J. Phycol. **39,** 481–489 (2003)

STRUCTURE AND FUNCTION OF BENTHIC ALGAL COMMUNITIES IN AN EXTREMELY ACID RIVER[1]

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The composition of algal species and pigments and the structural and functional characteristics of the algal community were investigated in an acid stream of southwestern Spain, the Río Tinto. The algal community had low diversity and showed few seasonal differences. It was mainly made up of *Klebsormidium flaccidum* Kütz. (Silva, Mattox & Blackwell) that produced long greenish or purplish filaments, *Pinnularia acoricola* Hust. (producing brown patches) and *Euglena mutabilis* Schmitz. The algal filaments made up a consistent biofilm that also included fungal hyphae, iron bacterial sheaths, diatoms, and mineral particles. HPLC analyses on Río Tinto samples showed that undegraded chl accounted for 67% of the total chl in the filamentous patches but were a minority in the brown patch (2.6%). The brown patch had a concentration of carotenoids eight times lower than that observed in the green patch. When chl concentrations were weighted for the proportion of the different patches on the streambed, undegraded chl *a* accounted for 89.2 mg chl $a \cdot m^{-2}$ of stream surface area (5.4 g $C \cdot m^{-2}$). This high algal biomass was supported by relatively high nutrient concentrations and by a high phosphatase activity (V_{max} = 137.7 nmol methylumbelliferyl substrate$\cdot cm^{-2} \cdot h^{-1}$, K_m = 0.0045 μM). The remarkable algal biomass in Río Tinto potentially contributed to the bacterial–fungal community and to the macroinvertebrate community and emphasizes the role that the algae may have in the organic matter cycling and energy flow in extreme systems dominated by heterotrophic microorganisms.

Key index words: **acid mine drainage; algal biomass; algal pigments; carotenoid; chlorophyll; diatoms; green algae; heavy metals; nutrients**

Abbreviations: **AMD, acid mine drainage; DOC, dissolved organic carbon; MUF, methylumbelliferyl; EDS, energy dispersive X-ray spectroscopy**

The most extreme acidic environments are usually the result of mineral acids entering the water, either naturally (e.g. hot springs, volcanic areas, acidic ponds) or induced by human activities (DeNicola 2000). Among the latter, the most common are those caused by acid mine drainage (AMD). Aquatic habitats having these origins show low pH values (<3.5) and, usually, high concentrations of heavy metals. Sulfuric acid and ferric ions are created by chemical oxidation and through the activity of sulfur-oxidizing bacteria (Kristjansson and Stetter 1992). Overall, the

[1] Received 15 July 2002. Accepted 17 February 2003.

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activity of these organisms creates strong oxidizing conditions that ultimately lead to metal leaching from the drained substrata, therefore enriching the water in dissolved heavy metals. Even though iron may be dominant in these environments (Robb and Robinson 1995), other heavy metals (e.g. zinc, lead, copper) may be toxic and may potentially affect the biota. Higher organisms are rare in these systems, with the exception of some invertebrates (Havas and Hutchinson 1983) and vascular plants (Fyson 2000), and only a reduced number of bacterial, fungal, and algal taxa persist (Whitton and Diaz 1981, DeNicola 2000, Gross and Robbins 2000, Robbins 2000).

The biomass, pigment composition, and fine structure that characterizes algal communities in these systems are largely unknown. Also, there is little information on the possible links these algal communities have with other components of the trophic web, namely the microbial heterotrophs and macroinvertebrates (Lessmann et al. 1999). This article is focused on the Río Tinto, a river that can be categorized as an AMD site for its low pH (between 1.5 and 2.5) and extremely high concentrations of heavy metals, mainly iron, zinc, and copper. In addition to the low pH and high heavy metal concentrations, the Río Tinto receives high inputs of solar radiation, has elevated water temperatures, and often experiences low discharge, making it an extreme habitat for algal development. Even so, algal communities develop as exuberant masses, in particular at the upstream localities. The ecological role the algae may play in this river is explored through their main structural and physiological features. In particular, the following questions guided the present study. What proportion of algal biomass that develops under these extreme situations is photosynthetically active? Which environmental or biological factors stimulate or constrain algal biomass development in this AMD site? Does the age of the algal community affect its pigment composition and metabolic requirements? Because Río Tinto shares characteristics with other AMD sites, the information gained from this case may contribute to the understanding of the functional characteristics of other analogous systems.

STUDY AREA

Río Tinto drains a significant part of the Iberian pyrite belt, a massive sulfide deposit in southern Spain and Portugal (van Geen et al. 1999). Early indications of mining (silver and copper) in the region date to 3000 BP. Mining was intense during the Phoenician and Roman periods and again during the 19th century. The study site was located at Peña de Hierro, in the Río Tinto headwaters, and was visited three times: July 1999, December 1999, and June 2000. The site is an ancient mining site, with various industrial ruins and ore dumps in the vicinity. Río Tinto headwaters drain the mine wastes accumulated throughout the area, being barren of vegetation except for some replanted eucalyptus located approximately 20 m away from the streambed. The substratum forming the streambed was half rocks and cobbles and half clay.

