Distribution and Seasonal Varibility in the Benthic Eukaryotic Community of Río Tinto (SW, Spain), an Acidic, High Metal Extreme Environment

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Running Title: Distribution of eukaryotes in an extremely acidic, high-metal river.

Abstract

The eukaryotic community of Río Tinto (SW, Spain) was surveyed fall, winter, and spring through the combined use of traditional microscopy and molecular approaches including Denaturing Gradient Gel Electrophoresis (DGGE) and sequence analysis of 18S rRNA gene fragments. We compared eukaryotic assemblages of surface sediment biofilms collected in January, May and September 2002 from 13 sampling stations along the river. Physicochemical data revealed extremely acidic conditions (pH ranged from 0.9 to 2.5) with high concentrations of heavy metals including up to 20 g l^{-1} Fe, 317 mg l^{-1} Zn, 47 mg l^{-1} As, 42 mg l^{-1} Cd, and 4 mg l^{-1} Ni. In total, 20 taxa were identified, including members of the *Bacillariophyta*, *Chlorophyta*, and *Euglenophyta* phyla as well as ciliates, cercomonads, amoebae, stramenopiles, fungi, heliozoan and rotifers. In general, total cell abundances were highest in fall and spring decreasing drastically in winter and the sampling stations with the most extreme conditions showed the lowest number of cells as well as the lowest diversity. Species diversity does not vary much during the year. Only the filamentous algae showed a dramatic seasonal change almost disappearing in winter and reaching the highest biomass during the summer. PCA showed a high inverse correlation between pH and most of the heavy metals analyzed as well as *Dunaliella* sp., while *Chlamydomonas* sp. is directly related to pH during May and September. Three heavy metals (Zn, Cu and Ni) remained separate from the rest and showed an inverse correlation with most of the species analyzed except for *Dunaliella* sp.

Keywords: acidophilic organisms, protists, acidic environment, pH, phytobenthos, water chemistry.

Introduction

The discovery of extreme environments and their endemic residents have recently taken on significance for several reasons: 1) modern extreme environments are believed to reflect both early Earth conditions as well as those that persisted for most of the planet's life history [40] and, 2) modern extreme environments may contain organisms that produce commercially important enzymes and cell products (i.e. thermostable DNA polymerases or heat-stable proteases) [31, 39].

One group of extremophiles that is becoming increasingly important is acidophilic microorganisms. These organisms thrive at low pH (<3), by maintaining their cytoplasm at the same pH as their neutrophilic relatives [29] through the use of mechanisms that may involve secondary proton uptake mediated by membrane-associated antiporters, unusual cell wall permeability properties, or high internal buffer capacity [34].

Since extreme acidic environments are often the consequence of anthropogenic influences (e.g., mining activity or acid rain), most ecological studies of acidic waters have been focused on environments affected by human activity. In this regard, Río Tinto (SW, Spain), is one of the most unique examples of extreme acidic environments, not only for its non-anthropogenic origin but for its peculiar microbial ecology [3, 13, 14]. The river originates in the massive bodies of iron and copper sulfides that make up the Iberian Pyritic Belt, and maintains a constant low pH (pH 1.0-2.5), buffered by ferric iron and with high concentrations of heavy metals that are toxic to numerous aquatic organisms [8, 13, 14]. These extreme conditions are the product of the metabolic activity of chemolitotrophic microorganisms, including iron- and sulfur-oxidizing bacteria that can be found in high numbers in its waters [17].

However, what makes Río Tinto a most unique extreme acidic environment is the unexpected degree of diverse eukaryotic organisms that are the principal contributors of biomass to the river, over 65% of the total biomass [3, 4, 25, 36]. Green algae, diatoms and euglenoids as well as ciliates, cercozoans, amoebae, stramenopiles, and fungi have been detected.

Thus, Río Tinto has attracted some attention in literature, mainly in the fields of geochemistry [13, 14] and microbial diversity [3, 17, 25]. The aim of this work is to complete the studies carried out until now in Río Tinto regarding the eukaryotic community. Although some recent papers have been published [1, 2], none of them reports a complete description of the eukaryotic species in relation with their spatial distribution along the river as well as their quantitative seasonal variation during one year. This study analyses also the temporal patterns in relation to physical and chemical conditions of the water, which is important to understand the ecology of these communities. In order to achieve this, several methods were used to assess the presence and degree of abundance of specific eukaryotic taxa. Traditional techniques involving species identification based on morphological and morphometric criteria as well as molecular techniques, such as DGGE or gene cloning, have been used. Multivariate statistics were used to determine trends and infer possible interactions between eukaryotic benthic communities and water physico-chemistry characteristics.

Materials and methods

Study site and environmental parameters

Río Tinto is located in southwestern Spain and can be divided into three main zones based on topological, geological and geochemical characteristics, northern, transitional and estuarial (Fig. 1) [13]. The headwaters of the river are characterized by

extreme physico-chemical conditions in terms of low pH, with an annual mean value of 2.2, and high concentrations of heavy metals, such as total Fe (up to 20 g l^{-1}), Cu (100 mg l^{-1}) and Zn (235 mg l^{-1}) [1]. Due to this fact, we concentrated our sampling in the upper part of the river.

A general description of the Río Tinto physico-chemical parameters as well as geological records and hydrochemistry conditions was carried out previously [1, 25]. Seasonal variations in geochemical conditions result from alternating wet and dry seasons, during the winter and summer months respectively. January rainfalls in excess of 120 mm are common, contrasting with the little or no rainfall observed from July through September.

