

# Prokaryotic community structure in algal photosynthetic biofilms from extreme acidic streams in Río Tinto (Huelva, Spain)

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**Summary.** Four algal photosynthetic biofilms were collected from the Río Tinto (SW Spain) at four localities: AG, *Euglena* and *Pinnularia* biofilms; ANG, *Chlorella* and *Pinnularia* biofilms; RI, *Cyanidium* and *Dunaliella* biofilms; and CEM, *Cyanidium*, *Euglena* and *Pinnularia* biofilms. Community composition and structure were studied by a polyphasic approach consisting of 16S rRNA analysis, scanning electron microscopy by back-scattered electron detection mode (SEM-BSE), and fluorescence in-situ hybridization (FISH). Acidophilic prokaryotes associated with algal photosynthetic biofilms included sequences related to the Alpha-, Beta-, and Gammaproteobacteria (phylum Proteobacteria) and to the phyla Nitrospira, Actinobacteria, Acidobacteria and Firmicutes. Sequences from the Archaea domain were also identified. No more than seven distinct lineages were detected in any biofilm, except for those from RI, which contained fewer groups of Bacteria. Prokaryotic communities of the thinnest algal photosynthetic biofilms (<100 µm) were more related to those in the water column, including *Leptospirillum* populations. In general, thick biofilms (>200 µm) generate microniches that could facilitate the development of less-adapted microorganisms (coming from the surrounding environment) to extreme conditions, thus resulting in a more diverse prokaryotic biofilm. [Int Microbiol 2008; 11(4):251-260]

**Key words:** algal biofilms · prokaryotic community · Río Tinto · SEM-BSE · FISH · 16S rRNA

## Introduction

Río Tinto headwaters provide a natural system to study iron-rich acidic waters and their relationship with the development of algal photosynthetic biofilms on the surface of solid substrates. Previous studies of the prokaryotic community found in acidic stream waters from Río Tinto focused on the microbial diversity

of the water column [22,29], the oxic-anoxic interfaces [31], and the floating macroscopic filaments [20]. In other acidic environments the analysis of the prokaryotic diversity is restricted to the subsurface part of the system, where precipitation of oxyhydroxides does not occur [11,17,42]. Algal photosynthetic biofilms have been described in Río Tinto [2–6,41] and in other acidic environments [8,12,36] but their interactions with the prokaryotic communities present has been seldom studied [40].

Natural biofilms are complex structures formed as a consequence of specific environmental conditions [19,23,47]. Previous studies have used microsensors to determine the existence of different microenvironmental conditions in acidic microbial mats [8]. The biofilm structure is thought to allow the formation of microenvironmental conditions that provide niches for microorganisms less adapted to the extreme conditions of their surroundings, thereby facilitating physiological activities

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that would not be feasible in the water column. The goal of this study was to describe the prokaryotic diversity associated with the algal photosynthetic biofilm communities growing in an acidic extreme environment. The thicknesses of these algal photosynthetic natural biofilms ranged from a few tens to several hundreds of micrometers, and previous studies indicated the presence of prokaryotic microorganisms within them [4]. To determine the structure and distribution of the diverse prokaryotic organisms inside the biofilms, we used a polyphasic approach consisting of 16S rRNA analysis, scanning electron microscopy by back-scattered electron detection mode (SEM-BSE), and fluorescence in-situ hybridization (FISH). The combined use of these techniques provided a better understanding of the ecology of these extreme algal photosynthetic acidic biofilms.

## Materials and methods

**Sampling location.** Four algal photosynthetic biofilms were studied in Río Tinto (Huelva province, southwestern Spain) in May, July, and October 2006. Physicochemical characterization and the microbial communities were described previously [2,3,5,6,22]. Table 1 shows the geographic coordinates of the study sites and the main physicochemical characteristics of the water at these four locations: AG, ANG, RI, and CEM, plus an additional one (FE) used as control. Redox potential and pH were measured with a Crison 506 pH/Eh meter (Crison, Barcelona, Spain), and conductivity with an Orion-122 conductivity meter (Thermo Scientific, Franklin, USA). Oxygen concentration was measured with an Orion 810 dissolved oxygen meter (Thermo Scientific, Franklin, USA). Semi-quantitative estimations of Fe (II) and Fe (total) iron concentrations were measured with a Reflectoquant analysis system (Merk, Darmstadt, Germany).

**Algal photosynthetic biofilm sample collection.** The FE sampling location was chosen as a control since biofilms did not develop there during the study period (Fig. 1A,B). Algal photosynthetic biofilm samples were collected from four sites: AG, *Euglena* and *Pinnularia* biofilms (Fig. 1C,D); ANG, *Chlorella* and *Pinnularia* biofilms (Fig. 1E,F); RI, *Cyanidium* and *Dunaliella* biofilms (Fig. 1G,H); and CEM, *Cyanidium*, *Euglena* and *Pinnularia* biofilms (Figs. 1I,J). Samples for DNA extraction were collected with pipettes and stored at  $-20^{\circ}\text{C}$  until further processing. Samples for FISH were immediately fixed with 4% formaldehyde and kept on ice. The samples were washed with acidic (pH 2) sterile distilled water and stored in filter-sterilized 50% ethanol. Samples for scanning electron microscopy were secured for transport with a thin layer of alginate acid (1%), gelled in place on the biofilm

surface after the addition of  $\text{CaCl}_2$  (1%). The samples were then fixed in the field with 2.5% glutaraldehyde in acidic (pH 2) sterile distilled water. The samples were kept cold ( $5^{\circ}\text{C}$ ) and in the dark until further processing.