Iron-oxidizing bacteria, sulfur-oxidizing bacteria, and filamentous fungi thrive in the Río Tinto (López-Archilla and Amils 1999, López-Archilla et al. 2001) and are largely responsible for the environmental conditions occurring in the river (Ehrlich 1996). The activity of these organisms leads to high concentrations of iron, copper, zinc, and lead (Table 1). The dominance of ferric iron (Table 1) and the turbidity confers a characteristic reddish color to the water (from which it derives the name Tinto, red wine in Spanish).

TABLE 1. Physical and chemical variables measured at Peña de Hierro in the three sampling periods.

Parameter	1 July 1999	20 December 1999	30 June 2000
Water flow ($L \cdot s^{-1}$)	1.8 ± 0.75	0.5 ± 0.98	0.4
Water velocity ($cm \cdot s^{-1}$)	4.2 ± 1.5	1.7 ± 2.5	3.0 ± 0.5
Irradiance ($\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$)	1414	1014	1195
pH	2.4	1.5	2.2
Water temperature (° C)	30.0	15.6	27.2
Dissolved oxygen (% sat.)	63.0	73.0	70.3
Water conductivity ($mS \cdot cm^{-1}$)	7.8	20.5	11.4
Suspended solids ($mg \cdot L^{-1}$)	58.3	24.0	94.0
Sulfates ($mg \cdot L^{-1}$)	9247	—	5483
Soluble reactive P ($\mu g \cdot L^{-1}$)	180	3250	200
NO_3-N ($mg \cdot L^{-1}$)	0.5	5.0	0.01
DOC ($mg \cdot L^{-1}$)	8.3	7.9	8.0
Fe ($mg \cdot L^{-1}$)	2630	3325	2498
Zn ($mg \cdot L^{-1}$)	168.1	82.1	129.7
Mg ($mg \cdot L^{-1}$)	392.0	248.8	301.4
Mn ($mg \cdot L^{-1}$)	142.2	82.1	122.9
Cu ($mg \cdot L^{-1}$)	43.8	34.9	28.9
Pb ($mg \cdot L^{-1}$)	0.7	1.8	3.7
Cd ($mg \cdot L^{-1}$)	0.8	0.01	3.4
Al ($mg \cdot L^{-1}$)	—	—	132.0

Water temperature, irradiance, dissolved oxygen, and pH were measured at noon.

MATERIALS AND METHODS

Field measurements. Streambed cross-sections, water velocity, and algal cover at Peña de Hierro were estimated along equidistant transects (four to five, depending on the visiting period) in a stream stretch 50 m long. Water velocity was measured with a propeller current meter (Schiltknecht, Zurich, Switzerland). Discharge was derived from the stream cross-sections and water velocity estimates. Values of discharge and velocity were given as averages for the whole stream stretch. Dissolved oxygen, pH, temperature, and conductivity were estimated *in situ* with appropriate electrodes at noon.

Water analyses. Water for chemical analyses was filtered immediately through Whatman GF/F filters, frozen in liquid nitrogen, and transported to the laboratory (12 hr of travel). Suspended solids in the water were measured by difference in weight of preweighed GF/F filters after filtration of a specific water volume. Nitrate was analyzed using a Skalar (Breda, The Netherlands) autoanalyzer. Soluble reactive phosphorus was analyzed with a Perkin-Elmer spectrophotometer Shelton, CT, USA (APHA 1989). Dissolved organic carbon (DOC) concentrations were measured by Pt-catalyzed high-temperature combustion (Shimadzu TOC 5000, Kyoto, Japan). Heavy metal concentration in the water was determined by flow injection, inductively coupled, plasma, mass spectrometry (APHA 1989).

Algal community analyses. Three distinct types of algal patches were observed in Peña de Hierro. Two of them were made of filaments and simply differed in their color, one being dark green and the other being purplish. These two patches occupied the main flowing water part of the stream. The filaments were attached to cobbles and rocks but produced long filaments that trailed into the flowing water. A third patch type was brown in color, partly underneath the filamentous masses and tightly adhered to the rocks and cobbles. The percentage cover and distribution of these algal patches was assessed with an underwater viewer (20 × 20 cm at its base). Two types of measurements were done. First, the percentage of streambed covered by the algae was estimated. Second, the relative fraction of each patch over the streambed was estimated. These two fractions expressed the percentage of area (qualitatively estimated) occupied by the algae as a whole and, subsequently by every algal patch, expressed as the average percentage across the complete transect. Finally, the average value of all transects was also calculated for every sampling date.

Samples for species composition and community structure were obtained qualitatively from the different patches visually identified at Peña de Hierro. Samples were separately collected for LM and SEM to, respectively, determine the community composition and structural architecture of the algal patches. Those samples for LM were preserved in 4% formaldehyde and stored at the dark until analyses. Samples for SEM were immediately frozen in the field using liquid nitrogen and stored in a freezer until analyses. Identification and counting were carried out using LM with a Reichert-Jung Polyvar microscope Wetzlar, Germany at 1200×. Diatoms were separately identified after being acid cleaned and mounted in Naphrax (refraction index, 1.74). Several aliquots from two different samples for every patch and sampling date were counted under the microscope, and the results (cell numbers contained in the microscope field) were expressed as a percentage of the total counted. Samples for SEM were freeze dried, carbon and gold coated, and observed with a Leica, Wetzlar, Germany scanning microscope operated at 2.0 kV. SEM observations were performed for the July and December 1999 samples. December 1999 samples were also analyzed for energy dispersive x-ray spectroscopy (EDS) coupled to a Jeol scanning microscope Wetzlar, Germany equipped with microprobe and operated at 25 kV to determine the location of metals over the biofilm surfaces.