Based on previous studies, 13 sites along Río Tinto (between 0 and 50 Km from its source) were selected for *in situ* measurements, water sampling and eukaryotic benthic communities collection [1] (Fig. 1). Samples were taken in January, May and September 2002. Measurements of conductivity, temperature (conductimeter Orion 122, Orion Research, USA), redox and pH (pHmeter Crison 506 pH/Eh), and Oxygen (Orion 810 oxymeter) were made in triplicate. Chemical analyses were carried out as described previously [25].

Sample collection

At each sampling site, two transects were defined perpendicular to the water current. Water depth was always between 1 to 5 cm. The sampling unit, a 10 × 10 cm quadrant with a grid of 100 equally spaced intersection points of $1cm^2$, was positioned along the transects as many times as it fit [41]. Each square of the grid was numbered and 10 of them were randomly chosen taking the sequence numbers from a random numbers table and ranking them from the smallest to the largest [27, 42]. The biofilm

occurring underneath each chosen square were resuspended in 1ml of filtered river water and combined. Two subsamples of 5 ml each were obtained in order to perform the microscopy identification and the DNA extraction.

Samples for light microcopy analysis were collected into sterile 1.5 ml tubes, fixed in 5% (wt/vol) formaldehyde and stored until processed. Samples for DNA extraction were also collected directly into sterile 1.5 ml tubes and kept at 4ºC until processed (within 3 days).

Microscopy and morphotype quantification.

Identification of algae and heterotrophic protists was carried out up to the lowest possible taxonomic level by direct microscopic observation using different phenotypic features based on previous studies of the eukaryotic communities in this river [3, 4, 25] as well as using traditional identification keys [7, 9, 22, 23, 28, 35, 43]. The microscope used was a Zeiss Axioscope 2 equipped with phase-contrast. Cell counts were performed in triplicate in a Sedwerick-Rafter chamber. For mean cell or filament volume estimates, at least 10 individuals where measured for size with a calibrated ocular micrometer , and volumes were calculated based on standard geometric formulas [18]. Biovolume of each species was the product of the count/liter and the mean volume.

DNA extraction

The Fast DNA Spin kit for soil (Bio 101, Carlsbad, CA, USA) DNA extraction was used according to the manufacturer's instructions. To disrupt the cells, the mixture of ceramic and silica beads provided in the kit and six pulses of 40 sec at speed 5.5 of the FastPrep bead beating instrument (Bio 101) were applied. Samples were washed

five times with TE Buffer (10mM Tris HCl [pH 8], 1mM EDTA) before DNA extraction in order to remove the natural acidic water.

PCR for DGGE analysis

Universal eukaryotic primers targeting 18S rDNA were used. For DGGE we tested the primers Euk20F (5'GTA GTC ATA TGC TTG TCT C 3') and Euk516r-GC (ACC AGA CTT GCC CTC CCG CCC GGG GCG CGC CCC GGG CGG GGC GGG GGC ACG GGG GG 3'), which amplifies fragments sized about 500 bp [2]. PCR were run in a Perking Elmer Cycler in 150 µl tubes using 100 µl reaction volumes. The reaction mixture contained 5 µl of template, 0.5 µl of both primers (50 µM), 1 µl of dNTPs 25 mM, 1x reaction buffer and 0.5 U of Taq DNA polymerase (Promega Co., Madison, USA). The program included an initial denaturation at 94ºC for 5 min, followed by 29 touchdown cycles of denaturation at 94ºC for 1 min, annealing at 64ºC (with the temperature decreasing 0.5ºC each cycle) for 1 min and extension at 72ºC for 3 min. Final extension was done at 72º for 6 min. Aliquots of 5 µl of the PCR product were run in 1% agarose gel at 130V for 1 hr, stained with ethidium bromide, and quantified by using a standard (Φ29 digested with *Hind*III).

DGGE electrophoresis

The DGGE was carried out as previously described [2]. Briefly, electrophoresis was performed with 0.75 mm thick 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide 37.5:1) and the denaturing gradient was urea 20% to 60%. The running buffer was TAE 1x (40 mM Tris, 40mM acetic acid, 1mM EDTA; pH 7.4) at 60ºC. Approximately 100 ng of PCR products were mixed with the same volume of loading dye (2% bromophenol blue, 2% xylene cyanol, 100% glycerol) and applied to

individual lanes. Gels were run at 60ºC for 11 hr at 100 V, stained with ethidium bromide and visualized under UV illumination.

DNA bands were cut from the gel with sterile razor blades and placed in sterilized vials, 20 µl of milliQ water was added. The DNA was allowed to diffuse into the water at 4ºC overnight. Five microliters of the eluate were used as a template DNA in a PCR with the primers described above.

Clone library construction

Clone libraries of complete 18S rDNA genes were generated from five environmental DNA templates collected at the following sampling stations AG, FE, ANG, EST and LPC. 18S rDNA genes were amplified from the environmental DNA extractions by PCR with two general eukaryotic primers, Euk1a (5' CTG GTT GAT CCT GCC AG 3') and Euk1800r (5' TCC GCA GGT TCA CCT AGC GA 3') [2]. PCR conditions were as follows, initial denaturation at 97ºC for 5 min, followed by 29 cycles of denaturation at 94ºC for 40 sec, annealing at 48ºC for 1 min, and extension at 72ºC for 1 min. The amplification products were used to construct a clone library with the TOPO 1 Kit (InvitroGen, Carlsbad, CA, USA). Positive transformants were checked for correct insert size by standard agarose gel electrophoresis.

