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METHODS

Extraction of extracellular polymeric substances from extreme acidic microbial biofilms

Angeles Aguilera · Virginia Souza-Egipsy · Patxi San Martín-Úriz · Ricardo Amils

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Abstract The efficiency of five extraction methods for extracellular polymeric substances (EPS) was compared on three benthic eukaryotic biofilms isolated from an extreme acidic river, Río Tinto (SW, Spain). Three chemical methods (MilliQ water, NaCl, and ethylenediamine tetra-acetic acid [EDTA]) and two physical methods (Dowex 50.8 and Crown Ether cation exchange resins) were tested. The quality and quantity of the EPS extracted from acidic biofilms varied according to which EPS extraction protocol was used. Higher amounts were obtained when NaCl and Crown Ether resins were used as extractant agents, followed by EDTA, Dowex, and MilliQ. EPS amounts varied from approximately 155 to 478 mg g^{-1} of dry weight depending on the extraction method and biofilm analyzed. EPS were primarily composed of carbohydrate, heavy metals, and humic acid, plus small quantities of proteins and DNA. Neutral hexose concentrations corresponded to more than 90% of the total EPS dry weight. The proportions of each metals in the EPS extracted with EDTA are similar to the proportions present in the water from each locality where the biofilms were collected except for Al, Cu, Zn, and Pb. In this study, the extracellular matrix heavy metal sorption efficiencies of five methods for extracting EPS from eukaryotic acidic biofilms were compared.

A. Aguilera (✉) · V. Souza-Egipsy · R. Amils
Centro de Astrobiología (INTA-CSIC),
Carretera de Ajalvir Km 4, Torrejón de Ardoz,
28850 Madrid, Spain
e-mail: aguileraba@inta.es

P. San Martín-Úriz · R. Amils
Centro de Biología Molecular (UAM-CSIC),
Universidad Autónoma,
C/ Nicolás Cabrera 1, Campus de. Cantoblanco,
28049 Madrid, Spain

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Introduction

The development of microorganisms forming biofilms has been well documented in aquatic environments (Lock 1993). They ubiquitously coat every wet surface acting as a trophic link between dissolved nutrients in the water column and the higher trophic levels of the ecosystem (Hynes 1970). Biofilms are usually a complex assemblage of microorganisms embedded within a matrix mostly composed of water together with extracellular polymeric substances (EPS) (Sutherland 2001).

EPS are microbial products located on or outside the cell surface that are formed by a complex mixture of proteins, neutral hexoses, acid polysaccharides, lipids, DNA, and humic acid substances, although, given the wide range of environments in which biofilms are found, it is extremely difficult to generalize about their structure, composition, and physiological activities (Jenkinson and Lappin-Scott 2001). Several studies indicate that EPS are not necessarily required for the initial attachment of microbial cells to surfaces (Allison and Sutherland 1987), but their production is essential for the development of the biofilm matrix, providing the framework into which microbial cells are inserted (Danese 2000).

Furthermore, EPS have also attracted attention because of their biotechnological potential, for instance for the removal of toxic heavy metals from contaminated waters. The affinity of the EPS anionic ligands for multivalent cations such as Ca^{2+}, Cu^{2+}, Mg^{2+}, and Fe^{3+} is very strong and favors mineral precipitation (McLean and Beveridge

1990; Beech and Sunner 2004). Moreover, wastewaters from chemical industries polluted with heavy metal ions represent an increasing hazard for all living organisms. As heavy metals cannot be degraded, they remain in the sediments and are slowly released into the water, creating a danger for ecosystems and human health (De la Noue and DePaw 1988). There are already numerous references to heavy metal elimination processes for industrial wastewaters with living cells, their components or their extracellular products (reviewed by Vierira and Volesky 2000), although most of these studies are related to seaweeds, yeasts, and bacteria isolated from environments polluted by industrial and domestic wastes (Volesky and Holan 1995).

We can also find unusually high levels of heavy metals in natural non-anthropogenic extreme acidic environments. These habitats tend to contain high concentrations of metals because their solubility increases markedly as the pH decreases (Norsdtrom and Alpers 1999). In this regard, Río Tinto (SW, Spain) is one of the most unique examples of extreme acidic environments. The river maintains a constant low pH (pH 0.9–2.5), buffered by ferric iron and with high concentrations of heavy metals that are toxic to numerous aquatic organisms. Concentrations of about 22 g l^{-1} of Fe, Zn at about 0.5 g l^{-1}, or Cd at about 70 mg l^{-1} can be found in its waters (Aguilera et al. 2006; Aguilera et al. 2007b).

Despite these extreme conditions, a number of prokaryotic and eukaryotic organisms have been isolated and identified in these environments (Nixdorf et al. 1998; Gross and Robbins 2000; López-Archilla et al. 2001; Amaral et al. 2002, Aguilera et al. 2007a). As the extracellular matrix is a complex and important component of all biofilms, it has to play a critical role in the development of eukaryotic microbial communities in extreme environments by providing mechanical stability and protection against the extreme external conditions. EPS are believed to play a substantial role in sorption of both organic and inorganic substances in biofilms, and they are able to protect cells against environmental parameters such as low pH or low temperature (García-Meza et al. 2005; Mancuso-Nichols et al. 2005). Thus, extreme acidic environments offer novel microbial biofilms that could produce varied EPS with different biotechnological applications (Umrania 2006).