Algal pigment analysis. Algal samples of the two most abundant patch types throughout the year (green and brown) were collected in the field for pigment analysis (June 2000). Algal collection was carried out by coring the algal mats with a small plastic cylinder to obtain a precise area (4.9 cm^2). The scarcity of studies of biomass and pigment composition for AMD communities may be related to the difficulty of obtaining reliable measurements of undegraded chl *a* in these systems. Because of the acid environment, chl is likely to be degraded when microhabitat conditions are altered (at the time of collection) or during the extraction. In this study, special care was taken to ensure that a correct measurement of undegraded and total chl was made. This included deep freezing in the field and addition of ammonium acetate buffer during the collection and the extraction, which enhanced the preservation of the chls. In detail, all collected samples were flooded with 0.3 M ammonium acetate buffer solution and immediately stored in liquid nitrogen. Before the extraction, the samples were freeze dried for 24 hr and referred to dry weight. Three replicates were analyzed for the field samples of June 2000.

To prevent chl degradation during extraction, we examined the suitability of using 90% acetone buffered with 0.15 M ammonium acetate (pH 7). The use of the buffered solution prevented chl losses by 20%–30% for chl *b* and by 30%–40% for chl *a*. Extraction was therefore carried out in 4 mL buffered 90% acetone after sonication (Sonopuls GM70 probe, Delft, The Netherlands). The extract was filtered through a Whatman ANODISC 25 filter Maidstone, UK (0.1 μm pore diameter) and analyzed immediately.

Pigment composition was analyzed by HPLC. The system consisted of a Waters 600E Milford, MA, USA Multisolvent Delivery System, an autosampler (Waters 717, Milford, MA, USA) equipped with a refrigeration unit (4°C), and a C-18 column (Spherisorb ODS-1 Milford, MA, USA, 250 × 4.6 mm, 5 μm particle size). Analytical separation was achieved by linear gradient (1.2 $mL \cdot min^{-1}$, 1400 psi) using a modification of the system described by Wright et al. (1991). After sample injection (40 μL of extract), pigments were eluted by linear gradient from 100% A (methanol/acetonitrile/MilliQ water [51:36:13] + 23.124 g ammonium acetate), to 75% A in 5 min followed by an isocratic hold for 5 min at 75% A and to 100% B (ethyl acetate/acetonitrile [70:30]) in 20 min. The solvent composition was returned to initial conditions with a 5-min gradient, followed by 5 min of system equilibration before the next sample injection. Detection was performed with a Waters 996 photodiode array detector, set at 440 and 660 nm for carotenoid and phorbin peak integration, respectively.

Identification of pigments was checked against a library of pigment spectra obtained from several extracts of pure algal cultures from the Culture Collection of Algae and Protozoa. Chl *a*, chl *b*, and beta-carotene were obtained from Sigma, San Francisco, CA, USA. Phorbin derivatives were prepared by acidification with 0.1 N HCl (Zapata et al., 1987). The extinction coefficients were obtained from Rowan (1989) and Wright et al. (1991).

Chl density in the stream was estimated by considering the percentage cover of the different patches and equating them with their respective chl concentration. Chl was converted to carbon using the empirical coefficient of 60 mg C:1 mg chl (Geesey et al. 1978, Romaní and Sabater 2000).

Analyses of algal communities developing in experimental streams. The use of an experimental stream allowed us to follow the evolution of several descriptors of the algal mats through time in environmental conditions mimicking those of Río Tinto. Pigment composition, metal accumulation in the algal community, and phosphatase activity of Río Tinto algae were analyzed in an artificial system in addition to the analyses made with field samples. Water and biofilms of the dominant patch (green patch) were collected in Río Tinto on 30 June 2000 and transported to the laboratory in a cooler equipped with a re-aerator (12 h of travel). Río Tinto water was used to feed the channel (6-L capacity) and was recirculated with a submersible pump. Water was fully replaced every 7 days. The channel was installed in a greenhouse to provide irradiance (maximum of 1400 μmol $photons \cdot m^{-2} \cdot s^{-1}$) and water temperature (20–30° C) analogous to that recorded at Río Tinto at the date of the collection. Algal mats collected at Río Tinto were placed at the top of channel and used as an inoculum for colonizing etched glass substrata

TABLE 2. Percentage of surface area of the streambed covered by the algal patches (upper row) and contribution (percentage) of the different macroscopically visible algal patches with respect to total algal cover at Peña de Hierro (Río Tinto headwaters) (lower rows).

Algal cover (%)	1 July 1999	20 December 1999	30 June 2000
Total	99 ± 2.2	39 ± 5.8	100
Green patch	76.7 ± 5.8	36 ± 19.5	48 ± 2.5
Purple patch	8.3 ± 10.4	54 ± 8.9	30 ± 1.1
Brown patch	15 ± 3.2	7 ± 9.7	22 ± 1.2

(1.4 cm^2), which were covering the bottom of the artificial channel. Physical and chemical conditions in the channel (conductivity, pH, dissolved oxygen) were monitored every 2–3 days.