Sequence analysis

The PCR products were directly sequenced with dye terminator cycle sequencing kit (Big-Dye 1.1 sequencing kit, Applied Biosystems) as described in the manufacturer's instructions. The sequences were aligned to 18S rRNA sequences obtained from the National Center of Biotechnology Information Database. The sequences were also checked for potential chimeras with the Bellerophon Chimera

Check program and were subsequently aligned with 18S rRNA reference sequences in the ARB package (http://www.arb-home.de). The rRNA alignment was corrected manually and alignment uncertainties were omitted. Only unambiguously aligned positions were used to construct phylogenetic trees with ARB. Distance analysis using the Jukes-Cantor correction [19] and bootstrap resampling (100 times) were performed, and the distance matrix was used to construct a tree via neighbor-joining method [37]. Parsimony and maximum likelihood analysis was performed using DNAPARS from the PHYLIP package [12]. Sequences have been deposited in GenBank under accession numbers EF591004-EF591019.

Statistical Analysis

All physical chemical and microbiological parameters for each sampling site and season were organized in a single matrix. An estimate of possible relations between physicochemical and biological parameters was developed by correlation analysis and Principal Components Analysis (PCA), performed with Statistica V.6.0 program. This analysis allows us to ascertain the origin of each element based on its level of association with the others and to determine the factors that control its behaviour. The method reduces the original variables to a smaller number of factors, those representing the original variables with a minimal loss of information.

Results

Sample locations and physicochemical parameters

Sample locations and sample types were chosen to cover most of the area under study and a range of the visibly different microbial assemblages that occur there. Water physicochemical measurements were taken from 13 locations distributed along the upper 50 km of the river (Fig. 1), together with samples of submerged eukaryotic mats

attached to the sediments. Zones A and B are the headwaters and can be considered the origin of the Río Tinto. Zone A includes site AG, where a small surface stream combines with water seeping out of a bank that has some of the most conspicuous biofilms on the river, and site 3.2, a small impoundment behind an old dam that is typically anoxic at the bottom. Zone B is the major headwater system (Fig. 2a); site Iz-Iz, nominally considered the "origin" of the river, is a trickle of very red water springing from the base of a large pile of rocks, and FE is a site located a few meters beyond where two other small streams join Iz-Iz.

Downstream from FE, site ANG is a spring coming from a pile of loose rocks; here the water temperature is high, presumably due to microbial metabolism inside the rock pile. Sites NUR and UMA are a seep and a small stream with moderate water flow that join the river downstream from ANG, while RI is located in the exit of a small tunnel with only periodic water flow associated with rainy periods. The drainages from Zones A and B merge just north of the town of Nerva into a small river flowing west of the town. Site CEM is located just downstream from the merging point (about 10km downstream from the origin); site STB is a small stream coming from a flooded mine shaft that joins the river and has conspicuous biofilms. At site EST, Río Tinto has become a wide stream with a high water flow all around the year.

The last two stations, BRR and LPC, are located at ca. 25 Km and 50 Km respectively from the origin of the river (Fig. 2b,c). At these locations, the river becomes wider and deeper, with an average water flow of 8.1 m^3/s throughout the year [25]. The slope of the river between these two stations is gentle, ca.0.56% average value, resulting in a moderate current speed that facilitates the settlement of dense microbial community mats covering large sections of the river course [25]. Sampling site BRR is a section where iron precipitates are beginning to make the water cloudy red

and by LPC the pH buffering capacity of the iron in the river is nearly exhausted, resulting in occasional spikes of higher pH where circumneutral side streams enter the main river.

Physical and chemical water data collected during this study are given in Table 1 and 2. The highest values for heavy metals were found in the stations located near the origin of the river. In this regard, Iz-Iz, RI, ANG and UMA were locations with lowest pH and highest concentrations of dissolved metals. Concentrations of most ions decreased downstream but proportions varied between sampling sites: Iz-Iz showed the highest concentration of Cd (more than 40 mg l^{-1}), FE had the highest Zn (more than 160 mg l^{-1}), RI had the lowest pH (1.2) and highest values of S (ca. 29 g l^{-1}), Fe (ca. 22 g l^{-1}), Co (61.9 mg l^{-1}), As (47.8 mg l^{-1}), and Cr (13.1 mg l^{-1}), while CEM was rich in Cu (278.3 mg l^{-1}) and Ni (7.9 mg l^{-1}).

The dominant characteristic of Río Tinto water chemistry is the extremely high proton concentration which influences other chemical parameters. Thus, extreme acidity is combined with high concentrations of calcium, silica, sulfate, manganese or arsenic and heavy metals as iron, zinc, cobalt, copper, chrome or cadmium. In general, concentrations of chemical elements rise during summer and fall and decrease in winter due to dilution by rain (data not shown).

Eukaryotic community structure and spatial distribution

The eukaryotic community is mainly distributed into biofilms of different thicknesses all over the riverbed (Fig. 2d-i). A total of 20 taxa were microscopically distinguished (Table 3).