It is well known that pH is an important physicochemical property of water that determines metal solubility and also the organic ligand adsorption capacities (Stone 1997). Thus, at low pH, the availability of negatively charged sites of the EPS, 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). Thus, the low pH of the environmental waters that surrounds the EPS in the living acidic biofilms could affect the charge of the functional groups of the EPS, changing their binding metal capability.

To evaluate the possible influence of low internal biofilm pH in the heavy metal sorption characteristics of the EPS as well as to their extraction effectiveness, five different extraction methods have been assayed. However, to date, no standard extraction procedure has been established for the preparation of EPS. To our knowledge, this is the first report regarding extraction of EPS from natural eukaryotic acidic biofilms.

Materials and methods

Sample collection Three eukaryotic biofilms (named RT, LZ, and ST) were taken from the bottom surface of the river (depth <5 cm) using a sterile plastic spatula and placed in 1.5-ml criotubes. The samples were immediately frozen in dry ice, freeze dried, and kept at −20°C until further use. In addition to the samples taken for EPS analysis, a subsample to identify the eukaryotic components of the biofilms was collected. Identification of algae and heterotrophic protists was carried out by direct microscopic observation up to the lowest possible taxonomic level using different phenotypic features based on previous studies of the eukaryotic communities of Río Tinto (López-Archilla et al. 2001; Amaral et al. 2002; Aguilera et al. 2006).

Water samples were filtered through 0.45-μm Millipore membranes. The total concentrations of ten recoverable metals were measured for each water sample (Al, Zn, Cu, Fe, Co, Ni, As, Cd, Cr, and Pb) using X-ray fluorescence reflection (TXRF) and inductively coupled plasma-mass spectrometry (ICP-MS).

Extraction of colloidal and capsular exopolymers The EPS of the three biofilms were extracted under five conditions. Figure 1 illustrates detailed procedures for each extraction method. Two fractions of exopolymers, colloidal and capsular, were extracted (Staats et al. 1999). The colloidal

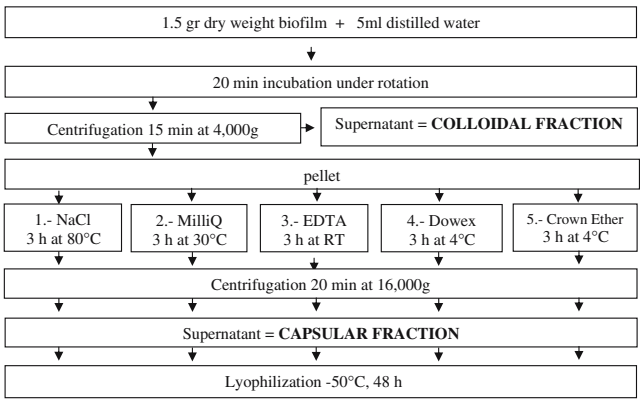

Fig. 1 Procedures for the five EPS extraction protocols

fraction includes carbohydrates and proteins that are loosely bound to microorganisms. The capsular fraction contains tightly bound compounds that require a lengthier extraction protocol (Hirst and Jordan 2003).

Briefly, each biofilm was freeze dried and divided into five subsamples of 1.5 g. Five milliliters of deionized water were added to each subsample and continuously agitated for 20 min at room temperature at 20–30 rpm (Hirst and Jordan 2003). Samples were then centrifuged (15 min, 4,000×*g*). The supernatant, colloidal EPS fraction, was removed for further analysis. The capsular fraction was then obtained by incubation with five different extractants: 5 ml of sodium chloride, (20 g l^{-1} for 3 h at 80°C; Rougeaux et al. 2001), 5 ml of MilliQ water (3 h at 30°C), 5 ml of ethylenediamine tetraacetic acid (EDTA; 10 mM, 3 h at room temperature; Staats et al. 1999), 2.5 g of Dowex cation exchange resin (Dowex 50×8, Fluka, USA) in 5 ml of extraction buffer for 3 h at 4°C (extraction buffer: 2 mM $Na_2PO_4{\cdot}12H_2O$, 4 mM $NaH_2PO_4{\cdot}H_2O$, 9 mM NaCl, 1 mM KCl, pH 7; Frolund et al., 1996). Finally, 5 ml of 30 mM Crown Ether complexing agent (dicyclohexyl-18-crown-6-ether, Sigma, Spain) in Tris buffer was incubated for 3 h at 4°C (Wuertz et al. 2001). All the incubations were performed in an orbital incubator at 20–30 rpm speed.

Samples were centrifuged at 16,000×*g* for 20 min (Mazor et al. 1996; Staats et al. 1999). Supernatants, capsular fractions, were removed for further analysis. The total amount of extracted EPS fractions was measured by weight after lyophilization (Liu and Fang 2002).