Three replicates (each consisting of several glass substrata) were collected at days 11, 39, 59, 67, and 82 after the addition of the inoculum and the material analyzed for algal pigments. Copper accumulation by the biofilm was determined from freeze-dried biofilms (five replicates) obtained from the channel on day 82. Copper was used as a representative of heavy metal accumulation by the biofilm. The analysis was completed after digestion in 70% HNO_3, using a microwave oven equipped with a temperature- and pressure-control program (MDS-200, CEM Laboratories, Matthews, NC, USA). Copper analysis in the biofilm was performed with flame and graphite furnace atomic absorption and emission spectra. Quality control of copper analysis was carried out by analyzing digestion blanks and reference material (NIST [SRM 2704]: Buffalo River Sediments, NIST, Gaithersburg, MD, USA). The measured values deviated less than 10% from certified values, and digestion blanks were near detection limits. Copper concentrations in the biofilm were expressed as micrograms of metal per gram dry weight of biofilm. Finally, the extracellular enzyme phosphatase was measured in colonized glass substrata obtained from the artificial channel on two occasions (days 26 and 59) to detect differences for this enzyme between young (day 26) and mature (day 59) algal communities. Glass substrata (five replicates) were incubated for 2 hr in the dark at river temperature immediately after sampling. Incubations were performed by using the fluorescent-linked substrate methylumbelliferyl (MUF) phosphate (Sigma). A range of substrate concentrations (0.1, 10, 100, 300, and 600 μM) were used to calculate the saturation curve for phosphatase in the colonized glass substrata. Blanks and standards of MUF (0–500 μM) were also incubated. Each glass substratum incubation was performed in 4 mL of filter-sterilized Río Tinto water (Whatman GF/F and 0.2 μm pore size cellulose nitrate membrane filters) to circumvent undesirable pH variations. Although MUF substrates show maximum fluorescence at pH 10 (Chróst and Krambeck 1986), the activity in the Río Tinto biofilms was high enough to measure MUF fluorescence at pH 2.5. The incubation was stopped with the addition of 100 μL of formaldehyde, and the fluorescence was measured at $^{365}/_{455}$ nm excitation/emission (Kontron spectrofluorometer SFM25 Eching, Germany).

Macroinvertebrate sampling. The macrobenthic community at Peña de Hierro was sampled in July and December 1999 to determine the possible links with the algal communities. Macroinvertebrates were always collected in the same 15-m stream stretch by using a semiquantitative method. A kick net (250 Fm mesh) was set in the stream, and the substrata in front of the net were washed up to free the macroinvertebrates. Heteroptera individuals occurring in the water surface were sampled with a hand net from the stream pools; therefore, the estimates for this group were qualitative.

RESULTS

Physical and chemical features. Discharge was low at the time of the three site collections (Table 1). Water temperature reached 30° C during one of the two summer visits, and irradiance over the algal mats was high. The pH ranged between 1.5 and 2.2. Total acidity was measured only in July 1999 and accounted for 4318 mg $CaCO_3 \cdot L^{-1}$ at the Río Tinto headwaters. Variations in water temperature and pH were monitored during 12 h (daylight) in July 1999. Over this period, water temperature rose from 21.8° C at 08:00 to the maximum of 30° C at noon. However, variations of pH were minimal, from 2.28 (08:00) to 2.31 (noon), decreasing later (19:00) to the initial value. Nutrient concentrations varied between the three sampling periods. Soluble reactive phosphorus had moderate values in the two summer visits and was extremely high in the winter visit (Table 1). These high soluble phosphorus concentrations during December 1999 corresponded with high nitrate concentrations. Iron was the most abundant heavy metal in Río Tinto waters, but magnesium, aluminium, copper, and lead also reached very high concentrations (Table 1).

TABLE 3. Algal and cyanobacterial composition at Peña de Hierro at the three sampling dates.

	July 1999			December 1999			June 2000		
	B	P	G	B	P	G	B	P	G
Bacillariophyta									
Pinnularia acoricola	98.1	0.9	5.8	92.0	84.1	59.3	17.2	0.3	1.7
Nitzschia aff. palea	0.3	—	—	—	—	—	—	—	—
Cyclotella kutzingiana	0.1	—	—	—	—	—	—	—	—
Nitzschia linearis	—	—	—	0.2	—	—	—	—	
Chlorophyta									
Klebsormidium flaccidum	0.2	98.2	93.0	—	—	—	77.2	97.8	96.8
Mougeotia sp. (4.5 μm)	—	0.7	—	—	—	—	—	—	—
Chlorococcal indet.	—	—	—	4.4	—	—	—	—	
Euglenophyta									
Euglena mutabilis	1.3	0.2	0.5	2.9	15.9	40.6	5.6	1.9	0.5
Cyanobacteria									
Gloeocapsa sp.	—	—	—	0.4	—	—	—	—	—
Dermocarpa sp.	—	—	0.8	—	—	—	—	—	0.9

The relative abundance of every taxonomic entity is given for the macroscopic patches visible at the site. B, brown; P, purple; G, green.