At sites Iz-Iz and FE, species related to different *Chlorophyta* genus (*Chlorella* sp., *Chlamydomonas* spp., *Mesotaenium* sp. and *Stichococcus* sp.) dominated the

phytobenthonic community (Fig. 3). *Dunaliella* sp. was the dominant species at Iz-Iz, as well as *Euglena* sp. and the filamentous algae, represented by the genera *Zygnemopsis* and *Klebsormidium*, were present at high numbers in FE, forming thick green filamentous biofilms all over the sediment (Fig. 2d). Although algae accounted for the greatest proportion of the biomass, there are other protozoan components present in lower proportions (usually less than 5% of the total cell number), resulting in increased biodiversity. Here we found one species of heliozoa, tentatively identified as *Actinophrys* sp., amoebas and three morphotypes of small flagellates belonging to the genera *Cercomonas, Bodo* and *Ochromonas*.

Stations AG, RI and ANG were also dominated by Chlorophyta species, however the diversity was higher in this part of the river (Fig. 3). AG was the first station with visible amounts of the diatom *Pinnularia* sp., along with *Cyanidium* sp. Euglenoids, and filamentous algae were also well represented. A completely different taxa composition was found at site RI. Here the predominant species were *Dunaliella* sp. and *Cyanidium* sp. (Fig. 2e) between them representing ca. 80% of the total biomass, followed by *Chlorella* sp. Amoebas and the cercomonad flagellates were also found in lower numbers. Site ANG was covered by green mats formed mainly by *Chlorella* sp., although *Pinnularia* sp. was also present forming small brown patches (Fig. 2f) as well as *Cyanidium* which makes up ca. 15 % of the total biomass. Amoebas, flagellates similar to *Bodo*, *Cercomonads* and *Ochromonas*, as well as the ciliate *Oxytricha* were also present at this site.

Sites 3.2, NUR and UMA were very different from each other (Fig. 3). The community present at 3.2 was mainly formed by euglenoids (ca. 90% of the biomass). Ciliates constituted ca. 5% of the total cell number, and amoebas and heliozoans were found in low numbers. This is the place closest to the origin of the river where a species

of bdelloid rotifer had been found. Site NUR is dominated by filamentous algae, euglenoids and diatoms which all together represent more than the 80% of the biomass. A thick green filamentous film covered all the sediment mixed with brown patches formed by diatoms (Fig. 2g). Ciliates, amoebas and heliozoans have also been found as well as small cercomonad flagellates. UMA showed a completely different community assemblage inhabited mainly by *Dunaliella,* small cercomonad flagellates, amoebas and *Oxytricha* ciliates. In this part of the river, no visible cell biofilms were found.

Near the town of Nerva, diversity increases at sites CEM and STB (Fig 3). *Chlorella* sp*., Pinnularia* sp., *Cyanidium* (Fig. 2h) and filamentous algae were most abundant. In addition, *Chlamydomonas* spp*., Euglena* sp*.,* amoebas and small flagellates also occurred at these places. Ciliates similar to *Colpidium* were observed along with *Oxytricha* ciliates. Site EST was mainly inhabited by *Euglena* and *Chlorella* (Fig. 2i, Fig. 3k). *Oxytricha* ciliates, amoebas and cercomonad flagellates contributed five species, but only 23% of the biomass.

Further downriver at site BRR, *Euglenas* dominated the community mixed with some amoebas and cercomonad flagellates (Fig. 3). No diatoms have been found at this site. LPC showed more diversity, with an increase in two green algae, *Chlamydomonas* and *Chlorella*. Other recorded groups included diatoms, *Zygnema* sp*., Stichococcus* sp., ciliates, amoebas, heliozoans and heterotrophic flagellates.

Total abundances and seasonal distribution

Seasonal variation of the eukaryotic communities as well as total cell numbers are shown in Fig. 3 and 4. Although cell numbers in free-flowing water were generally low ($<10^2$ cells ml^{-1}), in the biofilms they were considerably higher (up to 10^7 cells cm^2). Total cell abundances were generally highest in May and September decreasing

drastically in January. However, at five sampling stations the cell number remained relatively constant throughout the year (e.g. ANG, RI or CEM). The stations with the most extreme physicochemical conditions (Iz-Iz, RI and UMA, located at the origin of the river) showed the lowest number of cells (ca. 10^3-10^5 cells cm^{-2}) as well as the lowest diversity.

In general, although great differences were found in total cell number, the species diversity remained fairly constant during the year at most of the sampling stations (Fig. 3). Only the filamentous algae showed a dramatic seasonal change, almost disappearing in winter and reaching their highest cell number during the summer (e.g. FE and NUR sampling sites). There was also variability in the presence of heterotrophic flagellates. These groups typically increased their number during winter and spring to decrease in summer (e.g. Iz-Iz and UMA sampling stations). Ciliates were more common during summer and late summer, tracking the variation of the algae (e.g. 3.2 sampling site). Rotifers were found during the entire period from June to September decreasing substantially in winter. The heliozoans analyzed appeared in spring and disappeared in winter.