Chemical characterization of EPS The biochemical composition of the different EPS fractions was determined by the following colorimetric methods: Protein and humic acid contents were measured by the modified Lowry method (Frolund et al. 1995) using bovine albumin serum and humic acid as respective standards; neutral hexose sugars content was measured by the Dubois method (Dubois et al. 1956) using glucose as standard; DNA content was measured by the Burton method (Burton 1956) for nucleic acid content, using salmon semen DNA as standard.

The metal content present in the different EPS fractions was determined as follows (Guibaud et al. 2003): 5 ml of 0.5% HNO_3 and 0.5 ml of EPS fraction were mixed and agitated overnight. Solutions were centrifuged (20 min, 4,300×*g*) and filtered through a 0.2-μm Millipore membrane. The filtrate was recovered and the heavy metals analyzed by TXRF and ICP-MS.

To evaluate the possible contamination of the EPS with intracellular components due to cell lysis during the extraction processes, the glucose-6-phosphate-dehydrogenase (G6PDH) activity was measured (Platt et al. 1985), following manufacturer's instructions for the G6-PDH Trinity Biotech kit (St. Louis, MO, USA).

The results obtained from the biochemical analysis of the different EPS fractions were statistically compared by analysis of variance (ANOVA) using the Statistica V.6.0 program. All the analysis were carried out in triplicates.

Results

Eukaryotic community description of the biofilms

Although the biofilms present in the river are produced by a mixture of microorganisms, they are usually dominated by one or two species that can be easily differentiated by their color (Aguilera et al. 2007b). Thus, RT biofilm was mainly formed by the photosynthetic protist *Euglena mutabilis* (more than 80% of the cell counts), although about 15% of the cells corresponded to diatoms identified as *Pinnularia* sp. On the other hand, the Chlorophyta *Chlorella* sp. represented 95% of the total cell counts in the LZ biofilm. ST biofilm was dominated by filamentous green algae belonging to the genus *Zygnemopsis* (approximately 85% total cell counts) and unicellular red algae *Cyanidium* sp. (approximately 10% of the total cell counts). The remaining species found in all biofilms corresponded to amoebas (*Vahlkampfia* sp), flagellates (*Bodo* sp., *Cercomonas* sp.), and different minor unicellular green algae.

Extraction efficiency of EPS from acidic biofilms by different extraction techniques

Figure 2 summarizes the amounts of EPS extracted from the three acidic biofilms by the five processes compared in this study. Results show that the amount of EPS was strongly dependent upon the extraction method. There were statistically significant differences among methods and biofilms (ANOVA $P=0.002$ and $P=0.017$, respectively). Higher amounts of EPS were extracted from the three biofilms when NaCl and Crown Ether were used as extractant agents, followed by Dowex resin and EDTA. In all cases, the lower amounts of EPS were obtained when MilliQ water was used. The EPS amounts vary from 155 to 277 mg g^{-1} of dry weight (DW) in RT biofilm, from 187 to 478 mg g^{-1} of DW in LZ biofilm, and from 219 to 371 mg g^{-1} of DW in ST biofilm, depending on the extraction method.

A further analysis of the data suggested that colloidal/capsular ratios were similar in the three biofilms (approximately 2:1) except for the MilliQ treatment in RT and ST biofilms that reached a 6:1 ratio. Loosely bound colloidal EPS ranged from approximately 125 to 201 mg g^{-1} of DW in RT biofilm, corresponding to between 67% and 86% of the total EPS DW. Values for LZ biofilm colloidal EPS ranged from 123 to 301 mg g^{-1} of biofilm DW corresponding to about 60% of the total EPS DW. Colloidal

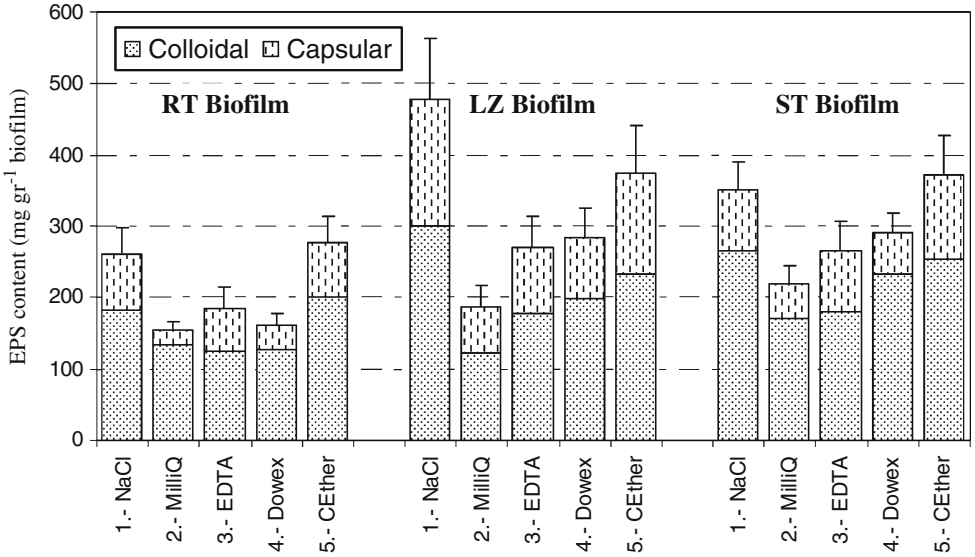

Fig. 2 EPS content (mg g^{-1} biofilm DW) of acidic eukaryotic biofilms by five extraction methods. *Error bars* represent standard deviations (n=3)