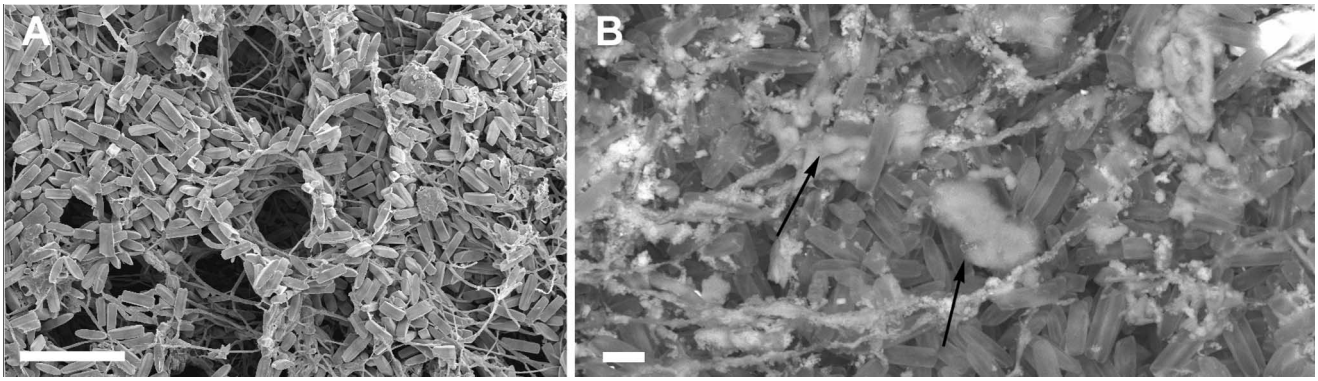

FIG. 1. (A) General view of the structural architecture of a portion of brown patch from Peña de Hierro (Río Tinto) in June 1999. The dominant component is *Pinnularia acoricola*, but filaments of *Klebsormidium* and fungi are also visible. The passage of microinvertebrate fauna through the algal patch could produce the empty spaces scattered throughout the structure. Bar, 50 μm. (B) EDS image of the same biofilm with indication of iron masses (arrows) accumulated on the algal and bacterial filaments. Bar, 10 μm.

Algal community composition and structure. The algal communities in Peña de Hierro (Table 2) were made up of a small number of taxa. *Klebsormidium flaccidum* (Kützing) Silva, Mattox & Blackwell (Chlorophyta, Ulothrichales) was the dominant alga in the green and purple filamentous masses in the two summer sampling periods (Table 3). Filaments from this species in Río Tinto ranged between 4 and 5 μm width, being in the lower size range for this species. Filaments of *Mougeotia* sp. (Chlorophyta, Zygnemales) also occurred interspersed with *Klebsormidium. Pinnularia acoricola* Hust. (Bacillariophyta, Pennatophyceae) was dominant in the brown patches (Table 3), where it accounted for nearly 100% of the total diatoms, and occurred in lower number in the filamentous masses. The euglenophyte *Euglena mutabilis* Schmitz was more commonly recorded in the brown patches and was less abundantly interspersed with the filaments. During winter, the green and purple patches were made up of bacterial and fungal filaments and mucilages. *Klebsormidium* was absent during that period, and *P. acoricola* and *E. mutabilis* grew interspersed among the nonalgal filaments (Table 3).

SEM observations showed that the algal filaments and cells were intertwined and produced a dense network. The filaments were predominantly *Klebsormidium* but also contained fungal hyphae, iron bacterial sheaths, diatoms, and mineral particles (Fig. 1A). These mineral particles had a crystalline structure and were identified by the EDS analysis as iron sulfur crystals that contained lead and copper. These granules accumulated on the filaments of iron bacteria and algae (Fig. 1B).

Biomass and pigment composition. The two main patch types (green and brown) showed a similar concentration of phorbins (by dry weight), but the proportion of undegraded versus degraded chl was different (Table 4). The green patch accounted for 114 $mg \cdot m^{-2}$ undegraded chl *a*, which represents approximately 64% of total chl *a* products, whereas the brown patch accounted only for 2.2 $mg \cdot m^{-2}$ chl *a* (2% of the total). The proportion of undegraded chl *b* was higher in the green patch (77%) than in the brown one (3%). The same occurred for chls *c1* and *c2*, which were detected in the brown patch but not quantified because of the low levels (Table 4). In both types of patches, there was presence of pheophorbide

TABLE 4. Phorbin composition in relation to dry weight (μg pigment·g dw^{-1}) determined by HPLC in the two algal patch types collected in Río Tinto headwaters on 30 June 2000 (above) and in the artificial channel at consecutive days (below).

