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Extracellular matrix assembly in extreme acidic eukaryotic biofilms and their possible implications in heavy metal adsorption

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ABSTRACT

To evaluate the importance of the extracellular matrix in relation to heavy metal binding capacity in extreme acidic environments, the extracellular polymeric substances (EPS) composition of 12 biofilms isolated from Río Tinto (SW, Spain) was analyzed. Each biofilm was composed mainly by one or two species of eukaryotes, although other microorganisms were present. EPS ranged from 130 to 439 mg g^{-1} biofilm dry weight, representing between 15% and the 40% of the total biofilm dry weight (DW). Statistically significant differences ($p < 0.05$) were found in the amount of total EPS extracted from biofilms dominated by the same organism at different sampling points. The amount of EPS varied among different biofilms collected from the same sampling location. Colloidal EPS ranged from 42 to 313 mg g^{-1} dry weight; 10% to 30% of the total biofilm dry weight. Capsular EPS ranged from 50 to 318 mg g^{-1} dry weight; 5% to 30% of the total biofilm dry weight. Seven of the 12 biofilms showed higher amounts of capsular than colloidal EPS ($p < 0.05$). Total amount of EPS decreased when total cell numbers and pH increased. There was a positive correlation between EPS concentration and heavy metal concentration in the water. Observations by low temperature scanning electron microscopy (LTSEM) revealed the mineral adsorption in the matrix of EPS and onto the cell walls. EPS in all biofilms were primarily composed of carbohydrates, heavy metals and humic acid, plus small quantities of proteins and DNA. After carbohydrates, heavy metals were the second main constituents of the extracellular matrix. Their total concentrations ranged from 3 to 32 mg g^{-1} biofilm dry weight, reaching up to 16% of the total composition. In general, the heavy metal composition of the EPS extracted from the biofilms closely resembled the metal composition of the water from which the biofilms were collected.

1. Introduction

With a length of ca. 100 km, Río Tinto (SW, Spain) provides one of the largest extreme acidic habitats for a broad range of organisms, including different species of bacteria, fungi, algae and protozoa (López-Archilla et al., 2001; Amaral et al., 2002; González-Toril et al., 2003; Gadanho et al., 2005; Aguilera et al., 2006a; Gadanho and Sampaio, 2006). These organisms can persist at the extremes of known physiological tolerance of organisms to low pH and high heavy metal concentrations, yet their survival mechanisms are not well understood.

Río Tinto flows through the Iberian Pyritic Belt, one of the richest metal sulfide ore deposits on Earth (Bourter, 1996), and the extreme conditions of its water are the product of the metabolic activity of chemolithotrophic microorganisms, mostly iron- and sulfur-oxidizing bacteria, that can be found in high concentrations in its waters. The iron-oxidizing metabolism is responsible for the solubilization of sulfidic minerals (mainly FeS_2) and the correspondent high concentration of ferric iron, sulfate and protons in the water column (Fernández-Remolar et al., 2003; González-Toril et al., 2003). The result is a strong acidic solution of ferric iron which dissolves other cationic metals into solution.

Besides the extreme physicochemical characteristics of water, what makes Río Tinto a unique extreme environment is that eukaryotic organisms are the principal contributors of biomass in the river, over 65% of the total biomass is due to the remarkable degree of eukaryotic diversity found in its waters (Amaral et al., 2002; Liu and Fang, 2002). However, despite extensive efforts devoted to studying the biodiversity and geochemistry of this system (López-Archilla et al., 2001; Amaral et al., 2002; Fernández-Remolar et al., 2003; González-Toril et al., 2003; Aguilera et al., 2006a), little is known regarding their ecophysiology.

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Most of the eukaryotic microbial communities found in the river are distributed in extensive biofilms along the riverbed. The macroscopic shape and species composition of the biofilms vary greatly throughout the river. Some of them adopt filamentous morphologies in flowing water while others form thick colourful patches firmly attached to the mineral substrates. Microbial biofilms at Río Tinto are three-dimensional structures that show a spectrum of structurally heterogeneous forms determined by the dominating organisms (Aguilera et al., 2007). In addition, the distribution of these communities seems to be more influenced by the presence of heavy metals than by the pH (Aguilera et al., 2006b). The analysis of similarities among different sampling sites showed three areas that indicated a gradient of pH and heavy metals along the river. Although the development of biofilms in aquatic and terrestrial extreme environments has been documented (Ferris et al., 1989; Hughes and Poole, 1989; García-Meza et al., 2005), the mechanisms of adaptation are not well understood. Previous studies have shown that the extracellular matrix could be partly responsible for the increased tolerance of biofilms, particularly to heavy metals (Flemming, 1993; Barranguet et al., 2000).

The biofilm matrix is a dynamic environment that organizes microbial cells (Wingender et al., 1999; Sutherland, 2001). Their major components, besides water, are extracellular polymeric substances (EPS). Microorganisms in natural environments produce EPS, which in turn determine the structural and functional integrity of the biofilms and are considered the key component responsible for their physicochemical and biological properties (Christensen and Characklis, 1990). The EPS consists of a complex mixture of proteins, carbohydrates, acid polysaccharides, lipids, DNA and humic acid substances, although the wide range of environments in which biofilms are found makes it extremely difficult to generalize about their structure and physiological activities (Jenkinson and Lappin-Scott, 2001). Polysaccharides are the most abundant component, generally representing 40–95% of the EPS (Flemming and Wingender, 2001).

The adsorption of heavy metals by EPS is attributed to their large number of negatively charged functional groups such as carboxyl, phosphate and sulfate at neutral pH (Bitton and Friehofer, 1978; Kaplan et al., 1987). However, the heavy metal complexing properties of the EPS may be altered with pH, since pH determines metal solubility and also the organic ligand adsorption capacities (Stone, 1997). In general, at acidic pH most heavy metals are in free cationic form, and more available to microorganisms, whereas at higher pH they tend to precipitate as insoluble compounds (Förstener and Prosi, 1979; Rai et al., 1981). In the same regard, low pH modifies the ionic status of the different EPS functional groups, changing their electrochemical properties (Ferris et al., 1989). Thus, at low pH the availability of negatively charged sites such as carboxylates and phosphates is greatly reduced so fewer metal cations are absorbed. The reduction of surface charge density with decreasing pH levels also serves to weaken electrostatic free energy contributions to metallic ion adsorption (Jefree and Read, 1991).