Principal Component Analysis (PCA)

Figure 5 shows the distribution of the variables in the space formed, during the year assayed, by the first two components of the PCA analysis. In January (Fig 5a), 63.66 % of the total variance is explained by the two first components of the analysis. In this season, five principal components (PC) with an eigenvalue >1 were extracted. PC1 explained 34.28 % of the observed variance and included the variables pH, conductivity, Fe, Co, As, Cd, Cr as well as *Dunaliella* sp. PC2 explained 29.38% of the observed variance and contained Zn, Cu, Ni and *Chorella* sp., Diatoms, Euglenoids,

Filamentous algae *Cyanidium* sp. and *Chlamydomonas* spp. PC3 (T^a, an heterotrophic flagellates) represented 12.6 % of the variance and PC4 and PC5 (Redox and Oxygen) were of less importance, explaining only 7.1 and 5.7 % of the variance. PC1 can be described as an abiotic factor as it contained most of the physicochemical parameters, while, PC2 had a strong biotic component including most of the species studied. Some variations were obtained when the variables corresponding to May were represented (Fig. 5b). In this case, although the total variance explained was similar to January, 57.65%, only four principal components (PC) with an eigenvalue >1 were obtained. The first component PC1 explained 36.49 % of the variance and included the same variables than in January besides Temperature. PC2 explained 21.26 % of the variance and contained Cu, Ni, Diatoms, Euglenoids, Filamentous algae and *Cyanidium* sp. PC3 (*Chlorella* sp., heterotrophic flagellates and Zn) represented 19.6 % of the variance and PC4 (*Chlaydomonas* spp., Redox and Oxygen), represented only 5.7 % of the variance. September showed different results (Fig. 5c). Total variance explained by the two first components of the analysis was 51.72%. Although six principal components (PC) with an eigenvalue >1 were obtained, more than 80% of the variance was explained by the first four PC. The first component PC1 explained 36.96 % of the variance and included the same variables than in January. PC2 explained 16.76 % of the variance and contained Diatoms, Euglenoids, Filamentous algae and heterotrophic flagellates. PC3 (Ni, Cu, Zn and *Chlorella* sp.) represented 15.6 % of the variance and PC4 (*Chlaydomonas* spp., Temperature, Redox and Oxygen), represented 12.26 % of the variance.

DGGE fingerprints analysis and cloning library construction

The potential of DGGE for identifying eukaryotic species was evaluated. Total nucleic acids were extracted from mat samples from each sampling station, and 18S rRNA gene segments were amplified using primer sets for eukaryotes. Amplification of the 18S rRNA gene was successful in all samples, and the DGGE generated band patterns that were characteristic for each sample (Fig. 5). A total number of 59 bands from the 13 samples were sequenced. The closest match and percentages of similarity for the sequences obtained were determined by BLAST searches (Table 4). In all samples, the number of species identified by DGGE was lower than the number of species detected morphologically, however the affiliations of all the provided sequences were consistent with microscopic observations. In addition, only eukaryotic sequences were recovered indicating the specificity of the primers. In general, the most intense bands obtained corresponded to the chlorophytas, *Chlamydomonas*, *Dunaliella* and *Chlorella*. Several other groups, such as diatoms, ciliates, amoebas and fungi were also detected. Percent similarities ranged from good (99%) for most of the species detected to poor (89%), usually for fungi. In general, the number of bands per sample was lower in samples that showed the most extreme physicochemical characteristics.

Cloning and Phylogenetic analysis

From each cloning reaction, ca. 30 clones were sequenced. None of the clone sequences were chimerical according to the Bellerophon program. Of those 150 clones, only 16 yielded unique species sequences. The phylogenetic tree obtained by using all the sequences available from natural samples is shown in Fig. 6. Most of the sequences grouped with chlorophytan and streptophytan algae, although other groups were also detected. In all cases, the affiliations of the sequences obtained provided results that were consistent with microscopic observations. Interestingly, the sequences related to

Chlamydomonas clustered into two groups, one group related to *Chlamydomonas noctigama* (i.e rtLPA6cla and rtAGA2cla) and other related to *Chlamydomonas pitschmanii* (i.e. rt32cla764 and rtUM32cla) not distinguished previously under the microscope since both species are phenotypically very similar.

The sequence alignment performed with ARB as well as the phylogenetic analysis showed that the sequences obtained for one particular species isolated from distant sampling sites were almost identical with an identity of over 98%. Thus, clones rtLPA6cla and rtAGA2cla were related to *Chlamydomonas noctigama* isolated from LPC and AG respectively clustered together as did clones rtUMH1dia and rtAGH5dia related to *Pinnularia* isolated from UMA and AG respectively.

Discussion

This study analyzed the diversity, spatial distribution and seasonal variation of the eukaryotic community in a natural extreme acidic river, Río Tinto (SW, Spain). There are relatively few reports on the biodiversity and abundance of acidophilic microorganisms in the literature, and most have focused exclusively on prokaryotes. In addition, few ecological studies have been performed in rivers, probably due to their complexity and the difficulty in obtaining integrated samples [21]. References regarding the use of molecular techniques to identify and estimate the eukaryotic species in these environments are also scarce. In the current work, molecular techniques were used in combination with more traditional methods including microscopy. The results revealed that, although molecular approaches facilitated the identification of the different species, these techniques should be complemented with microscopy observations in order to obtain more accurate data.