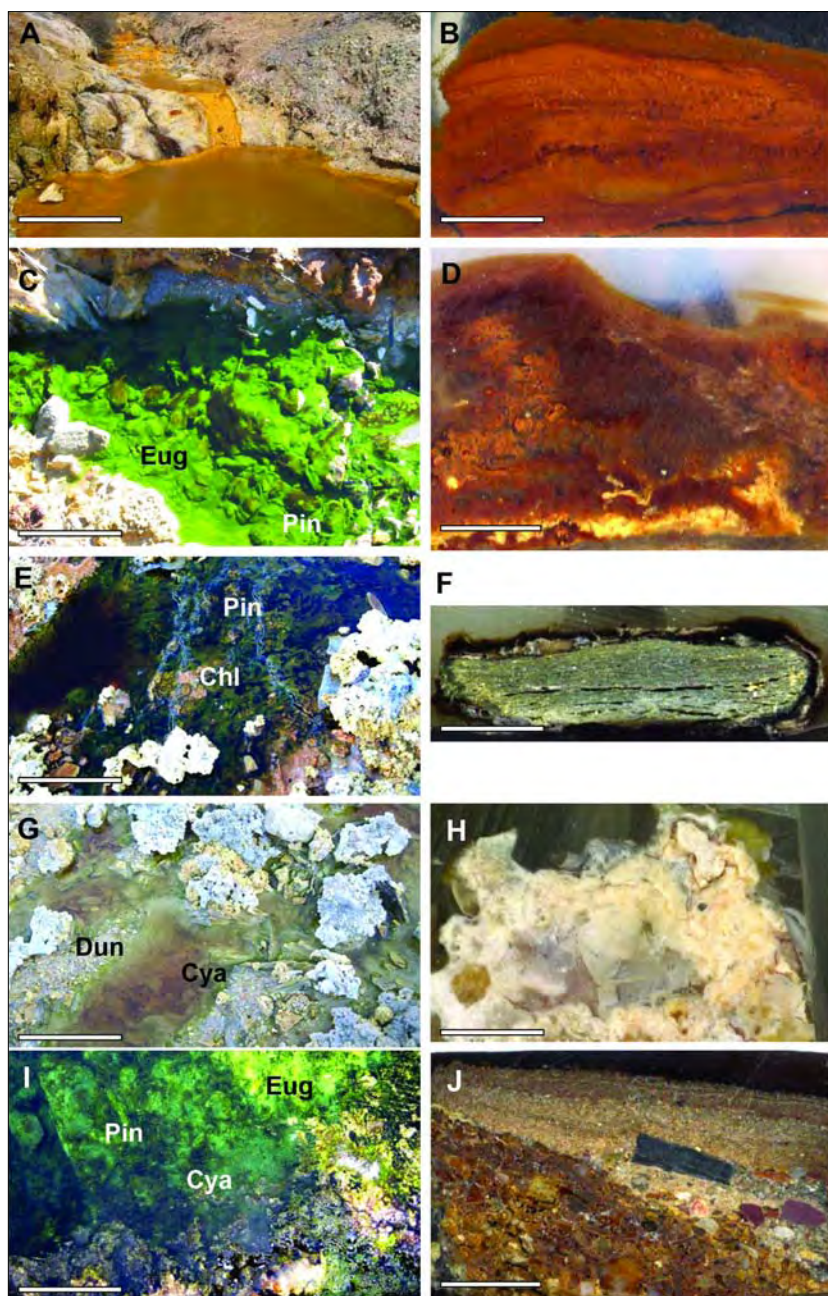
**Scanning electron microscopy in back-scattered electron detection mode.** The field fixed samples were washed in the laboratory with acidic sterile distilled water and postfixed with 1% osmium tetroxide in distilled water for a minimum of 8 h. Samples were dehydrated using an ascending series of ethanol (30, 50, 70, 90, and 100%) and then infiltrated with LR-white resin for 24 h. The infiltrated samples were polymerized at  $65^{\circ}\text{C}$  for 24 h after which the blocks were cut transversely using a diamond saw, fine polished and carbon-coated following the protocols described in a previous work [46]. Transverse sections of the polished surfaces of the blocks were examined using a Jeol 5600LV SEM equipped with a BSE detector.

**Fluorescence in-situ hybridization.** The fixed samples were spotted onto glass depression slides containing 0.8 % agarose previously melted at  $45^{\circ}\text{C}$ . Samples were slowly cooled and dehydrated for 30 s in 50, 80, and 100% ethanol. Hybridization and DAPI staining were performed as described previously [7]. Cy-3 labeled probes were provided by Bonsai Technology (Barcelona, Spain). Probes used in this work are shown in Table 2. To avoid fluorescence fading, Vectashield Mounting medium (Vector Laboratories, Burlingame, CA, USA) was added to the preparations. An 510 LSM laser-scanning confocal microscope (Zeiss, Oberkochen, Germany) equipped with an argon ion laser (450–530 nm) and two He/Ne lasers (543 and 633 nm) was used to visualize the hybridization results.

**DNA extraction, PCR amplification, and sequencing.** A Fast DNA spin kit for soil (Q-Biogene, Santa Ana, CA, USA) was used according to the manufacturer's instructions to extract DNA from the samples. Samples were washed five times with TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) before DNA extraction in order to remove remains of acidic water. Extracted DNA was purified by passage through a GeneClean Turbo column (Q-Biogene) and quantified by ethidium bromide-UV detection on an agarose gel. The 16S rRNA genes were amplified by PCR in mixtures containing 20–30 ng of DNA per 50- $\mu\text{l}$  reaction volume,  $1\times$  PCR buffer (Promega Biotech Ibérica, Madrid, Spain), 2.5  $\mu\text{M}$  of each of the deoxynucleotides (Amersham Biosciences, Alcobendas, Spain), 2.5 mM  $\text{MgCl}_2$ , 1 mg bovine serum albumin (BSA)/ml, 500 mM of each forward and reverse primer and 0.025 U/ $\mu\text{l}$  of *Taq* DNA polymerase (Promega Biotech Iberica, Madrid, Spain). In the amplification reactions, the reverse primer was the universal 1492r (5'-GGTTACCTTGTGTACGACTT-3') [1], and the forward primer was either *Bacteria*-specific 27f (5'-AGAGTTTGATCCTGGCTCAG-3') [28], or *Archaea*-specific 21f (5'-TTCCGGTTGATCCYGCCGGA-3') [16]. PCR conditions were as follows: initial denaturation at  $94^{\circ}\text{C}$  for 5 min, followed by 30 cycles of denaturation at  $94^{\circ}\text{C}$  for 40 s, annealing at  $52^{\circ}\text{C}$  for 1 min for the *Bacteria* domain primers and  $56^{\circ}\text{C}$  for the *Archaea* domain primers, and extension at  $72^{\circ}\text{C}$  for 1 min. PCR amplification products of 16S rRNA genes were purified by GeneClean San Diego, CA, USA). After minipreps were