We reported here a qualitative description of the extracellular matrix assembly and the evaluation of the importance of EPS of several eukaryotic biofilms collected from an extreme acidic environment in relation to their heavy metal binding capacity. For this purpose the biofilms were selected according to their dominant species composition and from locations along the river characterized by different physicochemical characteristics. Results may lead to a better understanding of the role of the extracellular matrix assembly and the importance of EPS in acidic environments in relation to their heavy metal adsorption capacities.

2. Materials and methods

2.1. Field sites and biofilm sampling

Six sites along Río Tinto were selected for *in situ* measurements, water sampling and biofilm collection (Fig. 1). The sampling sites were selected taking into account previous studies carried out in Río Tinto regarding their eukaryotic biodiversity and water physicochemical characteristics (Aguilera et al., 2006a,b, 2007). Samples were taken for all sites in May 2006 during the day (between 9.00 am and 11.00 am).

In situ measurements of water conductivity, temperature, redox potential and pH, were carried out as described previously (Fernández-Remolar et al., 2003). Water samples were filtered through 0.45 μm Millipore membranes. The total concentrations of nine recoverable metals were measured for each water sample (Zn, Cu, Fe, Co, Ni, As, Cd, Cr and Pb) using X-ray fluorescence reflection (TXRF) and inductively coupled plasma-mass spectrometry (ICP-MS).

Twelve eukaryotic biofilms were taken from the riverbed surface (maximum water depth <5 cm) using a sterile plastic spatula and then placed in 1.5 mL cryotubes. The samples were immediately frozen in dry ice and kept in the laboratory at −20 °C until the experiments were carried out. Besides the samples taken for the EPS analysis, a subsample was also taken for the microscopy identification of the eukaryotic species. Identification of algae and heterotrophic protists was carried out by direct microscopic observation down to the lowest possible taxonomic level using different phenotypic features based on previous studies of the eukaryotic

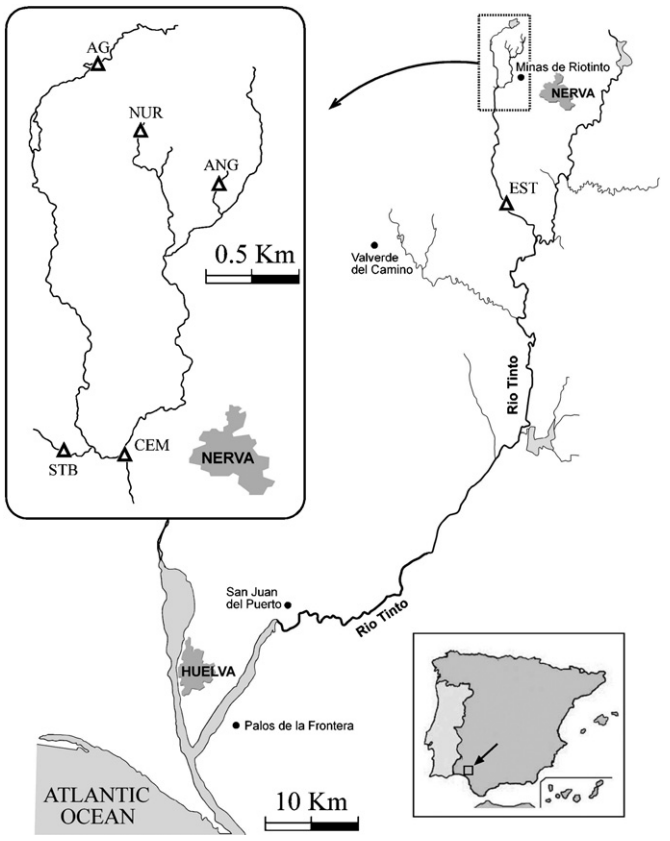

Fig. 1. Schematic map of the Río Tinto from the source near the town of Nerva to the ocean near the town of Huelva. The relative location of each sampling site is shown. Inset in lower right shows general location of the river in Spain, and at upper left is a detailed map of the headwaters.

communities in this river (López-Archilla et al., 2001; Amaral et al., 2002; Aguilera et al., 2006a,b).

2.2. Extraction of colloidal and capsular extracellular polymeric substances (EPS)

Two EPS fractions, called colloidal and capsular, were extracted from each biofilm (Staats et al., 1999). The colloidal fraction includes carbohydrates and proteins that are loosely bound to microorganisms. The capsular fraction contains tightly bound compounds that require a lengthier extraction protocol using EDTA (Hirst and Jordan, 2003).

Colloidal fractions were extracted using modified protocols previously described (Orvain et al., 2003). Briefly, 5 mL of deionized water were added to 500 mg of freeze-dried biofilm for 1 h at 30 °C, under continuous agitation. Samples were then centrifuged (10 min, 2000 × *g*) and 1 mL of the resulting supernatant was used for analysis of the colloidal fraction.

The capsular fraction was then extracted by adding 5 mL of 0.01 mol L^{-1} EDTA to the pellet, mixed thoroughly, incubated at room temperature for 3 h and centrifuged at 27,000 × *g* for 20 min. (Mazor et al., 1996; Staats et al., 1999). The supernatant was used for analysis of the capsular fraction. The total amount of extracted EPS fractions was measured by weight after lyophilization (Liu and Fang, 2002).

2.3. Biochemical characterization of EPS

The biochemical compositions of the different EPS fractions were determined for each biofilm by the following colorimetric methods: Lowry modified by Frolund (Lowry et al., 1951; Frolund et al., 1995) for protein content, bovine albumin serum as standard; Frolund method (Frolund et al., 1995) for humic acid content; Dubois method (Dubois et al., 1956) for total carbohydrate content, glucose as standard; Burton method (Burton, 1956) for nucleic acid content, DNA of salmon semen as standard.

The metal content present in the different fractions of the EPS was determined as follows (Guibaud et al., 2003): 5 mL of 0.5% HNO_3 and 0.5 mL of solution of EPS fraction were mixed and agitated overnight. Solutions were then centrifuged (20 min, 4300 × *g*) and filtered through a 0.2 μm Millipore membrane. The filtrate was recovered and the heavy metals analyzed by using X-ray fluorescence reflection (TXRF) and inductively coupled plasma-mass spectrometry (ICP-MS).

The results obtained from the biochemical analysis of the different EPS fractions were statistically compared by ANOVA using the Statistica V.6.0 program.