	Chl *a*	Σpheor *a*	Σphytin *a*	ΣdChl *a**	% dChl *a*	Chl *b*	Σphytin *b*	ΣdChl *b**	% dChl *b*	Chl *c1*	Chl *c2*	ΣChls *c*
Patch type												
Green	1162	20.1	64	1825	36	470	140	610	23	7.8	6.5	14.3
Brown	45.4	29.1	1866	1941	98	20.7	599	620	97	bq	bq	bq
Experiemntal stream (day)												
11	91.6	nd	64.1	156	41	62.9	nd	62.9	nd	nd	nd	nd
39	1042	nd	810	1853	44	567	42.3	609	7	nd	nd	nd
59	489	nd	639	1128	57	320	66.9	387	17	nd	nd	nd
67	327	nd	422	748	57	188	95.2	283	34	nd	nd	nd
82	933	nd	588	1521	39	331	57.1	389	15	nd	nd	nd

Chl *a*, concentration including allomers and epimers; Σpheor *a*, pheophorbides *a*; Σphytin *a*, pheophytins *a*; ΣdChl *a**, sum of a-phorbins; % dChl *a*, percentage of chl *a* detectable degradation products; Σphytin *b*, pheophytins *b*; ΣdChl *b**, sum of b-phorbins; % dChl *b*, percentage of chl *b* detectable degradation products; ΣChls *c*, sum of chl *c1* and *c2*.

nd, not detected pigment; bq, present but below quantifiable concentrations.

TABLE 5. Carotenoid composition in relation to dry weight (μg pigment·g dw^{-1}) in the two algal patch types collected in Río Tinto headwaters during 30 June 2000 and in the artificial channel at consecutive days.

	Alloxanthin	alpha-carotene	Fucoxanthin	Diatoxanthin	Diadinoxanthin	Neoxanthin	Violaxanthin	Lutein	Zeaxanthin	Beta-carotene	Σcarot-nd	Tcarotenoids
Patch type												
Green	6.5	nd	41.7	28.3	175.7	49.9	nd	21.1	8.3	14.7	12.1	358.3
Brown	3.5	nd	nd	5.2	4.7	6.5	nd	20.2	3.2	1.1	1.9	46.4
Experimental stream (day)												
11	nd	nd	nd	nd	44.9	8.6	nd	6.1	nd	nd	nd	59.6
39	6.0	5.5	nd	nd	63.3	130	8.6	322	91.9	69.6	61.4	758.6
59	2.4	2.6	nd	nd	13.6	52.0	9.4	118	nd	9.7	3.5	210.9
67	1.8	1.0	nd	nd	12.5	37.0	4.3	77.7	nd	10.9	5.1	150.4
82	6.4	4.3	nd	nd	37.7	83.0	12.5	207	18.1	40.0	29.7	438.3

Σcarot-nd, sum of carotenoids not identified; Tcarotenoids, total carotenoids; nd, not detected pigment.

a. As a whole, undegraded phorbins accounted for 67% of the total chl in the green patch but were a minority in the brown patch (2.6%).

The carotenoid composition was similar in the two patches, but their relative proportions diverged. Diadinoxanthin was the most abundant xanthophyll in the green patch (Table 5), followed by neoxanthin, fucoxanthin, diatoxanthin, and lutein. Other carotenoids that occurred in much lower concentrations were beta-carotene, zeaxanthin, and alloxanthin. The brown patch had a total carotenoid concentration eight times lower than that observed in the green patch. Lutein was the most abundant xanthophyll in this patch, followed by neoxanthin, diatoxanthin, and diadinoxanthin; alloxanthin, zeaxanthin, and beta-carotene occurred again in much lower concentrations.

Experimental channel analyses. The chl content in the experimental channels after 39 days of colonization reached comparable concentrations to those found in the green patch sampled on 30 June 2000 (Table 4). After a sharp decrease after 60 days, values again approached those in the field at the end of the colonization (Fig. 2). Degradation products of chl *a* (Fig. 2) and chl *b* increased throughout the colonization and were high with respect to total phorbins after 2 months. However, the percentage of active chl increased again at the end of the experiment, approaching those values of the early algal community. Total carotenoids followed the same pattern than that of the phorbins, with the highest concentrations after 39 days of colonization (Fig. 2). Carotenoid composition in the artificial channel was to some extent different from that encountered in the field, because fucoxanthin and diatoxanthin were not detected, whereas alpha-carotene and violaxanthin appeared in low amounts.

The amount of copper accumulated by the algal mats was measured in the experimental stream from newly colonized substrata. Total copper after 82 days of colonization accounted for 505 $\mu g \cdot g^{-1}$ dry weight. Compared with the copper concentration in the water (28.9 mg $Cu \cdot L^{-1}$), the concentration factor for this heavy metal was about one order of magnitude.

Phosphatase activity for young (day 26) and mature (day 59) algal communities was determined from algal mats developing in the artificial channel. The activity was high for both communities (average V_{max} = 239 nmol $MUF \cdot cm^{-2} \cdot h^{-1}$, SD = 52, and average V_{max} = 138 nmol $MUF \cdot cm^{-2} \cdot h^{-1}$, SD = 12, respectively). The K_m values were very low on both occasions (K_m = 0.041 μM, SD = 0.091 on day 26, K_m = 0.0045 μM, SD = 0.029 on day 59), indicating the high affinity of the enzyme for the substrate and suggesting potential phosphorus limitation. Substantial differences between the young and mature algal mats suggest that more reduced phosphorus availability occurred in the older algal mat.