Eukaryotic community and seasonal variability in Río Tinto

The high water table maintains the river flow even during the extremely dry summers, characterized by lack of rain and high evaporation rates. In general, the water temperature was highest in June and September with an average of 25 ºC. This corresponds to periods of lowest water flow and highest eukaryotic biomass. Dissolved oxygen rose in winter and then decreased during the dry summer and fall months. However, conductivity, redox and pH showed no notable changes over the year [1]. Ferric iron and sulfuric acid are the most common components found in this acidic environment. Ferric iron establishes an efficient buffer system at pH values of approximately 2.3. Ferric iron is produced by the metabolism of iron oxidizing microorganisms, which are very active in the aerobic part of the river, sulfuric acid originates from sulphides by chemical oxidation or the activity of sulfur-oxidizing microorganisms, depending on the sulfide mineral substrate [17]. The result is a strongly acidic oxidant solution of ferric iron which brings into solution other heavy metals, increasing their concentrations in comparison to neighboring rivers with higher pH [13].

Although some of the species found were previously identified using light microscopy [25] or molecular approaches [3, 4], our approach combining both methods revealed new taxa and details of the seasonal and spatial distributions of known species.

Microscopic observations of the biofilms revealed a variety of prokaryotic morphotypes, algae, protozoa or fungi. The whole community is usually embedded in a coating that may well protect the inner microbial community from external conditions [1]. Members of the *Chlorophyta* such as *Chlamydomonas*, *Dunaliella*, *Chlorella,* and *Euglena*, were the most frequent species, forming large green patches along the river bed. These species are known for their high metal and acid tolerance [15, 44] and show

the same patchy distribution found in other acidic environments [20]. The most acidic sampling station of the river, RI, is inhabited by an eukaryotic community dominated by two species related to *Dunaliella* and *Cyanidium.*

Members of the *Bacillariophyta* are the other eukaryotes able to produce visible brown mats in the river. One species closely related to the genera *Pinnularia* has been identified. The genera *Pinnularia* has been widely described in acidic environments [45]. The low number of taxa present in the river in comparison with the diversity found in neighboring freshwaters as well as in the literature, suggests that there is a threshold between pH 4.5 and 3.5 at which many species are eliminated [11]. There have been few experimental studies of the tolerance of diatom taxa to extremely low pH values. It has been reported that at pH values below 4.5 most species are not able to grow [30, 33].

The low species diversity of flagellates, dominated by cercomonads and stramenopiles, is also characteristic of these extreme environments [38]. These organisms employ the same ecological strategies as the phytoflagellates to overcome limitations in nutrient supply such mixotrophy and mobility which are in an important advantage in these environments [24].

The protistan consumer community was characterized by low diversity and the lack of some common groups such as corixids. At least two species of ciliates are quantitatively important members of the community. The dominating ciliate taxa belonged to the order *Hypotrichida*. Although two different species have been microscopically observed, only clones related to *Oxytrichia granulifera* were molecularly identified. The other morphotype could be tentatively assigned to the genera *Euplotes*. The reduction of species diversity and ciliate abundance with increasing acidity is well documented [5, 6]. The members of the order *Hypotrichida*

thrive predominantly in soils or benthos usually associated with algal clumps [16]. They can be found in almost all sampling sites in Río Tinto except in the upper headwaters and near Nerva. The ciliates found in the river were relatively large, slow-swimming organisms which may be attributed to lack of predatory impact. Amoebas can be found frequently even in the most acidic parts of the river eating large diatoms. *Valhkampfia* have been identified microscopically and several other species of amoeba have been observed, including lobosea-like and acanthamoeba-like amoebas. One species of heliozoan belonging to the genera *Actinophyris* was found at six sampling sites. Heliozoa seem to be characteristic top predators of the benthic food chain in the river. They are omnivorous [32], with the ability to overwhelm organisms larger than they are, including rotifers, algae and ciliates [46] that get stuck on their adhesive podiae. In Río Tinto, we have observed their ability to ingest algae, mainly *Chlorellas, Chlamydomonas,* and *Euglena.*

According to recently reviewed literature [10], few rotifers have been reported in waters of pH<3.0, which are typically dominated by one or two species. Our results in Río Tinto are in close agreement with this pattern as only one species of bdelloid rotifer related to the *Rotifera* genera has been observed at sampling sites 3.2 and LPC. This pioneer species can persist because of its high physiological tolerance of severe acidic stress and the lack of other more efficient competitors. Although this rotifer was observed ingesting algae, their potential impact on phytobentos seems to be negligible. Together with ciliates, amoebae, and heliozoans, rotifers are part of a simple food-web.

The abundance of eucarya show seasonal correlations with the physicochemical conditions of the water [1]. Spatially, low pH and heavy metal concentrations correlates with lower eukaryotic diversity and cell abundance at sites where the pH reached the lowest values such as RI and Iz-Iz where only *Dunaliella* and *Cyanidium* were able to

grow in appreciable amounts. In general, PCA showed a high inverse correlation between pH and most of the heavy metals analyzed as well as *Dunaliella* sp., while *Chlamydomonas* sp. is directly related to pH during May and September. Three heavy metals (Zn, Cu and Ni) remained separate from the rest and showed an inverse correlation with most of the species analyzed except for *Dunaliella* sp. The occurrence of filamentous species, *Zygnemopsis* and *Klebsormidium*, during the dry summer months, when ion concentrations were highest and most of the physicochemical parameters most extreme, suggested that these species have a competitive advantage under high-ionic-strength conditions. These species are attached to the sediments forming long filamentous biofilms on the water surface that reach 1 m in length (i.e. Fig. 3g).

While many species of fungi have been isolated from the river [25, 26], one species, related to the genus *Hobsonia,* has been identified in many parts of the river where it forms thick denditric macrofilaments closely associated with other protists present in the river (i.e. Fig. 3a,j). When the fungi is present, a whole community forms embedded in a mucilaginous substance that might protect the inner microbial community from the external conditions by creating different physico-chemical conditions.