2.4. Low temperature scanning electron microscopy (LTSEM)

Biofilm samples were collected 24 h prior to observation and kept in darkness and at 4 °C. The biofilm structures were fragmented (1–2 mm) with a spatula and then mounted and mechanically fixed onto a specimen holder at room temperature. The frozen samples were immediately plunged into slush nitrogen and directly transferred into the cryo-chamber, pre-cooled to −180 °C, via an airlock transfer device. The frozen sample was then fractured with a cooled blade and sputter coated with gold for 135 s at 10 mA. Then the sample was moved to the SEM-chamber pre-cooled to −160 °C where it was observed at an acceleration voltage of 15–20 kV. The instrument used was a CT 1500 Cryotrans system (Oxford Instruments) mounted on a Zeiss 960 SEM.

3. Results

3.1. Sampling site physicochemical conditions and biofilm description

Physical and chemical water data collected during this study are given in Table 1. Sampling points ANG and AG were the locations with the lowest and highest pH and conductivity values, respectively. The highest values for total heavy metal amounts were found in ANG and CEM. Concentrations of most ions decreased downstream, therefore the last sampling point EST showed the lowest concentration of total heavy metals. In the same regard, metal proportions varied between sampling sites: AG showed the highest Cd and Pb concentrations; ANG had the highest Fe, Co and Cr concentration values; CEM was rich in As, STB showed the highest Zn Cu and As concentrations, and EST had the highest amount of Ni.

Although prokaryotic organisms were present in the biofilms collected, eukaryotic species were the main contributors to the biofilm biomass. These eukaryotic biofilms are easily distinguished from the prokaryotic ones (usually called streamers) by their colour and texture. Table 2 shows the eukaryotic species composition of each biofilm analyzed. The biofilms were usually dominated by one or two phototrophic species easily differentiated by their colour. Thus, in order to evaluate possible changes in the extracellular matrix composition among different biofilms, the samples were selected taking into account their dominant species as well as the physicochemical water characteristics of their sampling sites. Three biofilms mainly formed by *Euglena mutabilis* were sampled from AG, NUR and STB. Four diatom biofilms were collected from AG, ANG, NUR and STB. The dominant species from two of the biofilms was the filamentous algae *Zygnemosis* spp., collected from NUR and CEM. Two biofilms dominated by *Chlorella* sp. and *Cyanidium* sp. were collected from ANG and CEM, respectively. Although Chlorellas represent 95% of the total cell counts in the biofilm, 25% of the composition of *Cyanidium* biofilm was shown to be made up of diatoms. Finally, the biofilm from the sampling point EST was dominated by fungi from the genera *Hobsonia*, although ca. 20% of the total cell number corresponded to Euglenas and Chlorellas.

3.2. EPS contents in different biofilms

Table 3 compares the amounts of EPS fractions extracted for each biofilm. Results show that the total amount of EPS ranged from a minimum of 130 (AG.Di) to a maximum of 439 mg g^{-1} dry weight (ANG.Di), representing 15% (i.e. AG.Di, NUR.Eu or CEM.Cy) to 40% (i.e. ANG.Di, NUR.Zy or EST.Fu) of the total biofilm dry weight. On the other hand, the cellular fraction constitutes the main part of the biofilm, varying from ca. 250 (i.e. AG.Eu or CEM.Cy) to 750 mg g^{-1} dry weight (i.e. STB.Di or NUR.Zy) corresponding to between 25% to over 75% of the dry weight of the biofilms.

Loosely bound colloidal EPS ranged ca. one order of magnitude among the different sites, from 42 mg g^{-1} dry weight in a *Euglena* dominated biofilm (NUR.Eug) to 313 mg g^{-1} dry weight in diatom dominated biofilms (ANG.Dia), corresponding to between ca. 10% to 30% of the total biofilm dry weight. More tightly bound capsular EPS ranged 6-fold, from ca. 50 mg g^{-1} dry weight in a diatom dominated biofilm (AG.Dia) to 318 mg g^{-1} dry weight in the fungi dominated biofilm (EST.Fu), reaching 5% to 30% of the total biofilm dry weight. Seven of the 12 biofilms showed higher amounts of capsular than colloidal EPS ($p < 0.05$), in a proportion ranging from 1.2 to 3 times higher concentration. *Euglena* dominated biofilms from different locations always showed higher amounts of capsular than colloidal fraction whereas diatom biofilms showed a higher amount of colloidal than capsular EPS, except in the biofilm from STB.Dia.

Table 1
Water physicochemical parameters from the different sampling sites

Location	pH	T[ª]	Cond.	Redox.	Zn	Cu	Fe	Co	Ni	As	Cd	Cr	Pb
AG	2.5 ± 0.2	20 ± 1.1	9.10 ± 1.6	393 ± 32	113.1 ± 5.7	28.51 ± 4.7	2294.9 ± 112	4.20 ± 0.6	0.75 ± 0.1	3.60 ± 1.0	0.5 ± 0.1	0.06 ± 0.01	0.50 ± 0.01
ANG	1.4 ± 0.2	27 ± 2.1	30.1 ± 4.0	457 ± 19	82.51 ± 8.1	83.22 ± 2.2	8967.9 ± 223	16.6 ± 4.5	1.13 ± 0.5	12.3 ± 2.8	0.4 ± 0.1	0.31 ± 0.08	0.02 ± 0.002
NUR	2.1 ± 0.3	20 ± 1.8	17.4 ± 3.6	522 ± 25	67.93 ± 9.1	39.70 ± 7.1	3765.4 ± 91	4.96 ± 1.1	0.73 ± 0.4	4.59 ± 1.0	0.2 ± 0.1	0.21 ± 0.03	0.02 ± 0.001
CEM	2.2 ± 0.1	17 ± 1.3	9.40 ± 1.3	455 ± 30	275.3 ± 9.9	441.2 ± 28	4763.2 ± 101	3.20 ± 0.7	1.11 ± 0.6	15.5 ± 3.6	1.2 ± 0.2	0.18 ± 0.09	0.06 ± 0.01
STB	1.9 ± 0.1	14 ± 2.4	18.3 ± 2.4	358 ± 14	356.6 ± 8.1	544.9 ± 15	2675.3 ± 10.2	3.31 ± 0.4	1.12 ± 0.4	15.8 ± 2.8	1.1 ± 0.3	0.19 ± 0.10	0.05 ± 0.02
EST	2.0 ± 0.3	21 ± 2.1	9.30 ± 1.1	465 ± 19	127.1 ± 22	187.3 ± 18	1414.1 ± 108	3.86 ± 1.1	1.43 ± 0.5	2.45 ± 0.8	0.8 ± 0.1	0.10 ± 0.02	0.02 ± 0.01

T[ª]: temperature in °C; Cond.: conductivity in mS cm^{-1}; Redox.: redox potential in mV; metals in ppm.