**Table 1.** Location of the studied sites and main physicochemical variables

| Sampling site | Geographic coordinates          | pH   | Redox (mV) | Conductivity (mS/cm) | $\text{O}_2$ ( $\mu\text{g}/\text{ml}$ ) | Fe(II) (mg/l) | Fe(III) mg/l | Fe(II) / Fe (total) (%) |
|---------------|---------------------------------|------|------------|----------------------|------------------------------------------|---------------|--------------|-------------------------|
| FE            | 37° 43' 19'' N<br>6° 33' 3'' W  | 2.79 | 413        | 5.2                  | 7.7                                      | 610           | 3090         | 16.48                   |
| ANG           | 37° 43' 14'' N<br>6° 33' 9'' W  | 2.19 | 471        | 30.8                 | 1.7                                      | 3000          | 12,600       | 19.23                   |
| AG            | 37° 43' 28'' N<br>6° 33' 36'' W | 3.05 | 384        | 5.3                  | 5.8                                      | 1140          | 480          | 71.25                   |
| RI            | 37° 43' 13'' N<br>6° 33' 15'' W | 1.80 | 460        | 38.9                 | 2.4                                      | 4100          | 28,900       | 12.42                   |
| CEM           | 37° 42' 1'' N<br>6° 33' 29'' W  | 2.68 | 446        | 11.4                 | 2.8                                      | 4400          | 1100         | 80.00                   |



**Fig. 1.** General aspects of field sites and collected biofilms. (A) The riverbed at site FE. Bar = 1 m. (B) Transverse section of the sediments from the riverbed at FE. Bar = 0.5 cm. (C) Biofilms on the riverbed at site AG. Different types of biofilms studied: dark green *Euglena* (Eug) and brown patches of the diatom *Pinnularia* biofilms (Pin). Bar = 25 cm. (D) Transverse section of fixed and embedded biofilms present on the streambed at AG. Bar = 0.25 cm. (E) Biofilms on the riverbed at site ANG. Different types of biofilms studied: green *Chlorella* biofilms (Chl) and brown patches of *Pinnularia* (Pin). Bar = 25 cm. (F) Detailed image of fixed and embedded transversal section of the biofilm communities present on the surface of pebbles from ANG. Bar = 0.2 cm. (G) Biofilms on the riverbed at site RI. Different types of biofilms studied: blue green biofilms of *Cyanidium* (Cya) and light green *Dunaliella* biofilms (Dun). Bar = 25 cm. (H) Detailed image of fixed and embedded sediments with biofilms from RI. Bar = 0.2 cm. (I) Biofilms on the riverbed at site CEM. Different types of biofilms studied: blue-green biofilms of *Cyanidium* (Cya); dark green biofilms of *Euglena* (Eug) and brown patches of *Pinnularia* biofilms (Pin). Bar = 25 cm. (J) Transverse section of fixed and embedded biofilms that developed on sediments at CEM. Bar = 0.4 cm.

prepared, the cloned inserts were directly sequenced with a Big-Dye sequencing kit (Applied Biosystems, Madrid, Spain) as described in the manufacturer's instructions. For sequencing, four primers were used: M13f (5'-TGTAACGACGCGCCAGT-3'), M13r (5'-CAGGAAACAGCTATGACC-3'), which amplified 700–1000-bp of the insert; GM5f (5'-CCTACGGGAGCAGCAG-3'), which amplified a 550-bp fragment of the 16S rRNA gene; and 907r (5'-CCGTCAATTCCTTTRAGTT-3'), which amplified a 520-bp fragment of the 16S rRNA gene [34].

**Phylogenetic analysis.** Sequences were analyzed using BLAST at the NCBI database [http://ncbi.nlm.nih.gov/BLAST] and added together with the most important BLAST hits to a database of over 50,000 homologous prokaryotic 16S rRNA primary structures by using the aligning tool of the ARB software package [30]. Phylogenetic trees were generated using parsimony and maximum-likelihood analyses with a subset of 100 nearly full-

length sequences (>1400 bp). Sequences obtained in this study were deposited in the EMBL sequence database under accession nos. EU370262–EU370323 and EU376020–EU376025.

## Results

**SEM-BSE study of the community ultrastructure in algal photosynthetic biofilms.** Río Tinto photosynthetic algal biofilms are three-dimensional structures that show a spectrum of heterogeneous forms and colors determined by the dominating photosynthetic organisms (Fig. 1 C–J). In each of