EPS concentrations in ST biofilm varied from 170 to 265 mg g^{-1} of biofilm DW, corresponding to between 77% and 75% of the total EPS DW. Total amounts of capsular EPS were lower in all cases, varying between 22 (RT biofilm, MilliQ method) and 177 mg g^{-1} total biofilm DW (LZ biofilm, NaCl method), which corresponds to between 14% and 38% of the total EPS DW.

EPS chemical characterization

Figure 3 compares the EPS constituents in the three acidic biofilms extracted by the various methods. As an ideal extraction method should cause minimal cell lysis, we measured the activity of G6PDH (Platt et al. 1985), a strictly intracellular enzyme, as a marker for intracellular

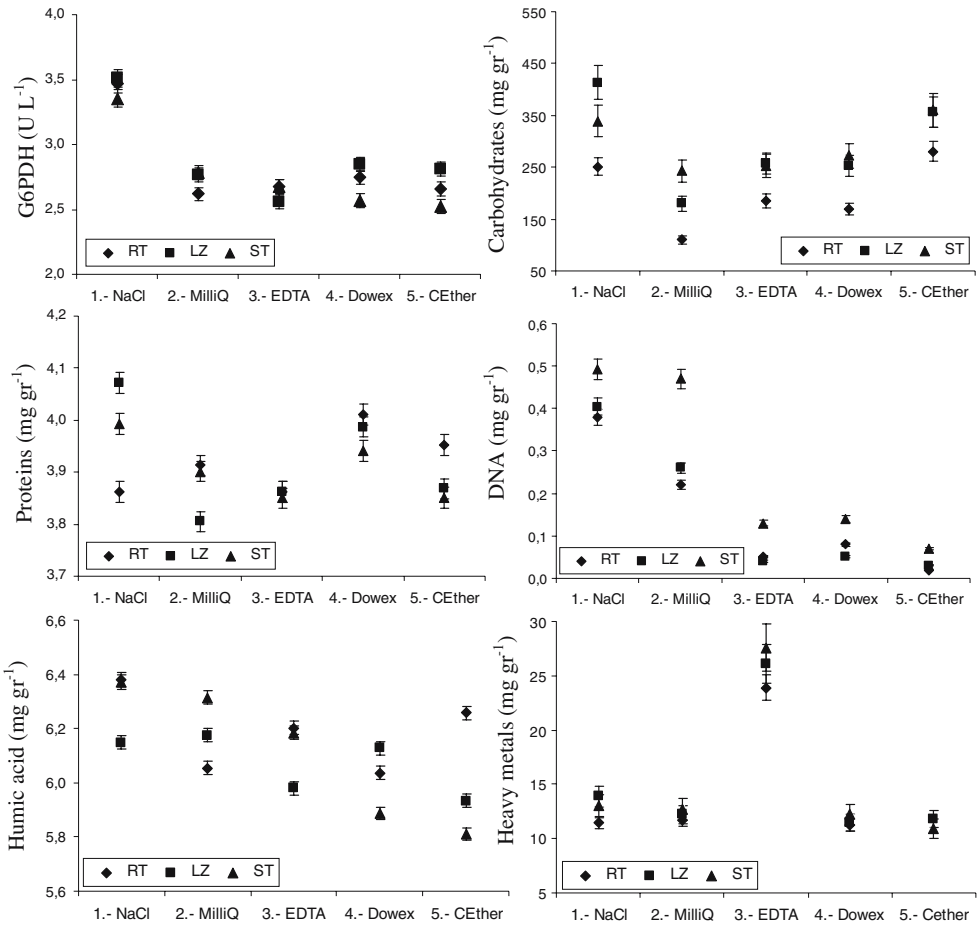

Fig. 3 Effect of the different extraction procedures on the EPS contents in the three biofilms analyzed. *Error bars* represent standard deviations (n=3)

contamination. Although no significant differences were found in the activity of G6PDH among biofilms (ANOVA, P=0.148), the NaCl extraction method showed the highest amount of G6PDH in the EPS, reaching up to 3.44±0.3 U l^{-1}. No statistically significant differences were found among the remaining methods (ANOVA, P=0.125).

