hustedt. and probably several varieties of *E. septentrionalis* Østrup (DeNicola, 2000). Detailed studies of *Eunotia exigua* showed very high variability in the size and shape of the frustules (Figs 18, 19-31), which makes this taxon identification hard. *Pinnularia acoricola* var. *acoricola* was firstly described from Java by Husted after Krammer (2000). This variety comprises the smaller forms of the species, up to 30 μm long, whereas the varieties lanceolata and *rostellata* are larger. According to Krammer (2000), *Pinnularia acoricola* var. *acoricola* (Figs 2-4, 18, 32-41) is a cosmopolitan taxon, uncommon, preferring electrolyte-poor waters, but its presence in the electrolyte-rich waters of São Domingos mine reveals that its distribution is more widespread than reported by that author. In Europe, it has been reported from the central mountain regions of Germany and Scotland, but also from lowland waters in the Alps. Negoro (1985) found it to be widespread in highly acidic habitats in Japan, with pH ranging from 2.0 to 4.0; however, according to Watanabe & Asai (1995) this taxon can occur in waters with pH values as low as 1.1. More recently, Löhr *et al*. (2006) also reported the presence of *P. acoricola* in water bodies with pH ranging between 2 and 3, in East Java, Indonesia. *Euglena mutabilis* was also found in abundance at São Domingos mine's drainage and it has been found to occur in similar acidic polluted waters (Hargreaves *et al*., 1975; Olaveson & Nalewajko, 2000; Warner, 1971). *Euglena mutabilis* is both acid-tolerant and capable of withstanding elevated metal concentrations, namely Fe and Al (Olaveson & Nalewajko, 2000). The closely related *E. gracilis*, in turn, although also an acid-tolerant species, was not found at São Domingos mine drainage, most probably because of its much lower tolerance to the high metal concentration in the medium (Olaveson & Nalewajko, 2000).

The water's extreme acidity and metal toxicity limit the algal diversity but also cause changes in the species chemistry and structure. For example, the chloroplasts of *Euglena mutabilis* from the S. Domingos mine drainage were usually smaller and dispersed throughout the whole cell, not adpressed to the cell walls, as in the original description (Schmitz 1884 after Starmach, 1983). Also, chloroplasts with major morphological alterations were observed in the filamentous green algae *Ulothrix tenerrima* and these chloroplast modifications make it difficult to recognize the taxon. In an apparently healthy condition were found the chlorococcal forms as well as the bacterium *Achromatium volutans* previously reported from polluted waters (Hausler, 1982); the specimens of *Chlamydomonas* spp. (Figs 9, 10) and of the rare, unidentified colourless flagellate (Fig. 8) also exhibited great vitality. Brightly green macroscopic forms resembling green jelly balls of *Ophridium versatile* (O. F Müller) Ehrenberg (Fig. 16) containing numerous diatoms in their interior (Fig. 18) were also found. They formed spherical assemblages of a few mm in diameter, attached to the branched parts of plant remnants present in the water. The diatoms found within the jelly balls were heavily pigmented, contrasting with the rather bleached appearance of

their free-living counterparts. Thus, the diatoms enclosure within the jelly balls assemblages seems to provide protection against the hostile environment and improves diatom cell status.

The extreme high acidity and metal concentration of the São Domingos mine's drainage constitute two immediately apparent limitations to biological growth and the organisms there present must be equipped with mechanisms to cope with these conditions. Some of these mechanisms are known and have been discussed elsewhere (Gross, 2000). In respect to the low pH, it is well established that acidophilic and acid-tolerant organisms possess membranes with very low proton permeability and maintain their cytoplasmic pH near neutral values by pumping out H^+, which poses additional energy demands on their cells. As to the mechanisms to deal with excess metals in the medium, it has been shown that acidophilic and acid-tolerant algae are remarkably tolerant to excess metals, resorting mainly to exclusion strategies to prevent their toxic effects in the protoplast.

Additional limitations to phototrophs imposed by these still, acidic and iron-loaded water bodies respect to their light intensity and quality as well as CO_2 availability. These water bodies are of a deep red colour and this attenuates the intensity and changes the spectrum of the light that penetrates them, causing a relative depletion of the photosynthetically important blue wavelengths absorbed by chlorophylls. It is plausible that the photosynthetic organisms found in such strongly coloured waters show some sort of special adaptative features regarding the chemical composition of their antenna pigments and this has been demonstrated for *Chlamydomonas acidophila* Negoro, for example (Gerloff-Elias *et al*., 2005). Also, in these extremely acidic waters, CO_2 constitutes the large majority of the dissolved inorganic carbon, but saturation occurs at a concentration that is not saturating for photosynthesis and this may limit autotrophic growth (Gross, 2000). In this respect, it is important to point out that the major phototrophic taxa identified here, including *Eunotia*, *Pinnularia*, *Euglena* and *Chlamydomonas* can also grow heterotrophically on a variety of organic compounds and this capacity may be critical for its viability during periods of limiting photosynthesis. The pond contains several plant remnants and other organic material that could sustain significant heterotrophy and further studies are necessary on the metabolic pathways used by the algae in this hostile environment.

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