**Table 2.** Fluorescence labeled oligonucleotide probes used for in situ hybridization experiments

| Probe               | Target | Sequence (5' to 3')      | FM (%) <sup>a</sup> | Specificity                                          | Reference             |
|---------------------|--------|--------------------------|---------------------|------------------------------------------------------|-----------------------|
| EUB338              | 16S    | GCTGCCTCCCGTAGGAGT       | 0–35                | <i>Bacteria</i> domain                               | [7]                   |
| ALF968              | 16S    | GGTAAGGTTCTGCGCGTT       | 20                  | Alphaproteobacteria                                  | [Neef A] <sup>c</sup> |
| ACD638              | 16S    | CTCAAGACAACACGTCTC       | 20                  | <i>Acidiphilium</i> spp.                             | [22]                  |
| ACD821              | 16S    | AGCACCCCAACATCCAGCACACAT | 35                  | <i>Acidiphilium</i> spp.                             | [22]                  |
| BET42a <sup>b</sup> | 23S    | GCCTTCCCACCTTCGTTT       | 35                  | Betaproteobacteria                                   | [32]                  |
| GAM42a <sup>c</sup> | 23S    | GCCTTCCCACATCGT TT       | 35                  | Gammaproteobacteria                                  | [32]                  |
| THIO1               | 16S    | GCGCTTTCTGGGGTC TGC      | 35                  | <i>Acidithiobacillus</i> spp.                        | [Stoffels, unpb.]     |
| NTR712 <sup>d</sup> | 16S    | CGCCTTCGCCACCGGCCT TCC   | 35                  | <i>Nitrospira</i> group                              | [15]                  |
| LEP154              | 16S    | TTGCCCCCCCTTTCGGAG       | 35                  | <i>Leptospirillum ferriphilum</i>                    | [22]                  |
| LEP634              | 16S    | AGTCTCCCAGTCTCCTTG       | 35                  | <i>Leptospirillum ferrodiazotrophum</i>              | [22]                  |
| LEP636              | 16S    | CCAGCCTGCCAGTCTCTT       | 35                  | <i>Leptospirillum ferrooxidans</i>                   | [22]                  |
| FM732               | 16S    | GTACCGGCCAGATCGCTG       | 35                  | <i>Acidimicrobium</i> sp., <i>Ferrimicrobium</i> sp. | [10]                  |
| FMR0732             | 16S    | GTGTCGGCCCCAGATTGCT G    | 35                  | Isolates KP1 and CS11                                | [25]                  |
| ACM1160             | 16S    | CCTCCGAATTAACCTCCGG      | 35                  | <i>Acidimicrobium</i> spp.                           | [22]                  |
| TMP654              | 16S    | TTCAACCTCACCCGGTCC       | 35                  | <i>Thermoplasma</i> spp., <i>Picrophilus</i> spp     | [22]                  |
| FER656              | 16S    | CGTTTAACCTCACCCGATC      | 35                  | <i>Ferroplasma</i> spp.                              | [18]                  |
| NON338              | –      | ACTCCTACGGGAGGCAGC       | 35                  | Negative control                                     | [7]                   |

<sup>a</sup>Formamide percentage (vol/vol) in the hybridization buffer.

<sup>b</sup>Used in conjunction with an unlabeled competitor probe, GAM42a.

<sup>c</sup>Used in conjunction with an unlabeled competitor probe, BET42a.

<sup>d</sup>Used in conjunction with an unlabeled competitor probe, NTR712c.

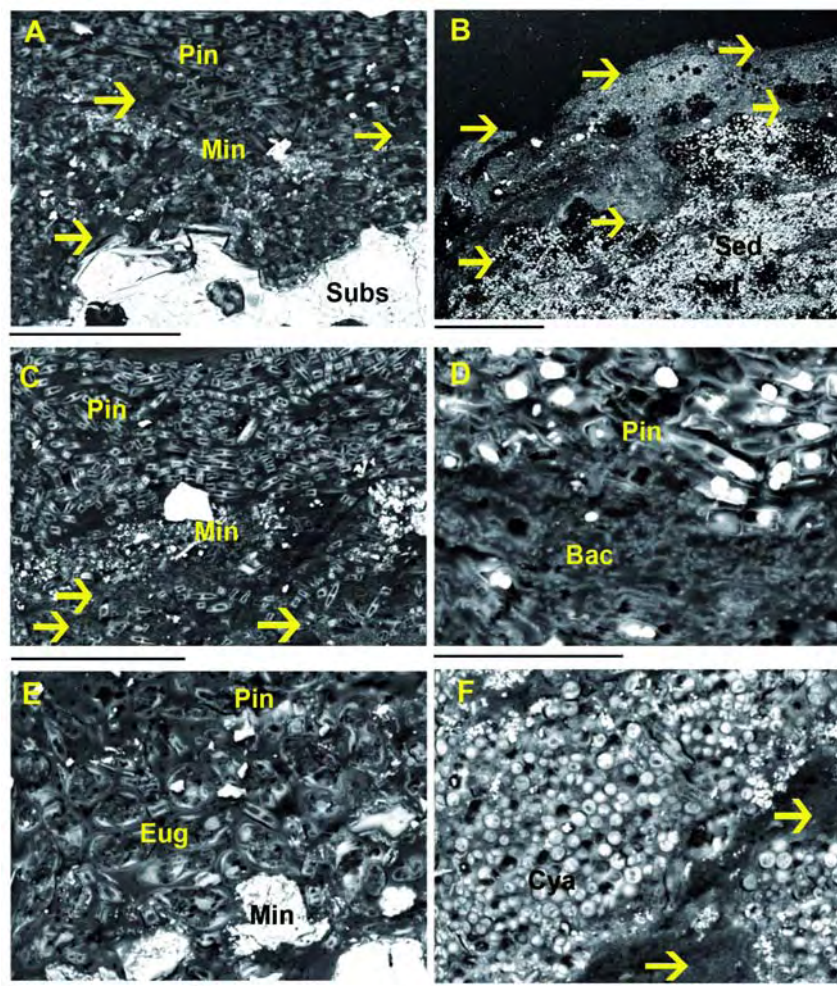
<sup>e</sup>[Neef A (1997) PhD Thesis. Technical University of Munich, Germany.]

the four localities, photosynthetic biofilms were distributed on the riverbed as independent patches (Fig. 1C,E,G,I) that could be clearly identified by their texture and color, although after microscopy some areas indicated mixing of communities mostly at the interface with the substrate. The *Pinnularia* and *Euglena* biofilms from the AG site were mixed with layers of minerals (Fig. 1D, and Min in Fig. 2A). The thickness of the biofilm varied among the samples studied (from 100 µm to 200 µm); in general there were very few clusters of bacteria associated with these biofilms (arrows in Fig. 2A). The thickest were algal photosynthetic biofilms from site CEM, several hundreds of micrometers thick (Fig. 1J and arrows in Fig. 2B). In the case of *Pinnularia*-dominated biofilms (Pin in Fig. 2C), several layers of diatoms were intermixed with minerals (Min in Fig. 2C) and a large number of bacterial clusters (arrows in Fig. 2C). In some areas, bacteria formed layers around *Pinnularia* cells (Bac in Fig. 2D). *Euglena* dominated biofilms (Eug in Fig. 2E) showed few clusters of bacteria; at site CEM, these were in close contact with *Pinnularia* biofilms (Pin in Fig. 2E) and intermixed with minerals (Min in Fig. 2E). The *Cyanidium* biofilms from CEM (Cya in Fig. 2F) were located in shaded areas, forming dense layers and surrounded by clusters of bacteria (arrows in Fig. 2F).