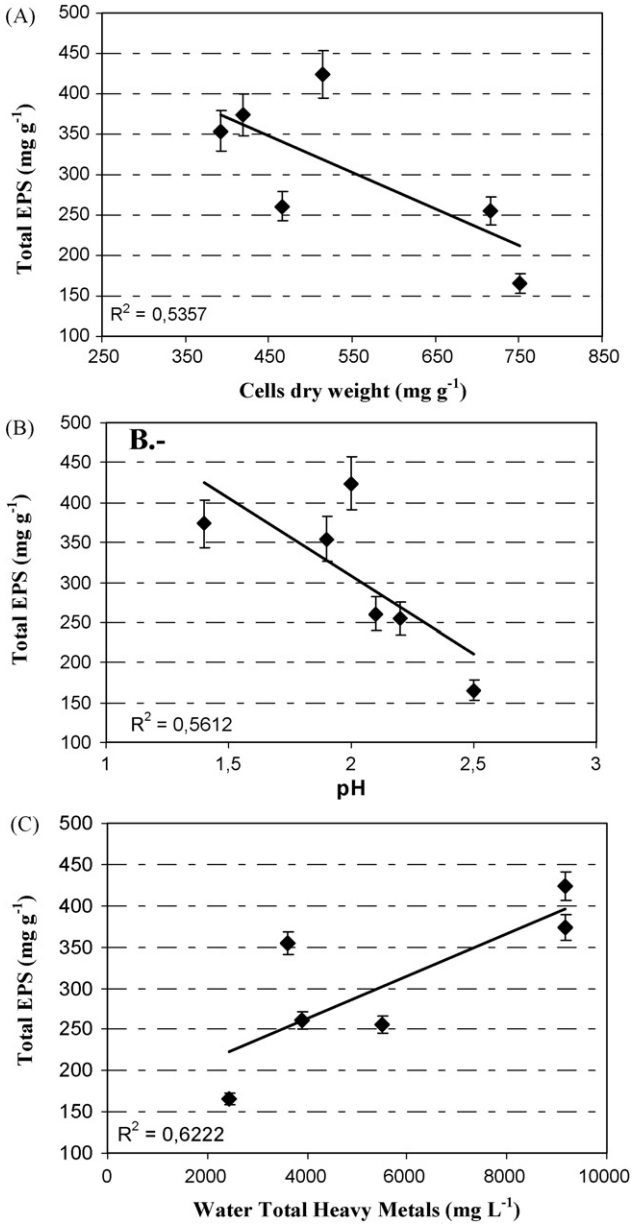

Fig. 2. Relationships between the total EPS extracted and (A) the cellular fraction, (B) the total concentrations of heavy metals in the water, (C) the pH. Each point represents the mean value of the total EPS (colloidal and capsular) for all the biofilms isolated at each sampling point.

Significant differences ($p < 0.05$) were found among the total EPS extracted from biofilms dominated by the same species at different sampling points (Table 3). Thus, total amounts of EPS mainly formed by *Euglenas* were statistically significantly higher in the biofilm isolated from STB than the ones collected at AG or NUR ($p = 0.044$ and $p = 0.047$, respectively). Similar results were obtained in the biofilms formed by diatoms These biofilms showed significant higher amounts of EPS at STB and ANG sampling sites than the ones collected at NUR, or AG.

In the same manner, statistically significant differences were found among the different biofilms collected from the same sampling location (Table 3). For example, at NUR sampling site, the biofilm mainly composed by *Zygnemopsis* spp. (NUR.Zy) almost doubled the amount of EPS produced by the diatoms (NUR.Di, $p = 0.044$) or *Euglenas* (NUR.Eu, $p = 0.045$).

Table 2
The percentage contribution of eukaryotic taxa to the total cell number in the different biofilms at each sampling site as determined from microscope counts

Location	Biofilm	Diatoms (%)	Euglenoids (%)	Filamen (%)	Chlorella (%)	Cyanidium (%)	Others (%)
AG	AG.Eu	15	**83**	–	–	–	2
	AG.Di	**93**	5	–	–	–	2
ANG	ANG.Ch	4	–	–	**95**	–	1
	ANG.Di	**91**	–	–	8	–	1
NUR	NUR.Zy	–	5	**89**	3	–	3
	NUR.Di	**96**	–	1	1	–	2
	NUR.Eu	–	**98**	–	–	–	2
CEM	CEM.Cy	25	–	–	–	**72**	3
	CEM.Zy	11	6	**81**	–	–	2
STB	STB.Eu	2	**94**	–	1	–	3
	STB.Di	**96**	1	–	2	–	1
EST	EST.Fu	–	10	–	12	–	**78**

Diatoms: *Pinnularia* sp.; Euglenoids: *Euglena mutabilis*; Filamen: Filamentous algae (*Zygnemopsis* sp.); Chlorella: *Chlorella* sp.; Cyanidium: *Cyanidium* sp.; Others: Amoebas (*Vahlkampfia* sp.), flagellates (*Bodo* sp., *Cercomonas* sp.). The biofilm EST-Fu was mainly composed of a fungi belonging to the genera *Hobsonia*. The dominant species in each biofilm are in bold.

Total amounts of EPS decreased when the total number of cells and pH increased ($r^2 = 0.53$ and $r^2 = 0.56$, respectively; Fig. 2A and B). However the correlation was higher and positive when the amount of EPS was compared with the total concentration of heavy metals in the water ($r^2 = 0.62$, Fig. 2C, $p = 0.048$).

3.3. EPS characterization

Results in Fig. 3 show that EPS in all biofilms were composed of carbohydrates, heavy metals and humic acid, plus small quantities of proteins and DNA. Carbohydrate concentrations (colloidal + capsular fraction) varies from ca. 30 (CEM.Cy) to 300 mg g^{-1} dry weight (CEM.Zy), which correspond from the 15% to more than 85% of the total biofilm dry weight. In general, the colloidal EPS fraction had a higher carbohydrate concentration than the capsular fraction ($p < 0.05$), in a proportion ranging from 1.1 to 3. Only three exceptions were found, CEM.Zy, NUR.Eu and EST.Fu.

Heavy metals were the second main constituents of the EPS in the acidic biofilms. Their total concentrations ranged from ca. 6 to 36 mg g^{-1} biofilm dry weight, reaching up to ca. 10% of the total EPS composition. As with the carbohydrates, significant differences ($p < 0.05$) were found among the heavy metal concentrations in both EPS fractions. In most cases, colloidal EPS fractions showed higher values of heavy metals than the capsular fraction, with a mean proportion 4 times higher. Only two exceptions were found, *Euglena* and diatom dominated biofilms from AG and the biofilm from EST. In addition, only As and Pb were found in higher concentrations in the capsular fraction of the EPS for almost all biofilms.