Conclusions

The Río Tinto is a unique natural extreme environment where eukaryotic organisms are the main contributors of biomass and where eukaryotic diversity is greater than prokaryotic diversity [17, 25]. The results obtained using different methodological techniques with environmental samples demonstrate that only an integrated approach combining molecular techniques, microscopic observations and

new isolation strategies will guarantee a more thorough knowledge of the microbial diversity of any given ecosystem.

Understanding the ecology of highly acidic environments requires investigation of metabolic pathways and survival strategies. Although highly acidic environments occur all over the Earth, and interest in the ecology of acidophilic organisms has increased significantly over the past decade, the answers to many questions about them remain elusive. One of the most intriguing questions is how acidophilic species colonize new habitats. Are they contained as endemic species and therefore isolated for long periods of time, forming different strains with different genotypes? We hope to address some of these topics in future research on Río Tinto.

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References

[1] A. Aguilera, S.C. Manrubia, F. Gómez, N. Rodriguez, R. Amils, Eukaryotic Community Distribution and their Relationship with the Water Physicochemical Parameters in an Extreme Acidic Environment, Río Tinto (SW, Spain), Appl Environ Microbiol 72 (2006) 5325-5330.

[2] A. Aguilera, F. Gómez, E. Lospitao, R. Amils, A molecular approach to the characterization of the eukaryotic communities of an extreme acidic environment: methods for DNA extraction and Denaturing Gradient Gel Electrophoresis analysis, Syst Applied Microbiol 29 (2006) 593-605.

[3] L.A. Amaral-Zettler, F. Gómez, E. Zettler, B.G. Keenan, R. Amils, M.L. Sogin, Eukaryotic diversity in Spain's river of fire, Nature 417 (2002) 137.

[4] L.A. Amaral-Zettler, M. Messerli, A. Laatsch, P. Smith, M.L. Sogin, From genes to genomes: Beyond biodiversity in Spain's Río Tinto, Biol Bull 204 (2003) 205-209.

[5] J.R. Beaver, T.L. Crisman, Acid precipitation and the response of ciliated protozoans in Florida Lakes, Verh Int Ver Limnol 21 (1981) 353-358.

[6] R.W. Bienert, J.R. Beaver, T.L. Crisman, The contribution of ciliated protozoa to zooplankton biomass in an acidic subtropical lake, J Protozool 38 (1991) 352-354.

[7] P. Bourrelly, In: Les Algues Deau Deuce. Initiation a la Systematique, Editions N, Boubee & Cie, Paris, 1966, pp. 544.

[8] P.G.C. Campbell, Interactions between trace metals and aquatic organisms, In: A. Tessier, D.R. Turner (Eds.), Metal Speciation and Bioavailability in Aquatic Systems, John Wiley, New York, 1995, pp. 45-102.

[9] C.C. Davis, In: The Marine and Freshwater Plankton. Michigan State University, East Lansing, MI, 1955, pp. 332.

[10] R. Deneke, Review of rotifers and crustaceans in highly acidic environments of pH values <3, Hydrobiologia 433 (2000) 167-172.

[11] D.M. De Nicola, A review of diatoms found in highly acidic environments, Hydrobiologia 433 (2000) 111-122.

[12] J. Felsenstein, PHYLIP phylogeny inference package, Cladistics 5 (1989) 164-166.

[13] D.C. Fernández-Remolar, N. Rodríguez, F. Gómez, R. Amils, Geological record of an acidic environment driven by the iron hydrochemistry: the Tinto river system, J Geophys Res 108 (2003) 5080-5095.

[14] D.C. Fernández-Remolar, R.V. Morris, J.E. Gruener, R. Amils, A.H. Knoll, The Río Tinto basin, Spain: mineralogy, sedimentary geobiology and implications fro interpretation of outcrop rocks at Meridiani Planum Mars, Earth and Planetary Sci Lett 240 (2005)149-167.

[15] M. Fisher, A. Zamir, U. Pick, Iron uptake by the halotolerant alga *Dunaliella* is mediated by a plasma membrane transferring, J Biol Chem 273 (1998) 17553-17558.

[16] W. Foissner, H. Blatterer, H. Berger, F. Kohman, In : Taxonomische und Okologische Revision der Ciliaten des Saprobiensystems-Band I: Cyrtophorida, Oligotrichida, Hypotrichia, Colpodea. Informationsberichte des Bayer. Landesamtes für Wasserwirtschaft, München, Heft 1/91, 1991, pp. 478.

[17] E. González-Toril, E. Llobet-Brossa, E.O. Casamayor, R. Amann, R. Amils, Microbial ecology of an extreme acidic environment. The Tinto River, Appl Environ Microbiol 69 (2003) 4853-4865.

[18] H. Hillebrand, C. D. Durselen, D. Krischtel, U. Pollinger, T. Zohary, Biovolume calculations for pelagic and benthic microalgae, J Phycol 35 (1999) 403-424.

[19] T.H. Jukes, C.R. Cantor, Evolution of protein molecules. In: H.N. Munro (Ed.), Mammalian Protein Metabolism, Academic Press, New York, 1969, pp. 21-132.