The biofilms obtained from sites ANG and RI were thinner (Fig. 1E,G). At ANG, there were very few bacterial clusters (arrows in Figs. 3A,B) inside the structures of the *Pinnularia* (Pin in Fig. 3A) and *Chlorella* biofilms (Chl in Fig. 3B). *Dunaliella* biofilms from site RI were the thinnest (less than 100 µm) and had clusters of bacteria in contact with minerals from the substrate (Subs in Fig. 3C). Microscopy revealed that the algal photosynthetic biofilms consisted mainly of algae (Dun in Fig. 3C). Fungi and prokaryotes were preferentially located at the interface with the substrate (arrows in Fig. 3C). It was possible to differentiate bacteria from mineral particles by their shape and different contrast properties under back-scattered electron detecting mode (Bac and Min in Fig. 3D).

#### **FISH analysis of the prokaryotic community in algal photosynthetic biofilms.**

Results from the FISH analysis of the studied biofilms are summarized in Table 3. In general, hybridizations with specific probes revealed that the prokaryotic communities in the biofilms were mostly identified with the *Bacteria* domain probe (EUB338). The amount of bacteria in the different biofilms varied from low (Fig. 4A) to abundant (Fig. 4B).



**Fig. 2.** SEM-BSE images of algal photosynthetic biofilms studied from sites AG and CEM. (A) A transverse section shows *Pinnularia* (Pin), which dominated the biofilm obtained from AG above the rock fragments on the riverbed (Subs). Small clusters of bacteria are present inside the biofilm (arrows) between layers of mineral fragments (Min). Bar = 100  $\mu$ m. (B) Low-magnification image of the biofilms at CEM. These were the thickest biofilms (arrows) observed above the sediments from the riverbed (Sed). Bar = 1 mm. (C) Features of the different layers of *Pinnularia* (Pin) and minerals (Min) in biofilms from CEM. Bacterial populations were located between the layers of diatoms (arrows). Bar = 100  $\mu$ m. (D) Detailed image of bacterial communities (Bac) between layers of *Pinnularia* (Pin). Bar = 30  $\mu$ m. (E) Detailed image of a *Euglena* dominated biofilm between minerals (Min) and diatoms (Pin), obtained from the CEM site. Bar = 60  $\mu$ m. (F) Detailed image of a *Cyanidium* biofilm from CEM; the presence of bacteria is indicated by arrows. Bar = 40  $\mu$ m.

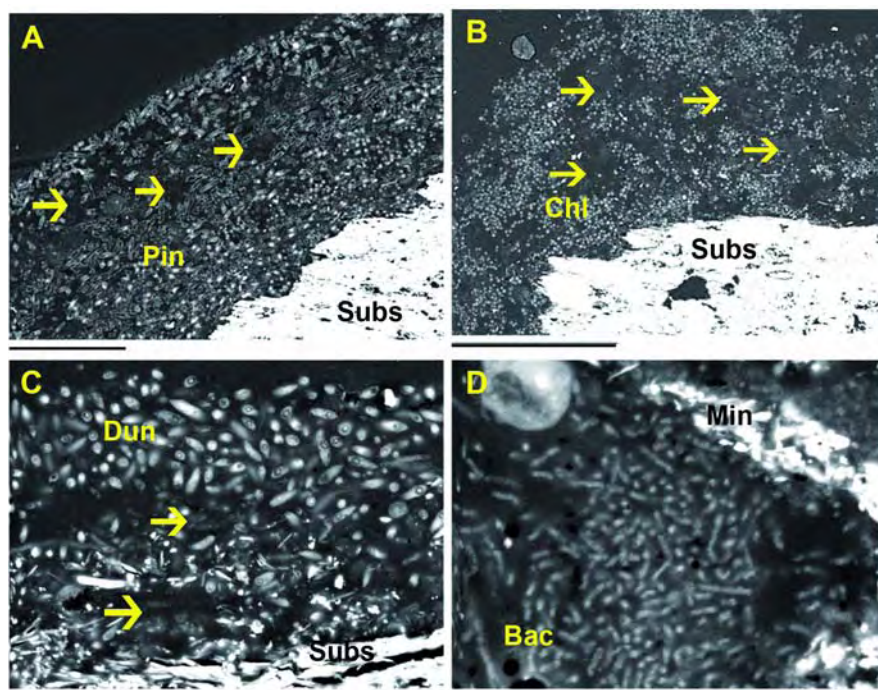
A comparison of biofilms from the same locality showed that ANG biofilms were associated with few bacteria clusters. The majority of the cells that hybridized with the Alpha-proteobacteria probe were identified as *Acidiphilium* spp. Gammaproteobacteria were present in the *Chlorella* biofilms and hybridized with the THIO1 probe (genus *Acidithiobacillus*). The *Nitrospira* probe gave positive results in both the *Pinnularia* and the *Chlorella* biofilms (Fig. 4E). *L. ferrooxidans* was the only *Leptospirillum* detected in the *Pinnularia* biofilms whereas all the other leptospirilli were found in the *Chlorella* biofilms. The *Archaea* domain probe gave positive results only in the *Pinnularia* biofilms (Table 3).

Bacteria detected in the *Euglena* and *Pinnularia* biofilms from AG were identified as Alpha- and Gammaproteobacteria (Fig. 4C). Use of the ACD638, ACD821 probes showed the presence of *Acidiphilium* spp. in the biofilms obtained from those two sites. However, the THIO1 probe showed that *Acidithiobacillus* spp. were present only in *Pinnularia* biofilms. Probe LEP634 showed the presence of *L. ferrodiazotrophum* only in *Euglena* biofilms, while hybridization with probe LEP636 detected the

presence of *L. ferrooxidans* in *Pinnularia* biofilms. With the *Actinobacteria* and *Archaea* probes, positive results were obtained only in the *Pinnularia* biofilms from this locality (Table 3).