Humic acid was the third most abundant component of the EPS (Fig. 3). Their concentrations ranged from 0.2 to ca. 11 mg g^{-1} dry weight (EST.Fu) reaching up to almost 4% of the total EPS amount. As usual, the colloidal fraction showed significantly higher levels ($p < 0.05$) than the capsular EPS, except for five biofilms; *Cyanidium* and *Zygnemosis* biofilms collected at CEM, STB.Di, NUR.Eu and EST.Fu. Finally, proteins and DNA showed the lowest values in the EPS composition. Protein concentrations ranged from 0.9 to ca. 7 mg g^{-1} dry biofilm weight, constituting up to ca. 2% of the total EPS. However, in this case, most of the biofilms showed higher amounts of proteins in the capsular than in the colloidal fraction. DNA concentrations were always lower than 0.8 mg g^{-1} dry weight, which means less than 0.5% of the total EPS. In this case, no statistically significant differences were found between capsular and colloidal fraction ($p > 0.05$) except for the biofilm EST.Fu.

We have also analyzed the possible relation between the EPS heavy metal content and the heavy metal composition of the water from which each biofilm was collected (Fig. 4). In general, the concentration of heavy metals extracted from the EPS fraction from biofilms collected at ANG and NUR resembled greatly the heavy metal composition of the water analyzed in both places. No statistically significant differences ($p > 0.05$) were found among them except for the Pb in the NUR.Eu biofilm, in which the amount was higher than in the water ($p < 0.05$). Similar results were found at AG and CEM sampling sites, no statistically significant differences were found ($p > 0.05$) among the heavy metals, except for the Pb that showed higher levels in the extracted EPS than in the water ($p < 0.05$).

On the other hand, EPS of the biofilms collected from STB showed significantly lower amounts of Cu and Zn than expected. In the water at STB, 15% of the heavy metal content was due to Cu and 10% corresponded to Zn, while the percentages of these two metals were ca. 7% and 5%, respectively, in the extracted EPS. In addition, EPS showed ca. 86% Fe in their metal composition, although the Fe present in the water reached only ca. 75% of the total heavy metals. Related results were found at EST sampling location. The EPS at this point accumulated ca. 10% more Fe as well as half of the amount of Cu and Zn than expected from the water analysis.

3.4. LTSEM analysis

Fig. 5 shows photomicrographs of different biofilms and the microstructure of the extracellular matrix as seen with low temperature scanning electron microscopy (LTSEM). The first

Table 3
Amounts of extracted EPS and cellular fractions (mg g^{-1} DW)

Biofilm	Colloidal EPS	Capsular EPS	Cell fraction	Unknown fraction
AG.Eu	75 ± 4.3	125 ± 9.8	725 ± 84	75.7 ± 14
AG.Di	78 ± 2.1	52.2 ± 2.9	775 ± 43	93.7 ± 11
ANG.Ch	247 ± 14	60.6 ± 2.3	364 ± 13	327 ± 32
ANG.Di	313 ± 21	126 ± 9.0	473 ± 21	86.7 ± 12
NUR.Zy	181 ± 17	215 ± 19	272 ± 18	329 ± 26
NUR.Di	120 ± 11	88.1 ± 7	358 ± 11	432 ± 38
NUR.Eu	42.3 ± 5	132 ± 10	769 ± 35	55.9 ± 9
CEM.Cy	71.4 ± 5	119 ± 11	785 ± 24	23.8 ± 7
CEM.Zy	171 ± 10	148 ± 13	645 ± 19	34.3 ± 9
STB.Eu	153 ± 18	161 ± 10	538 ± 37	146 ± 10
STB.Di	168 ± 11	224 ± 19	247 ± 13	359 ± 29
EST.Fu	106 ± 17	318 ± 34	515 ± 22	60.6 ± 14

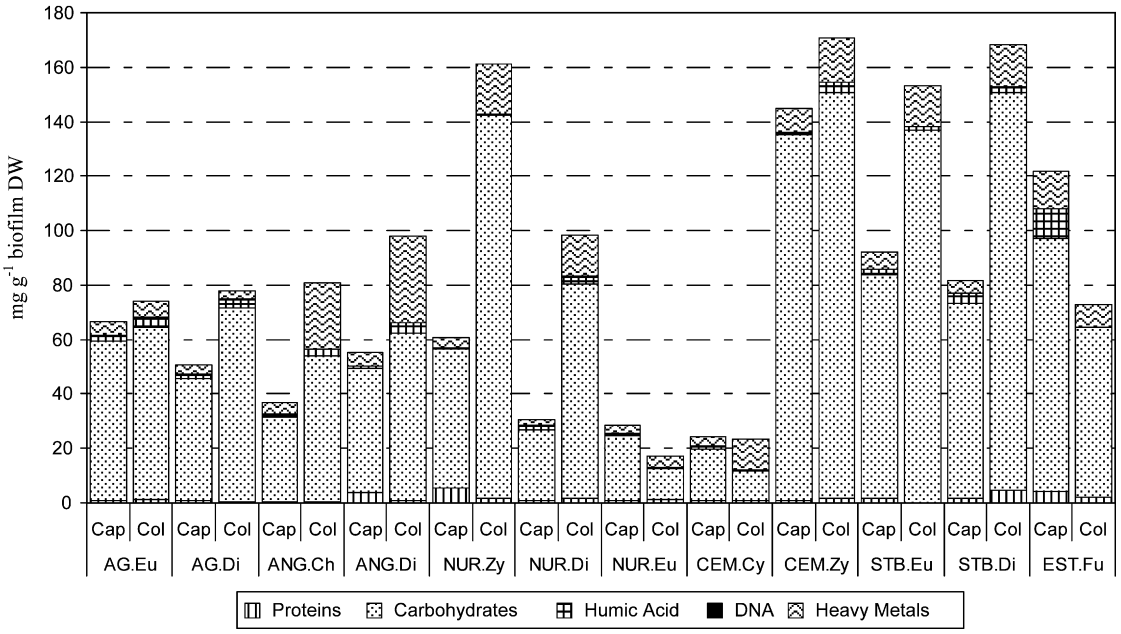

Fig. 3. Percentages of the different EPS components in the capsular (Cap) and colloidal (Col) fractions.

photomicrograph (Fig. 5A) shows the ultrastructure of a *Chlorella* biofilm (ANG.Ch) and the progressive saturation of colloidal EPS alveolar structure with iron minerals from the surrounding water. Arrows point to the areas where the fibrillated texture of the EPS is becoming impregnated with iron minerals. The onset of mineral precipitation is initiated within the EPS matrix. Fig. 5B is a higher-magnification picture showing progressive deposition of minerals on the capsular fraction of the EPS of the cell walls and the enlargement of the deposits growing from the colloidal fibrillated texture of the matrix. The small deposits grow larger forming smooth plates between the cells. Fig. 5C shows a diatom biofilm from ANG.Di embedded in EPS matrix with no significant mineral precipitation. The picture is taken in an active part of the biofilm not in contact with the substrate. Only in some areas is it possible to locate deposits of minerals between the cells (arrows Fig. 5C). A photomicrograph of a *Euglena* biofilm from AG.Eu (Fig. 5D) shows both types of situation, areas with mineral precipitation indicating the presence of EPS with saturated binding capacity (arrows) and areas were the fibrillated EPS are embedded in frozen water (asterisks).