For hexose sugar concentrations (colloidal + capsular fraction), the amounts obtained with MilliQ water extractions were the lowest at least for two of the biofilms assayed (RT and LZ). In addition, although no statistically significant differences were found among the remaining methods (P=0.679), differences were found among biofilms, with RT having the lowest hexose sugar content (P=0.017). Hexose sugar concentrations varied from 110 mg g^{-1} biofilm DW in RT using MilliQ to 413 mg g^{-1} biofilm DW in LZ using the NaCl method.

Protein content of extracted EPS showed no significant differences among biofilms or methods (P=0.723 and P=0.828, respectively), reaching values up to 3.9±0.07 mg g^{-1} biofilm DW. Each extraction method yielded different amounts of DNA. NaCl and MilliQ methods were the most effective in extracting DNA (P=0.002) for all biofilms analyzed, yielding from two to five times more DNA than the remaining methods. In addition, no significant differences were found in EPS DNA content among biofilms (P=0.004).

Concerning the humic acid extracted, no significant differences were found among biofilms or methods (P=0.757 and P=0.496, respectively), reaching values between 5.81 and 6.38 mg g^{-1} biofilm DW. On the contrary, the heavy metal content was different and depending on the extraction method. For all biofilms assayed, the EDTA method showed the highest extraction rate, yielding values more than two times higher than the other methods. No significant differences were found among biofilms (P=0.497).

Biochemical composition of EPS

Results in Fig. 4 show that the EPS in all biofilms were primarily composed of hexose sugars, followed by smaller quantities of heavy metals and humic acids. Proteins and DNA were also present, although in lower concentrations than the other components. For all biofilms and methods analyzed, hexose sugar concentrations represented over 90% of the total EPS DW. In general, the colloidal EPS fraction had a higher hexoses concentration than the capsular fraction (Fig. 5, P=0.002), in a proportion ranging from 1.2 (i.e., RT-EDTA) to 3.6 (i.e., RT-Dowex). Humic acid and heavy metals were the second main constituents of the EPS in these acidic biofilms. Their total concentrations reached up to approximately 7% of the total EPS composition in some cases (Fig. 4). As with the hexoses, significant differences (P=0.01) were found among the heavy metal concentrations in both EPS fractions, colloidal

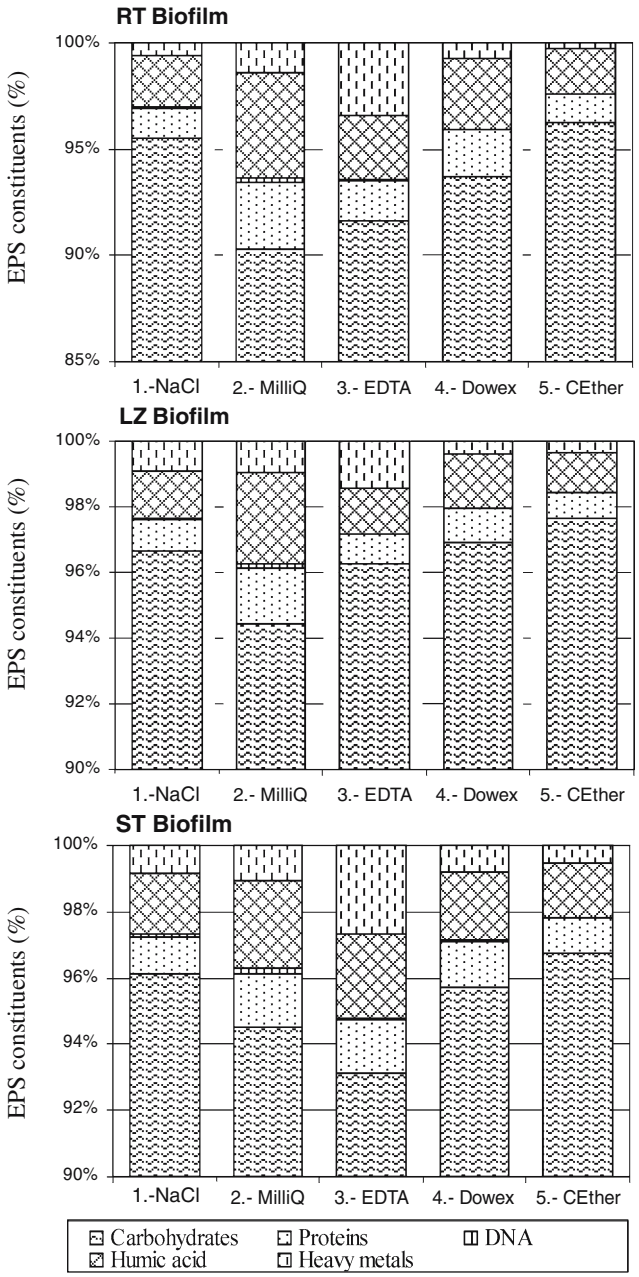

Fig. 4 Percentages of the different EPS components of the three biofilms extracted by the different methodologies. *Error bars* represent standard deviations (n=3)

and capsular (Fig. 5). In general, colloidal EPS fractions showed higher heavy metal values than the capsular fraction, with a mean proportion of about two times higher.