The most abundant prokaryotic community of all the biofilms analyzed was found in the algal photosynthetic biofilms from site CEM (Table 3, Fig. 4B,D). Clusters of Alphaproteobacteria were identified as *Acidiphilium* spp. Most of the bacteria forming long chains and identified as Gammaproteobacteria gave specific hybridization signals with the probe for *Acidithiobacillus* spp. (Fig. 4D). Of the *Nitrospira* phylum, only members of the *L. ferrodiazotrophum* group were identified in all the biofilms from this site. No positive hybridization signals were detected with probe LEP636, specific for *L. ferrooxidans*. In addition, probe LEP154 (specific for *L. ferriphilum*) gave positive hybridization signals only in the *Euglena* biofilms. Some clusters of Betaproteobacteria were identified in *Pinnularia* and *Euglena* biofilms. The *Actinobacteria* probes showed positive hybridization signals only in the *Euglena* biofilms from site CEM (Table 3).

Algal photosynthetic biofilms in site RI had the fewest prokaryotic clusters. Alpha- and Gammaproteobacteria were only



**Fig. 3.** SEM-BSE images of algal photosynthetic biofilms studied from sites ANG and RI. (A) *Pinnularia* (Pin)-dominated biofilm on a rock fragment as a substrate (Subs) at site ANG. Between the diatoms there are clusters of bacteria (arrows). Bar = 100  $\mu$ m. (B) *Chlorella*-dominated biofilms were also found at site ANG on the rock fragments (Subs). Clusters of bacteria are present inside the biofilm (arrows). Bar = 200  $\mu$ m. (C) A biofilm of *Dunaliella* (Dun) from RI. Notice the thinness of the biofilm above the substrate (Subs). Bar = 40  $\mu$ m. (D) Detailed aspect of the bacteria (Bac) found in the *Dunaliella* biofilms from RI. Note the different shapes in contrast to those of the mineral particles (Min). Bar = 10  $\mu$ m.

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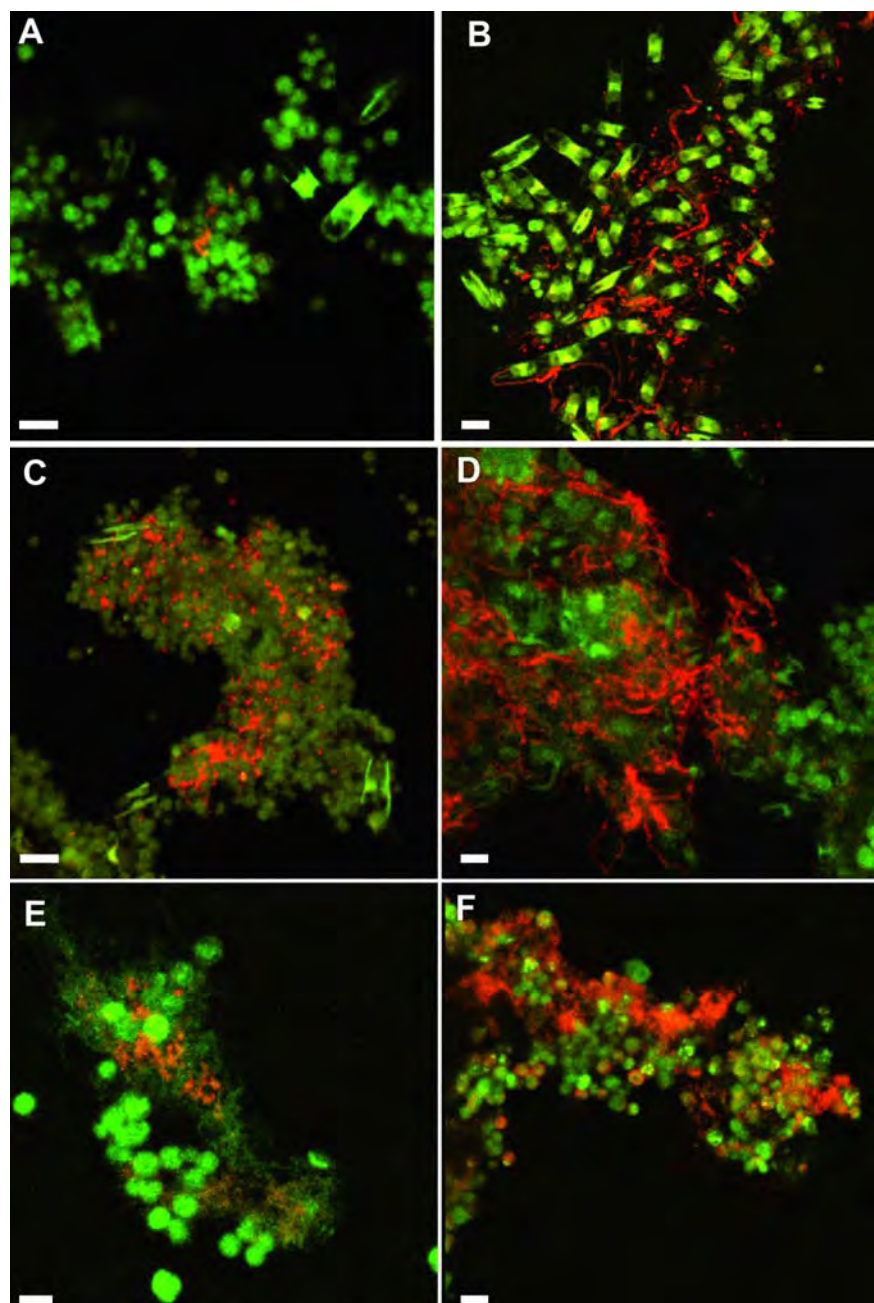
identified with FISH probes in the *Cyanidium* biofilms. Positive hybridization signals were obtained for all the leptospirilli species in the *Dunaliella* and *Cyanidium* biofilms. The *Actinobacteria* probes yielded positive results only in the *Dunaliella* biofilms from this sampling site. Members of the *Nitrospira* phylum were the most abundant prokaryotes in RI, as evidenced by the positive results obtained with probe NTR712 (Fig. 4F).