The EPS structure of the three eukaryotic communities differed markedly, although two of the biofilms (ANG.Ch and ANG.Di) were collected from the same sampling location. Images of *Chlorella* biofilm revealed the presence of mineral depositions on the cell wall of the cells surrounded by a fibrillated texture identified as an exopolymer matrix on the basis of its physical aspect. These structures were not observed on the other diatom biofilm isolated from the sample location, where the matrix was more continuous. These facts could be due to the different EPS composition of the biofilms. Although no significant differences were found in the amounts of colloidal EPS, the concentration of capsular EPS in the *Chlorella* was

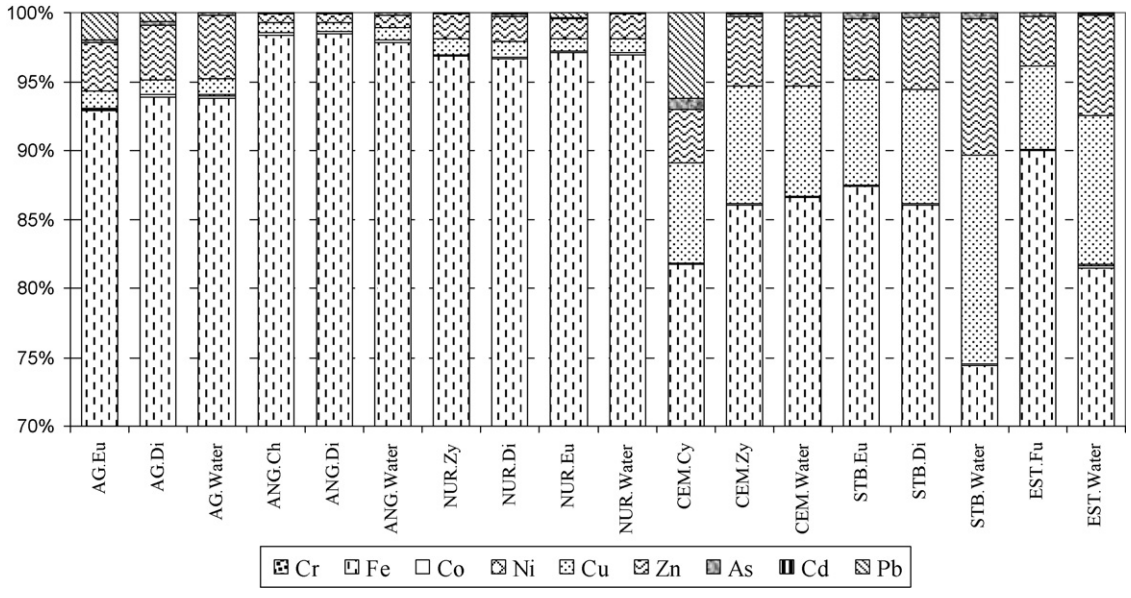

Fig. 4. Percentages of the heavy metal composition of the EPS extracted and the heavy metal distribution in the water samples.

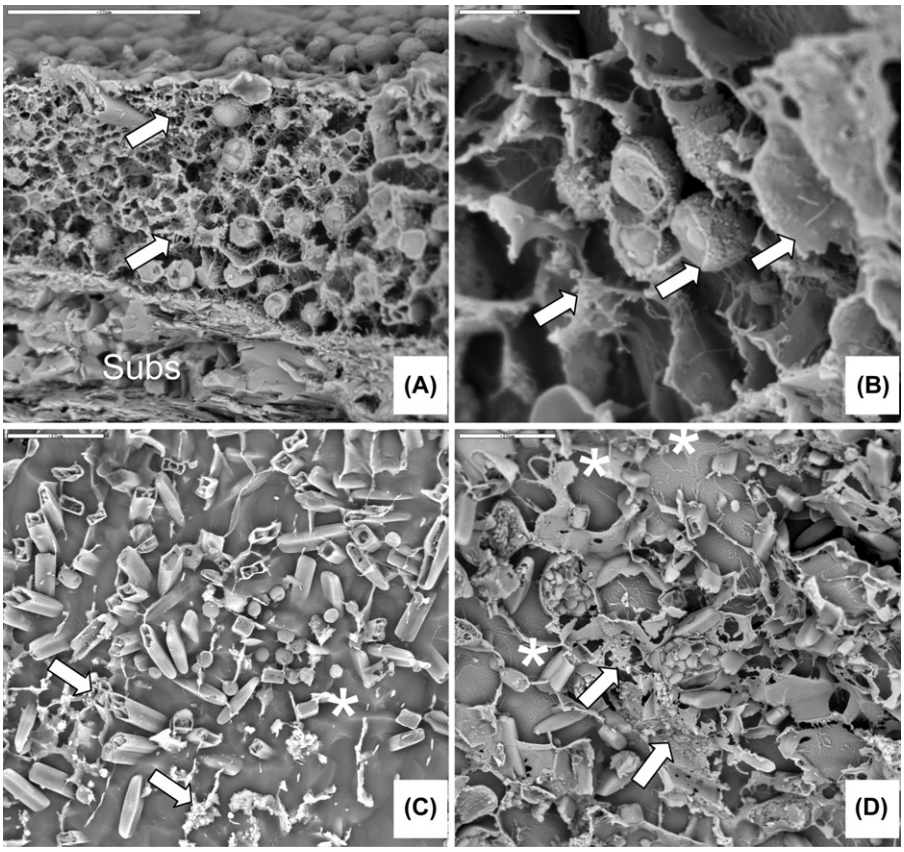

Fig. 5. (A) LTSEM image of the ANG.Ch biofilm in contact with the substrate (Subs). *Chlorella* cells are surrounded by EPS encrusted with mineral precipitates. Arrows point to the fibrillated EPS material. Bar = 20 μm. (B) Detailed image of the encrustation of the cell walls of the *Chlorella* cells. Arrows point to the progressive encrustation of the cell walls, fibrillated material and dense mineral deposits between the cells. Bar = 5 μm. (C) Detailed image of the ANG.Di diatom biofilm with very little encrustation around it and in the EPS matrix. Arrows point to the rare mineral deposits between the frozen water and the EPS matrix (asterisk). Bar = 20 μm. (D) Detail of the AG.Eu *Euglena* biofilm with encrustation of the EPS and very little on the surface of the cells. Bar = 20 μm. Arrows point to the mineralized EPS zone. Most of the EPS are not mineralized and appeared embedded in the frozen water (asterisks).