Finally, proteins and DNA showed the lowest values in the EPS composition. Protein concentrations ranged from 3.86 mg g^{-1} (i.e., RT-EDTA) to 4.07 mg g^{-1} biofilm DW (i.e., LZ-ClNa), constituting up to about 2% of the total EPS DW. DNA concentrations were always lower than 0.5 mg g^{-1} biofilm DW, which means less than 0.4% of the total EPS DW. In this case, no statistical significant

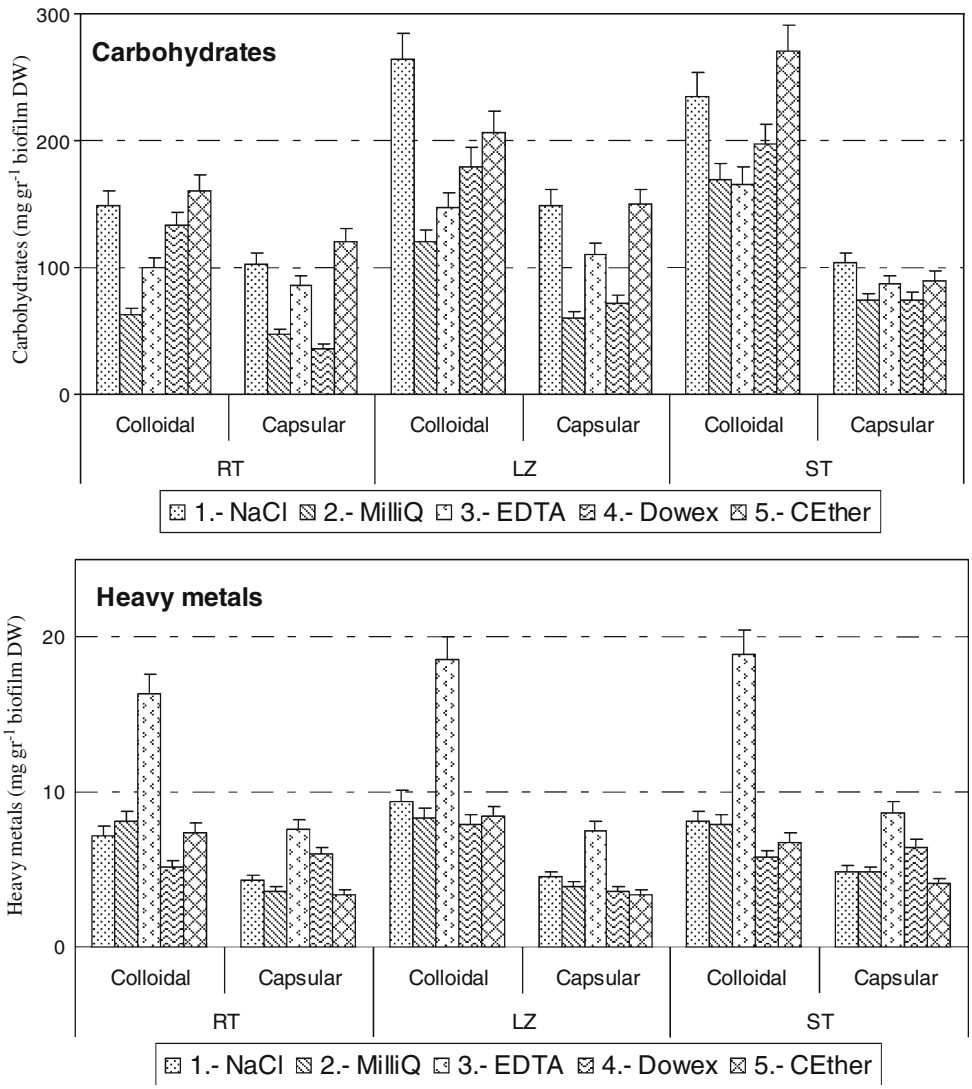

Fig. 5 Effect of extraction protocols on the contents of hexose sugars and heavy metals in the capsular (Cap) and colloidal (Col) fractions of the three biofilms analyzed. *Error bars* represent standard deviations (n=3)

differences were found in the percentages of DNA among biofilms (P=0.851).

We have also analyzed the possible relation between the EPS heavy metal distribution and the heavy metal composition of the water from which each biofilm was collected (Fig. 6). For most of the heavy metals analyzed, the EPS extracted from the biofilms with EDTA greatly resembled the metal composition of the water analyzed. Thus, the proportion of each metal in the EPS is similar to the proportion of each metal present in the water from each locality where the biofilms were collected. No statistical significant differences (P=0.776) were found among them except for Al, Cu, Zn, and Pb. EPS capsular fractions from all biofilms showed significantly higher amounts of Al than the expected, taking into account the level of this heavy metal in the water. Thus, 15% of the total heavy metals in RT and ST biofilm capsular fractions was Al, while the water they came from only reached values of about 1% to 4%. On the other hand, Cu showed half the expected values in the capsular fractions of RT and ST biofilms. At these sampling points, Cu reached values between 4% and 8% of the heavy metals in the water, whereas the percentages of this metal were approximately 2% and 4%, respectively, in the extracted EPS.