Macroinvertebrate community. The macroinvertebrate community had both a low diversity and density of individuals (Table 6). Macroinvertebrates were only sampled in July and December 1999. One genus

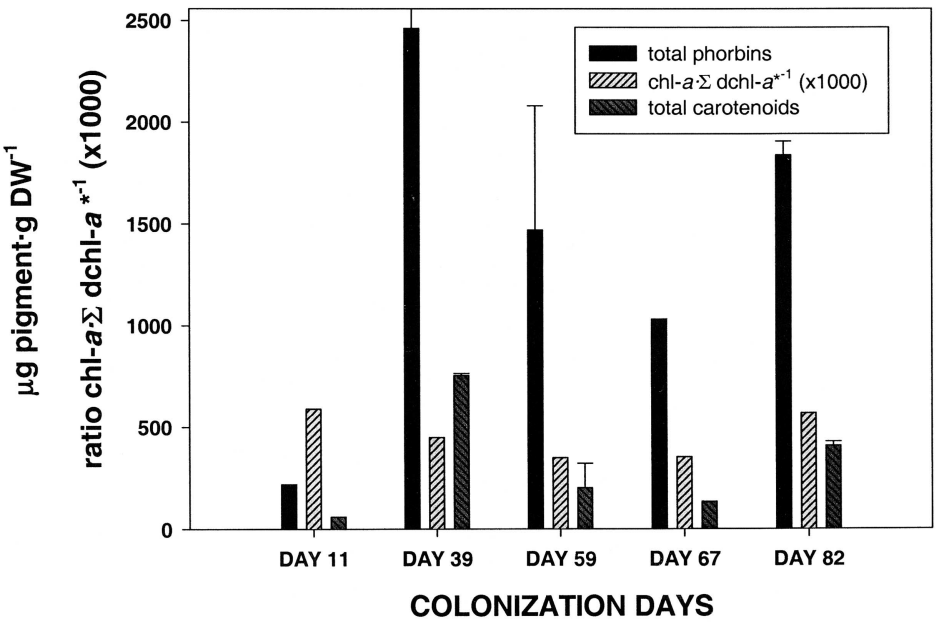

FIG. 2. Evolution of total phorbins and total carotenoids (averages and SDs) in the artificial channel from days 11 to 82. The ratio between active chl *a* and the sum of phorbins (Σdchl *a** × 1000) is also indicated.

of Chironomidae (genus *Lymnophyes*) occurred at extremely low densities (approximately 2 individuals·m^{-2}). The chironomid species found in Río Tinto was green pigmented, that is, without hemoglobin, in contrast to other observations that indicate that red-colored chironomids dominate in AMD sites (Havas and Hutchinson 1982, Lessmann et al. 1999). Macroinvertebrate densities were higher in some quiet water areas (15 individuals in a pool of 0.04 m^2). Individuals of Coleoptera and Hidracarina were present but rare. Three *Heteroptera* genera (belonging to the families Corixidae and Netonectidae) were observed, mainly in summer pools.

DISCUSSON

The role of benthic algae in AMD sites could be regarded as marginal, in the context of the microbial-based functioning of these systems (Ehrlich 1996). However, this concept is challenged when the undegraded fraction of chl (i.e. photosynthetically active chl) reaches significant values, implying that a quantifiable fraction of the organic matter cycling flows through the algal compartment. In Río Tinto, undegraded chl per stream surface area accounted for 89.2 mg chl-*a*·m^{-2} (representing 5.4 g organic carbon·m^{-2}). This level of algal biomass (obtained when chl concentration was weighted for the proportion of the two dominant patches in Río Tinto) is close to that observed in nutrient-rich river systems elsewhere (Dodds et al. 1998, Romani and Sabater 2000).

Algal biomass in AMD sites have been correlated with increases in acidity (Mulholland et al. 1986), but the large variation in algal biomass among AMD sites (Kapfer 1998, Verb and Vis 2000) indicates that local conditions may be important in algal abundance. Woelfl et al. (2000) observed a phytoplankton bloom in an acidic lake with a large concentration of heavy metals and where the nutrient concentrations were extremely high. Phosphorus availability in Río Tinto seemed related to algal demand for this nutrient. When algal mats dominated by *Klebsormidium* completely covered the riverbed (July 1999 and June 2000), reactive phosphorus was much lower than when algal cover was reduced (December 1999) and *Klebsormidium* was absent from the algal community. In Río Tinto, the high phosphatase activity and, especially, the high affinity for the substrate highlighted the role of inorganic phosphorus in constraining algal biomass development. Río Tinto algal mats showed similar V_{max} values but clearly lower K_m than the phosphatase activity in algal biofilms from a calcareous stream with phosphorus limitation (Romani 2000). Such a high affinity at low substrate concentrations may indicate that Río Tinto biofilms are subject to an especially low availability of inorganic phosphorus.

TABLE 6. Macroinvertebrates collected in Río Tinto (Peña de Hierro) on July 1999 and December 1999, indicated as number of individuals per square meter.

	July 1999	December 1999
Diptera		
F. Chironomidae, g. *Lymnophyes* (larvae, pupae and adults)	2	0.3
Coleoptera	0.06	
Heteroptera		
Parasigara sp.	+	
Notonecta maculata	+	
Anisops marazanofi	+	
Hidracarina		0.06

Presence of individuals during the collection is indicated as +. Macroinvertebrates were not sampled in June 2000.