much lower that in the diatom one. In the same regard, carbohydrate and protein content were also significantly higher in the diatom EPS that in the chlorella biofilms.

4. Discussion

The purpose of this study was to investigate the EPS composition of different eukaryotic biofilms collected from an extreme acidic environment, the Río Tinto (SW, Spain), as well as their ability to bind heavy metals. Although there are numerous reports documenting these topics in several aquatic and terrestrial environments (Hughes and Poole, 1989), to our knowledge, this is the first study carried out on natural biofilms from an extreme environment characterized by its low pH and high concentration of heavy metals. In addition, since most of these previous studies have focussed exclusively on prokaryotes (Ferris et al., 2004), references to the contribution of biofilm-forming eukaryotic microorganisms to increased solid phase partitioning of dissolved metals are even more scarce (Brake et al., 2004).

4.1. EPS contents and composition from acidic biofilms

Our results shows high concentrations of EPS in all samples examined, always reaching mg g^{-1} amounts of biofilm dry weight. Although it is difficult to compare the results obtained in this study to the literature data due to the variety of environments and microorganisms studied and the lack of reports carried out in acidic habitats, most of the data reported in neutral microphytobenthic environments showed magnitudes of μg g^{-1} (Hirst and Jordan, 2003; Orvain et al., 2003). At the same time, high production of EPS has been described for other extreme environments, such as arctic sea ice or deep-sea hydrothermal vents (Krembs et al., 2002; Mancuso-Nichols et al., 2005).

We have also found that total amounts of EPS showed positive correlation with the total concentration of heavy metals present in the water (Fig. 2C). These results are comparable to those measured in other environments (Hill and Larsen, 2005) and with the observations made by García-Meza et al. (2005) using photosynthetic biofilms from mine tailings, habitats in which the concentration of heavy metals is also high. Using mesocosms formed by biofilms mainly composed of *Chlorococcum* sp. (Chlorophyta) and *Phormidium* sp. (Cyanophyta), García-Meza et al. (2005) demonstrated that metal exposure increased EPS production, although their experiments were carried out at neutral pH 7. In our case, the negative correlation between the EPS and the pH is also significant (Fig. 2). At higher pH the total mount of EPS decreased. These results are in agreement with the well-documented fact that, at low pH level, the availability of negatively charged sited such as carboxylates and phosphates is greatly reduced (Ferris et al., 1989). Thus, fewer metal cations could be adsorbed and higher EPS amounts are necessary to chelate the higher amount of metals in localities with lower pH.

From our results, we cannot conclude which EPS fraction, colloidal or capsular, is more abundant in the eukaryotic acidic biofilms. Seven biofilms showed higher amounts of capsular EPS while the remaining biofilms showed higher amounts

of colloidal fractions. Colloidal fraction is the supernatant fraction obtained during extraction and referred to as the soluble, labile or liquid phase (Underwood et al., 1995). Capsular fraction is the sediment fraction and is referred to as the bound or solid phase. Although these two general fractions are operational classifications that depend on aqueous extraction and centrifugation, discrete fractions or physical states of EPS do not exist in nature (Decho, 1994). Instead, a continuum exists of tertiary states ranging from highly condensed gels, loosely conformed slimes, to colloidal solutions. This continuous distribution of both fractions makes their differential extraction and correct quantification difficult.

Results of this study also found that carbohydrates were the main constituents of EPS, as reported for most environments and cultures (Sutherland, 1999, 2001), reaching between 15% and 90% of the total EPS amounts (Fig. 3). However, our results for protein contents differ strikingly from those reported for a wide range of algal species reaching up to 60%, especially in nutrient-rich environments (Decho and López, 1993; Flemming et al., 1999). In our case, the protein content of the EPS never exceeded 3% of the total dry weight. Nevertheless, EPS extracted from natural environments are generally believed to contain few or no proteins (Hirst and Jordan, 2003), and previous studies carried out in prokaryotes from other extreme environments had shown similar low amounts of proteins in their EPS (Mancuso-Nichols et al., 2004; Raguenes et al., 2003).

Humic acids were also found at lower concentrations than reported in previous studies from other environments (Liu and Fang, 2002). Humic substances are thought to be formed during the microbial degradation of organic matter and they have been related to nutrient depletion and heavy metal complexation (Suteerapataranon et al., 2006). Our results are supported by previous studies on acidic environments in which the total concentration of humic substances in the water is also scarce probably influenced by the low pH (Stevenson, 1994).

In general, concentrations of carbohydrates, humic acid and heavy metals were significantly higher in the colloidal fraction (Fig. 3). Little is known about the ecological significance of either fraction, although it is generally considered that colloidal fractions are related to the mechanical stability of the sediment and locomotion (Winder et al., 1999), and capsular EPS may aid in the adsorption of essential nutrients and trace metals, protecting against desiccation and toxic metals (Decho, 1990). Our observations regarding the high amounts of heavy metals in the colloidal fraction strongly support their significant protective contribution against their toxic effect.

On the contrary, proteins and DNA showed higher concentrations in the capsular fraction for most of the biofilms analyzed. Since proteins and DNA results could be interpreted as contamination of intracellular components, we measured the activity of glucose-6-phosphate-dehydrogenase (Platt et al., 1985). As this enzyme is strictly intracellular, we could use it as a marker for contamination from the cellular fraction. The results were negative in all the samples indicating that the DNA and proteins were extracted from the extracellular fraction (data not shown). These results are in agreement with Flemming and Wingender's model (2001) of a matrix architecture which arranges the proteins in proximity to the cells preventing the washing out of exozymes which would be lost easily under conditions of water flow. The presence of DNA in the extracellular space of microbial communities has been described previously (Corinaldesi et al., 2005; Steinberger et al., 2002; Steinberger and Holden, 2005). In some cases the presence of DNA was involved in the flocculating ability of photosynthetic marine bacterium (Nishimura et al., 2003) and in some cases was necessary for bacterial biofilm formation (Whitchurch et al., 2002). For the moment the DNA has not yet been analyzed to know whether it is eukaryotic or bacterial in origin but the presence of bacterial communities inside the studied eukaryotic biofilm have been described in previous works (Aguilera et al., 2007). The role of extracellular DNA in the microbial ecology of diverse environments and the ecological importance in biofilms of mixed of eukaryotic and bacterial populations remains unknown.