On the contrary, Zn showed higher amounts than expected in the capsular fraction of the EPS from the RT and ST biofilms. In these cases, the percentages of Zn in the water were approximately 4% and 5%, respectively, while the percentages of this metal were about 9% and 10.5%, respectively, in their extracted capsular EPS. Finally, Pb was also accumulated in higher concentrations than expected in the colloidal fractions of RT and LZ biofilms. Both water samples showed less than 0.01% of Pb, while their percentage increased up to about 4% in the colloidal fractions analyzed.

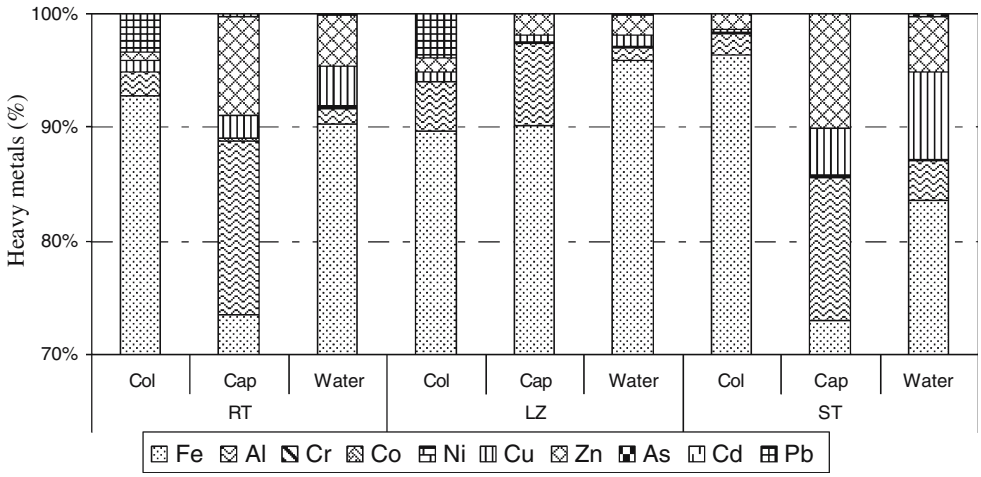

Fig. 6 Percentages of the heavy metal composition of the EPS extracted, using EDTA, and the heavy metal distribution in the water samples

Discussion

Significant efforts have been made toward developing and comparing different EPS extraction methods to find the most effective technique for various aquatic and terrestrial environments. However, little is known about biofilms formed mainly by eukaryotic organisms, not to mention biofilms located in extreme acidic environments, as these studies are even more scarce. pH is one of the most important physicochemical parameters affecting the efficiency of most of the EPS extraction methods based on chemical reagents or exchange resins. The main objective of this work was to examine the efficacy of different methodologies in the extraction of EPS from different eukaryotic biofilms isolated from an extreme acidic environment with high heavy metal content in Río Tinto (SW, Spain). To our knowledge, this is the first study of this type carried out on these natural acidic biofilms.

Extraction method efficiency

This study shows considerable differences in extraction efficiency results among the five methods and the biofilms used (Figs. 2 and 3). This fact supports the statement postulated by Novak and Haugan (1981) suggesting that there is no universal method for providing quantitative extraction of exopolymers. In general, we can conclude that extractions using NaCl and the complexing agent Crown Ether yielded the highest amounts of total EPS. Although NaCl extraction has been used for EPS extraction in natural freshwater microbial mats (Rougeaux et al. 2001), in our case, this method also showed the highest levels of G6PDH (Fig. 3), meaning a higher level of cell lysis during extraction. This fact is also corroborated by the higher amounts of proteins and DNA measured with this method in our samples, parameters usually used as induced cell lysis indicators (Brown and Lester 1980; Gehr and Henry 1983).

In contrast to other published results (Wuertz et al. 2001), Crown Ether yielded EPS amounts significantly higher than those obtained with the ion exchange resin DOWEX 50×8, and both methods showed the same ratios of heavy metal recovery (Fig. 3). The unexpected difference between the two resins could be explained by the low pH of our biofilms (approximately 2.5). These cation exchange resins, widely used in EPS extraction from activated sludge, removes cations (usually Ca^{2+}) from the biofilm matrix, leading to breakup of the biofilm flocs and a subsequent release of EPS (Frolund et al. 1996). Both resins remove calcium ions without affecting the distribution of metals under investigation (Wuertz et al. 2001). Taking into account that pH is one of the most important physicochemical factors that determine metal solubility (Ernst 1998), the characteristics of these resins may be modified under low pH. Unfortunately, to our knowledge, all the studies regarding the relation between cation exchange resins and optimal pH thus far have been carried out at circumneutral pH (Wuertz et al. 2001).