**Phylogenetic analysis of the prokaryotic community in algal photosynthetic biofilms.** The acidophilic prokaryotes associated with algal photosynthetic biofilms included sequences related to the classes Alpha-, Beta-, and Gammaproteobacteria (phylum Proteobacteria) as well as the phyla Nitrospira, Actinobacteria, Acidobacteria, and Firmicutes. Sequences from the *Archaea* domain were also identified. The 66 sequences of 16S rRNA genes retrieved from the algal photosynthetic biofilm samples were grouped in seven OTUs based on 97% sequence similarity. No more than seven distinct lineages were detected in any biofilm except for those from RI, in which the diversity was lower.

Within the Gammaproteobacteria, the 13 most abundant phylotypes were related to *Acidithiobacillus ferrooxidans* with the exception of site RI, from which *At. ferrooxidans* sequences were not retrieved. The sequences retrieved from the *At. ferrooxidans* group clustered into the four groups previously described using FISH probes [22]. Sequences related to groups *a* and *b* were not detected in the biofilms from ANG, whereas sequences belonging to groups *c* and *d* were present in all sites.

Within the Beta- and Gammaproteobacteria, two groups of sequences related to uncultured bacteria from other acidic mine drainage habitats were retrieved. Two sequences clustered next to the 16S rRNA gene sequence of the Gammaproteobacteria WJ2, an iron-oxidizing bacteria isolated from moderate acidic mine drainage waters [24], and next to bacterial streamers in acidic waters [20,25]. Five sequences retrieved from CEM and AG biofilms were found to be related to sequences previously detected in Río Tinto streamers [20] and to uncultured bacteria from forested wetlands soils impacted by reject coal [13].

With respect to the Nitrospira phylum, most of the retrieved sequences (twenty-one) clustered with *Leptospirillum* strains. Sequences related to *L. ferrooxidans* were retrieved from all four localities studied, while sequences from *L. ferriphilum* and *L. ferrodiazotrophum* were retrieved only from the ANG and RI biofilms. Within the Alphaproteobacteria, the most abundant phylotypes (eight) were related to uncultured bacteria belonging to Acetobacteriaceae, isolated from acid mine drainage habitats [24]. These sequences were retrieved from all sampling sites except RI. A group of these sequences (three) retrieved from CEM was related to the genus *Acidiphilium*. Sequences related to other heterotrophic acidophilic bacteria, such as *Acidocella* and *Acidosphaera*, normally present in other acidic environments [26,39], were not detected but the rest of the sequences (five) were determined to be related to uncultured bacteria from acid mine drainage environments. The absence of other heterotrophic acidophilic bacteria might be explained by the low amounts of organic carbon sources present in the river.



**Fig. 4.** Laser-scanning confocal images from algal photosynthetic biofilms hybridized with bacteria-specific Cy3 labeled probes. The bacteria are shown in red; the green fluorescence is an artifact of formaldehyde fixation. Bar = 5  $\mu$ m. (A) *Chlorella* biofilm from ANG hybridized with the universal probe for *Bacteria* EUB338. (B) *Pinnularia* biofilm from CEM hybridized with the universal probe for *Bacteria* EUB338. (C) *Pinnularia* biofilm from AG hybridized with the GAM42a probe, specific for Gammaproteobacteria, distributed as small clusters. (D) Laser-scanning confocal image of a *Euglena* biofilm from CEM hybridized with the THIO1 probe, specific for the genus *Acidithiobacillus*. (E) *Chlorella* biofilm from ANG hybridized with a probe specific for the Nitrospira phylum (NTR712). (F) The Nitrospira phylum probe NTR712 was also positive in *Cyanidium* biofilms from RI.

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Regarding the Firmicutes phylum, one sequence from RI was related to the genus *Sulfobacillus* and two sequences from AG and CEM were related to gram-positive acidophilic iron-oxidizing bacteria of the genus *Alicyclobacillus* [27] and to uncultured endolithic communities from geothermal environments [44]. Within the Actinobacteria phylum, two groups of sequences were identified. The first set (three sequences), retrieved from sites ANG and CEM, clustered with uncultured Actinobacteria related to *Microthrix*, present in the rhizosphere bacterial community of *Erica andevalensis*, an endemic heather from the Iberian Pyrite Belt [33], and with uncultured bacteria

sequences from freshwater environments [38,45]. Five of the second set of six sequences were related to "*Ferrimicrobium acidiphilum*" and were present in all the localities studied; one sequence was related to uncultured bacteria also found in an acid mine drainage system located in the Iberian Pyrite Belt [40]. Sequences within the Acidobacteria were related to an uncultured soil bacterium from the rhizosphere of the above-mentioned *Erica andevalensis* [33] and to other sequences from forested impacted acidic soils or polluted soils [35]. This group of sequences was detected only in samples from the CEM and AG localities.

**Table 3.** Relative abundance of the main groups of prokaryotic microorganisms using fluorescence in situ hybridization with probes with different specificity

| Dominant alga in biofilm | EUB 338 <sup>a</sup> | ALF 968 | ACD 638<br>ACD 821 | GAM 42a | THIO1 | NTR 712 | LEP 154 | LEP 634 | LEP 636 | BET 42a | FM 732<br>FMR 0732<br>ACM 1160 | TMP 654<br>FER 656 |
|--------------------------|----------------------|---------|--------------------|---------|-------|---------|---------|---------|---------|---------|--------------------------------|--------------------|
| <i>Chlorella</i> (ANG)   | +                    | +       | ±                  | +       | +     | +       | ±       | +       | ±       | –       | +                              | –                  |
| <i>Pinnularia</i> (ANG)  | +                    | ++      | ++                 | –       | –     | ++      | –       | –       | ±       | –       | +                              | +                  |
| <i>Euglena</i> (AG)      | +                    | +       | ±                  | +       | –     | ±       | –       | ±       | –       | –       | –                              | –                  |
| <i>Pinnularia</i> (AG)   | +                    | +       | ±                  | +       | ±     | ±       | –       | –       | ±       | –       | +                              | +                  |
| <i>Cyanidium</i> (RI)    | +                    | ±       | –                  | ±       | –     | ±       | ±       | ±       | ±       | –       | –                              | –                  |
| <i>Dunaliella</i> (RI)   | +                    | –       | –                  | –       | –     | ±       | ±       | ±       | ±       | –       | ±                              | –                  |
| <i>Cyanidium</i> (CEM)   | +                    | –       | ±                  | ±       | ±     | ±       | –       | ±       | –       | –       | –                              | –                  |
| <i>Pinnularia</i> (CEM)  | ++                   | ++      | ++                 | ++      | ++    | ±       | –       | ±       | –       | ±       | –                              | –                  |
| <i>Euglena</i> (CEM)     | ++                   | ++      | ++                 | ++      | ++    | +       | +       | +       | –       | ±       | +                              | –                  |

Key: (++) high number of cells; (+) one order of magnitude lower number of cells; (±) low amount of cells; (–) no hybridization signal.