4.2. Heavy metal composition

In addition to carbohydrates, the EPS from the acidic biofilms also contained substantial amounts of heavy metals, reaching up to 10% of the total EPS dry weight (Fig. 3). Although this fact has been well documented in neutral environments, to our knowledge this is the first report addressing this statement in extreme acidic habitats. The capability of these EPS in binding significant amounts of heavy metals is particularly relevant since, at low pH levels, the availability of negatively charged sited is drastically reduced, decreasing the number of metal cations that can be adsorbed (Ferris et al., 1989).

Previous studies have shown that biosorption of heavy metals as a result of interaction with anionic ligands in biopolymers (Lion et al., 1987) offers many advantages to the microbial community (Stone, 1997). These processes include highly efficient metal removal and the low operational cost compared to other conventional physico-chemical metal removal technologies (Eccles, 1999). Thus, the role of EPS in the extraction of potentially toxic metals is even more important in acidic environments, usually characterized by their high levels of heavy metals as well as their high bioavailability. Metal toxicity depends on bioavailability, and this accessibility is mainly determined by water physicochemical factors, the presence of chelators and the concentration of free ions (Sunda and Huntsman, 1998). pH is an important physicochemical property of water that determines metal bioavailability. In general, at acidic pH most heavy metals are in free cationic form, and more available to microorganisms, whereas their bioavailability decreases in alkaline conditions, thereby exerting less toxicity (Ernst, 1998).

Since the EPS of each biofilm is made up of different components that may interact more strongly with some metals than with others, we have also investigated the distribution of the metals in the extracted EPS. In general, metal distribution in EPS correlates well with the metal composition of the water from which they were isolated, except for Pb. This metal is extensively accumulated in two of the three *Euglena* biofilms and in the EPS extracted from the *Cyanidium* biofilm. In lake biofilm systems adsorption studies showed that Pb binding to metal oxides was greater than that estimated for organic phases (Nelson et al., 1999). Thus it is possible that these localities had a higher presence of metallic oxides in their extracellular matrix assembly increasing the adsorption of Pb. Fe and Mn oxides could have an important effect on the adsorption of Pb from natural environments (Dong et al., 2000). The precipitation of iron oxyhydroxides and hydroxysulfates is a common process in acid mine waters and they likely contribute to increase the solid phase partitioning of dissolved metals in the Tinto river system (Ferris et al., 2004). Further studies describing the speciation of the metals present in the eukaryotic extracellular assembly matrix need to be done to explain these results.

In addition, the biofilms isolated from CEM and STB sampling locations, were able to accumulate only half the Cu and Zn found in the water. These two sampling stations have higher Cu and Zn concentrations in the water and yet the EPS extracted from their biofilms adsorpted less Cu and Zn than did biofilms from other areas less saturated with these metals. This suggests a competitive threshold in the binding of metallic ions, also reported by García-Meza et al. (2005) for these two metals in biofilm mesocosms studies. These results support the idea suggested by Aguilera et al. (2006b) that, based on PCA analysis, these two particular metals play a

substantial role in controlling the epiphytic eukaryotic diversity and distribution in the river and is the reason why not all the biofilms are equally distributed along all the localities studied.

4.3. LTSEM structure analysis

Although the ultrastructure of some of these biofilms has been already described using scanning electron microscopy in backscattered electron mode (Aguilera et al., 2007), low-temperature scanning electron microscopy is the technique chosen for this study in order to preserve the structure of hydrated samples (Jefree and Read, 1991). LTSEM visualisation of the biofilms retains structural water and therefore preserves the natural fabric of the matrix, including the EPS polymers of interest to the present study (Defarge et al., 1996; Défarge, 1997). The EPS structure of the three eukaryotic communities studied differed markedly, although two of the biofilms (ANG.Ch and ANG.Di) were isolated from the same sampling location (Fig. 5). Images of *Chlorella* biofilm (Fig. 5A and B) revealed the presence of mineral depositions on the cell wall of the cells surrounded by a fibrillated texture identified as an exopolymer matrix on the basis of its physical aspect. These structures were not observed on the other diatom biofilm isolated from the same sampling site, where the matrix was more continuous (Fig. 5C). These facts could be due to the different EPS composition of the biofilms. Although no significant differences were found in the amounts of colloidal EPS, the concentration of capsular EPS in the *Chlorella* was much lower than in the diatom one. In the same regard, carbohydrate and protein content were also significantly higher in the diatom EPS that in the chlorellas. These observations strongly suggest that EPS composition has a significant impact on the assemblage and architecture of biofilms in extreme acidic environments. The role of EPS in mineral precipitation is ambiguous because of its ability to inhibit or promote mineral nucleation in specific situations. Initially the EPS matrix acts as a cation absorbent inhibiting mineral precipitation by removing cations from solution (Dupraz et al., 2004). Precipitation is induced when the metal availability exceeds the binding capacity (Fortin et al., 1997; Little et al., 1997).

5. Conclusion

To date little is known regarding the role of photosynthetic eukaryotes in shaping the varied ecosystems that occur in the Río Tinto acidic environment and less about whether photosynthetic biofilm communities have microenvironmental conditions that increase the survival of their members, or the interaction between different organisms enhances colonization of others.

In general, we have found significant differences among the total EPS extracted from biofilms dominated by the same species at different sampling points, and among different biofilms collected from the same sampling location, pointing out the importance of the microorganism assemblage within the biofilms in the EPS production. In the same regard, total amounts of EPS decreased when total cell numbers and pH increased, and the cells produces more EPS when the heavy metal composition of the water is higher. Besides, after carbohydrates, heavy metals were the second main constituents of the EPS analyzed, highlighting their ability of binding metals even at low pH. Since the extracellular matrix is a vital and complex component of all biofilms, it has to play an even more important role in the development of eukaryotic microbial communities in extreme environments by providing a suitable architectural structure, mechanical stability and protection against external conditions, and be able to selectively accumulate metals such as Pb from the surrounding water.

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