Similar amounts of EPS were extracted when EDTA was used as extractant agent, although, in this case, the amounts of heavy metals recovered were significantly higher (Figs. 2 and 3). This could be due to the chelating effect of EDTA against divalent cations, which includes most of the heavy metals analyzed (Nowak et al. 1996). The incubation of the biofilms with EDTA could release metals from the cellular fraction to the EPS, increasing their concentrations. Even though we have used a 10-mM EDTA solution to avoid possible intracellular contamination (ten times lower than with marine materials; Platt et al. 1985), extraction with EDTA could release metals from the cell walls and membranes, increasing their amounts in the EPS significantly. These results are in agreement with the analysis performed by Wuertz et al. (2001) suggesting that metals in biofilms are usually sorbed to cellular material.

Our results also suggest that not only the quantity but also the biochemical characteristics of the EPS extracted by

different methods differ (Fig. 4). Thus, hexoses and heavy metal concentrations were almost two times higher when NaCl and EDTA were, respectively, used, and DNA was most effectively extracted by NaCl or MilliQ water. Consequently, EPS extracted by a single method is not representative of the real composition of EPS in biofilms, and further, the quantity of total EPS extracted by different methods should not be used to compare extraction efficiency or EPS composition, as suggested by Park and Novak (2007).

Extraction with EDTA increased the hexose sugars yield probably by chelating metal ions that form interchain links between carbohydrates (Underwood et al. 1995). Therefore, it has been suggested that EDTA extraction methods may release more tightly bound EPS (capsular EPS) into the colloidal phase (Decho 1990). However, this is not true in our case, where EDTA extraction yielded the lowest amounts of colloidal EPS after the MilliQ water extraction (Fig. 2). The effect of EDTA depends on the concentration of metal ions present in the sample. As the presence of metals in acidic samples is extremely high (Aguilera et al. 2007b), higher concentrations of EDTA should be needed to overcome the binding of EDTA to metal ions present in the media (Decho 1990; Underwood et al. 1995).

Regardless of the protocol used, concentrations of hexoses and heavy metals were significantly higher in the colloidal than in the capsular fractions (Fig. 5). Little is known about the ecological significance of both fractions, although it is generally assumed that colloidal fractions are implicated in the mechanical stability of the sediment (Winder et al. 1999), while capsular EPS help in the adsorption of essential nutrients and trace metals, as well as in the protection against desiccation and toxic metals (Decho 1990). Our observations regarding the high amounts of heavy metals in the colloidal fraction strongly support the significant protective contribution of EPS against their toxic effect.

Heavy metal analysis

Besides low pH, acidic environments tend to contain unusually high concentrations of heavy metals, because their solubility increases markedly as the pH decreases (Norsdtrom and Alpers 1999). One of the main mechanisms of metal accumulation used by benthic communities is through adsorption in EPS. For this reason, we were particularly interested in the relationship between EPS and heavy metals. 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; Brown and Lester 1979; Kaplan et al. 1987). However, the reaction between metallic ions and polymeric organic substances is strongly influenced by the pH (Stumm and Morgan 1985). Low pH modifies the ionic status of the EPS functional groups, changing their characteristics (Ferris et al. 1989). The reduction of surface charge density with decreasing pH levels also serves to weaken electrostatic free energy contributions to metallic ion adsorption (James and Healey 1972). The ability of EPS fractions from the acidophilic eukaryotic biofilms analyzed to bind significant amounts of heavy metals (up to10% of the total EPS DW) is particularly relevant, as at low pH levels (i.e., pH of about 2.0), the availability of negatively charged sites is drastically reduced, decreasing the number of metal cations that can be adsorbed (Ferris et al. 1989). A possible reason for this behavior could be the precipitation of metal oxides in the extracellular matrix that favor the adsorption of other metals.

For this reason, we analyzed the possible relation between the distribution of the different heavy metals in the EPS and the heavy metal composition of the water from which each biofilm was collected (Fig. 6). In general, the heavy metal composition in the extracted EPS greatly resembled the metal composition of the water except for Al, Cu, Zn, and Pb. This suggests a competitive threshold in the binding of metallic ions, reported also by García-Meza et al. (2005) for Cu and Zn in biofilm mesocosms studies. Surprisingly, recent studies based on principal component analysis carried out in Río Tinto suggested that these two particular metals play a substantial role in controlling the epiphytic eukaryotic diversity and distribution in the river (Aguilera et al. 2006). In the same regard, Holding et al. (2003) have also reported no significant correlations between Pb in EDTA-extractable biofilms and the sediments from where they were collected.

In summary, this study showed that no single extraction method can extract all the potential EPS components with the same efficiency. Intracellular contamination was detected only when NaCl was used, probably due to the subsequent incubation of the biofilms at 80°C. Combined extractions are necessary for a more precise characterization of EPS. The analysis revealed that acidic EPS are mainly composed of hexoses, proteins, humic acids, and DNA. The EPS also accumulate substantial amounts of heavy metals, and their distribution is highly correlated with the heavy metal composition of the water in which they develop. We can conclude that the EPS extraction protocol used affects, to a greater or lesser extent, the quantity and composition of the EPS extracted from acidic biofilms. Thus, the functional characterization of EPS depends on the extraction protocol used.

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