The highest affinity was observed in the aged algal mats (59 days old), indicating that lower phosphorus availability occurs in higher biomass algal communities where nutrient diffusion may be more limiting. Inorganic phosphorus therefore could be a limiting nutrient in this acidic environment, and organic phosphorus could provide an additional source of reactive phosphorus (Whitton 1991). Phosphorus limitation has been described in acidic environments, especially when there is a high concentration of aluminum in the river water (as in the Río Tinto), because it causes precipitation of orthophosphates (Gross 2000) with consequent potential limitation of algal growth.

Only a small number of specialized algal and invertebrate taxa may develop in the harsh conditions of AMD systems. Verb and Vis (2000) observed that AMD stream diatom assemblages exhibited lower species diversity and richness than other non-AMD sites and showed little or no variation in floristic seasonality. This is also the case for Río Tinto. The three most common taxa in Río Tinto, *K. flaccidum* (green alga), *P. acoricola* (diatom), and *E. mutabilis* (euglenophyte), are common elsewhere in AMD sites. Several species of *Klebsormidium* have been observed in a variety of AMD sites in northern Europe, the United States (Whitton and Diaz 1981), and Australia (Lottermoser et al. 1999). Whitton and Diaz (1981) concluded that this alga occurred at a pH as low as 2.3 (in the case of *K. rivulare* Kütz.), but our observations in Río Tinto waters (where *K. flaccidum* was recorded in pH of 1.5) suggest that the pH threshold for their distribution may be much lower. The most abundant diatom in Río Tinto, *P. acoricola*, is also a common inhabitant of acidic environments, specifically in AMD sites (Whitton and Diaz 1981). This taxon has a variable morphology and closely resembles *P. obscura* Krasske (Carter 1972), both having been recorded in several sites in North America, England, and South Africa (DeNicola 2000). In Río Tinto this diatom formed single-species cushion-like brown patches. Finally, *E. mutabilis* has an optimal pH for growth between 2.5 and 6 (Olaveson and Nalewajko 2000), but its pH tolerance can be as low as 1 (Whitton and Diaz 1981). This adaptation may explain why it is the most widespread taxa in AMD sites elsewhere. The extreme environmental conditions also dictated that the macroinvertebrate community at Río Tinto had a very low density and diversity, being dominated by a single Chironomid genus. Decreases in density and diversity of macroinvertebrates has been shown to occur with increases in stream acidity and iron abundance (Havas and Hutchinson 1983, Rasmussen and Lindgaard 1988, Smith and Cranston 1995).

Severe conditions for algal development were evidenced by the existence of a high fraction of degraded pigments, which not only changed according to the age of the algal mass, but also with the patchy distribution of the algal community in the stream. The comparison between the two algal patch types observed in Río Tinto indicates that microenvironmental conditions could determine the physiological fate of these communities. The filamentous algae (green patches), which had the higher proportion of active chls, were in large part located in the free-flowing water part of the stream. However, the brown patch showed clear senescent properties, possibly because their position in the stream (underneath the filamentous algal patch) made light penetration difficult or limited the diffusion of gases and nutrients. Observations of EDS coupled to SEM indicated that crystals, including heavy metals (i.e. mineral nucleation sites), were abundant along bacterial sheaths and algal filaments in the algal patches, possibly having an effect on resource availability.

Several clues indicate that the algal component may be important for the trophic web in Río Tinto. First, the high DOC present in Río Tinto waters, which is produced in the absence of allochthonous sources in the watershed, suggests the role of algae as producers of part of the DOC. Because part of this algal-produced DOC may be readily consumed by the heterotrophs (Kaplan and Bott 1982, Sobczak and Burton 1996, Romani and Sabater 2000), the algae may be essential for the heterotrophic bacteria and fungi of the river (Gross and Robbins 2000). Second, if the algal community does have a substantial role in contributing dissolved organic matter for the decomposers, its relationship with the macroinvertebrate community is no doubt also important. In principle, the density of the macroinvertebrate community is far too low to significantly affect the algal community. However, signs of microinvertebrate activity in the algal patches suggest that the algal masses may provide (at least) refuge for the animals. On the other hand, grazing activity on the algal mats is suggested by the abundance of pheophorbides in the pigment extracts (Brotas and Plante-Cuny 1996). Other consumers (i.e. ciliates, observed in Río Tinto) may also exert a substantial grazing pressure on the algal community. Algae may also indirectly provide resources (organic carbon) for bacteria inhabiting Chironomid larvae guts (Smith and Cranston 1995). These bacteria could, at the end, provide an additional food source to the larvae.

The large biomass and good quality of the photosynthetic pigments of the algal community in Río Tinto indicate these primary producers could contribute both to the bacterial–fungal community and to the freshwater community of insects. The data presented here emphasize the potential role algae may have in extreme systems such as AMD.

Supported in part by the Programmes Microbenthos (ENV4-CT96-0298) and Biofilms (EVK1-CT1999-00001) of the European Community. The Scientific Services of the University of Barcelona provided assistance for the chemical analyses. Dr. J. Casas (University of Almeria) provided the taxonomical identity of some macroinvertebrate individuals from Río Tinto. We appreciate the help of Vicenç Acuña and Elisabet Vilalta during the field work. Dr. Simon Roberts (Monash University) made

helpful comments to the manuscript. The critical comments of two anonymous reviewers helped to improve the manuscript.

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