<sup>a</sup>This column indicates general abundance of bacteria detected with the universal probe for *Bacteria* EUB338.

In the domain *Archaea*, three different sequences from sites AG and CEM were found to be related to uncultured *Archaea* from streamers collected in Río Tinto waters [20] or other acid mine drainage habitats [9]. These sequences are related to those of the Thermoplasmata, a group detected in other acid mine drainage environments [10].

## Discussion

Algal photosynthetic biofilm communities in the acidic waters of Río Tinto seem to play two contrasting and indirect roles in controlling redox transformations of iron in water. On the one hand, by oxygenating water they promote iron oxidation; on the other hand, they provide organic carbon via their associated exopolysaccharides, which can be used by iron-reducing heterotrophic acidophiles. This second aspect is clearly related to the presence of *Acidiphilium* spp. in all the localities except RI (Table 3). *Acidiphilium* spp. can use ferric iron as an electron acceptor for anaerobic respiration of reduced organic compounds. The amount of organic biomass coming from the thinnest algal photosynthetic biofilms at the most extreme location, i.e., RI, was lower than in any of the other sampling sites.

In addition, the structure of the biofilms at AG and CEM apparently included microniches that could facilitate the development of less extreme-adapted organisms coming from soil habitats. In these algal photosynthetic biofilms we found

sequences related to uncultured organisms present in acidic or contaminated soil habitats, such as Betaproteobacteria and Acidobacteria. Previous studies of prokaryotic communities associated with the rhizosphere of plants that grow along the banks of Río Tinto have shown that the main groups present are Actinobacteria and the Acidobacteria [34]. The existence of microniches with different environmental conditions might explain why, to date, the highest prokaryotic diversity is associated with localities in which algal photosynthetic biofilms develop [21,22]. Following this argument, it seems that thinner algal photosynthetic biofilms, such as those present in the most extreme study location, i.e., RI, have less influence in the formation of microniches; consequently, their prokaryotic community is more closely related to the one present in the water column. Site ANG is probably an intermediate case: its *Pinnularia* biofilms contained more diverse prokaryotic communities, with few clusters of *L. ferrooxidans* and *Acidiphilium* spp.; Actinobacteria and *Archaea* were found in the area in contact with the substratum. The prokaryotic communities of the *Chlorella* biofilms, obtained from the ANG site, were more similar to those in the water column, with clusters of *Leptospirillum* spp., *At. ferrooxidans*, and *Acidiphilium* spp.

Previous studies have found that one of the most representative bacterial species in the water column belongs to Gammaproteobacteria, whose main representative is *At. ferrooxidans*, related to the oxidation of iron [22,31]. This group was also present with high sequence diversity and abundance in the algal pho-

tosynthetic biofilms studied except in those from RI, where the most abundant bacterial group was *Leptospirillum* spp. The capacity of *L. ferrooxidans* to grow at a lower pH than *Acidithiobacillus* was noted in other studies [38,43] and may explain the absence of *At. ferrooxidans* in the most extreme (pH 1.8) location studied (RI).

Of the three known leptospirilli species, only *L. ferrooxidans* has been identified as an important member of the prokaryotic community present in Río Tinto's waters [20–22]. *L. ferriphilum* [14] and *L. ferrodiazotrophum* [43] were only detected in low numbers in the water column and sediments of some locations [20]. However, the presence of all three types of leptospirilli was observed in subaerial lithotrophic biofilms in other extreme acid mine drainage sites [11]. It should be noted that all *Leptospirillum* species were well-represented in the algal photosynthetic biofilms from RI and in the *Chlorella* biofilms from ANG, indicating more favorable conditions for *Leptospirillum* in environments with algal photosynthetic biofilms and more extreme conditions of pH. Although sequences related to *L. ferriphilum* and *L. ferrodiazotrophum* were not recovered in the thicker algal photosynthetic biofilms from CEM, FISH analysis indicated that some bacterial clusters were identified as belonging to *L. ferriphilum* and *L. ferrodiazotrophum*. This suggests the presence of only a few restricted microenvironments in the algal photosynthetic biofilms in which these species might develop [37].

A comparison of the *Pinnularia* biofilms present in most of the sampling locations (ANG, AG, CEM) showed that they were consistently the most diverse biofilms in terms of their prokaryotic content. These results indicate that the different layers of diatoms intermixed with sediments form thicker biofilms and generate specific conditions facilitating the growth of microorganisms that otherwise would be unable to develop. The lowest diversity was found in the *Cyanidium* biofilms from RI and CEM. Since the environmental conditions of the water column are rather different in those two locations, biodiversity is more likely related to the habitat selected by *Cyanidium* in the formation of biofilms in acidic environments. A similar case was seen in the *Euglena* biofilms from AG and CEM, in which very different levels of diversity were observed, low for AG biofilms and high for CEM one. The presence of the highly diverse *Pinnularia* biofilms in close contact with *Euglena* biofilms might explain the increase in prokaryotic communities at the CEM site.

Environmental sequences obtained by cloning and FISH probes can be designed to determine the abundance and spatial location of microbial populations in specific microenvironments in these and other extreme environments. FISH is a very promising technique to evaluate the presence and distribution of specific prokaryotic microorganisms in algal photosynthetic acidic biofilms. This, in turn, should facilitate in-situ structure/function analyses by combining FISH with ultrastructural descriptions of the biofilms in their natural habitats.

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