[20] M. Kapfer, Assessment of the colonization and primary production of microphytobenthos in the litoral of acidic mining lakes in Lusitania (Germany), Wat. Air Soil Pollut. 108 (1998) 331-340

[21] T. Kenzaka, N. Yamaguchi, K. Tani, N. Nasu, rRNA-targeted fluorescent in situ hybridization analisis of bacterial community structure in river water, Microbiol. 144 (1998) 2085-2093

[22] J.L. Lee, S.H. Hutner, E.C. Bovee, In: An Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence, 1985, pp. 332.

[23] G.F. Leedale, In: Euglenoid Flagellates. Concepts of Modern Biology Series. Prentice-Hall Inc, Englewood Cloffs, NJ, 1967. pp. 221.

[24] D. Lessmann, A. Fyson, B. Nixdorf, Phytoplankton of the extremely acidic mining lakes of Lusatia (Germany) with pH<3, Hydrobiol. 433 (2000) 123-128.

[25] A.I. López-Archilla, I. Marín, R. Amils, Microbial community composition and ecology of an acidic aquatic environment: the Tinto river, Spain, Microbial Ecol. 41 (2001) 20-35

[26] A.I. López-Archilla, A.E. González, M.C. Terrón, R. Amils, Diversity and ecological relationships of the fungal populations o fan acidic river of Southwestern Spain: the Tinto River, Canadian J. Microbiol. 50 (2005) 923-934.

[27] R.L. Lowe, Y. Pan, Benthic algal communities and biological monitors. In: R.J. Stevenson, M. Bothwell, R.L. Lowe (Eds.), Algal Ecology: Freshwater Benthic Ecosystems, Academic Press, San Diego, USA, 1996, pp. 705-39.

[28] L. Margulis, J.O. Corliss, M. Melkonian, D.J. Chapman, In: Handbook of Protocista, Jones and Barlett Publ, Boston, 1990, pp. 210.

[29] M.A. Messerli, L.A. Amaral-Zettler, E. Zettler, S.K. Junh, P.J. Smith, L.M Sogin, Life at acidic pH imposes an increased energetic cost for a eukayotic acidophile, J. Exp. Biol. 208 (2005) 2569-2579.

[30] B. Moss, The influence of environmental factors on the distribution of freshwater algae: An experimental study. II The role of pH and the carbon dioxide-bicarbonate system, J Ecol 61 (1973) 157-177.

[31] F. Niehaus, C. Bertoldo, M. Kähler, G. Antranikian, Extremophiles as a source of novel enzymes from industrial application, Appl Microbiol Biotechnol 51 (1999) 711-729.

[32] C. Page, F.J. Siemensma, In: Nacke Rhizopoda und Heliozoa, Gustav Fischer Verlag, Stuttgart, 1999, pp. 297.

[33] R. Patrick, The effect of changes in pH on the structure of diatom communities, Notuale Naturae 426 (1968) 1-15.

[34] U. Pick, Dunaliella acidophila a most extreme acidophilic alga, In: J. Seckbach (Ed.), Enigmatic Microorganisms and Life in Extreme Environments. Kluwer, Dordrecht, 1999, pp. 478.

[35] F.E. Round, R.M. Crawford, D.G. Mann, In: The Diatoms, Biology and Morphology of the Genera, Cambridge University Press, Cambridge, 1990, pp. 198.

[36] S. Sabater, T. Buchaca, J. Cambra, J. Catalán, H. Guasch, N. Ivorra, I. Muñoz, E. Navarro, M. Real, A. Romaní, Structure and function of benthic algal communities in an extremely acid river, J Phycol.39 (2003) 481-489.

[37] N. Saito, M. Nei, The neighbour-joining method:a new method for reconstructing phylogenetic trees, Molecular Biology and Evolution. 4 (1987) 406-425.

[38] D.W. Schindler, S.K. Holmgren, Primary production and phytoplankton in the experimental lakes area, NW Ontario and other low carbonate waters, an a liquid scintillation method for determining C14 activity in photosynthesis, J. Fish. Res. Bd. Can. 28 (1971) 189-201.

[39] C. Schiraldi, M. De Rosa, The production of biocatalysts and biomolecules from extremophiles. TRENDS Biotechnol, 20 (2002) 515-521.

[40] J.W. Schopf, M.R. Walter, Origin and early evolution of cyanobateria: the geological evidence, In: N.G. Carr, B.A. Whitton (Eds.), The Biology of Cyanobacteria, Oxford, Blackwell Sci. Publ., 1982, pp. 543-564.

[41] P. Snoeijs, F. Snoeijs, A simple sampling device for taking quantitative microalgal samples from stone surfaces, Arch. Hydrobiol. 129 (1993) 121-126.

[42] R. Jan Stevenson, Periphyton protocols. In: M.T. Barbour, J. Gerritsen, B.D. Snyder, J.B. Stribling (Eds.), Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish, Second Edition, EPA 841-B-99-002, U.S. Environmental Protection Agency; Office of Water, Washington, 1999, pp.234-250.

[43] G. Tell, V. Conforti, J. Cramer, In: Euglenophyta Pigmentadas de la Argentina. Bibliotheca Phycologica, Berlin, 1986, pp. 433.

[44] I. Visviki, D. Santikul, The pH tolerance of *Chlamydomonas applanata* (Volvocales, Chlorophyta), Arch Environ Contam Toxicol 38 (2000) 147-151.

[45] T. Watanabe, K. Asai, *Pinnularia acoricola* Hustedt var. *acoricola* as an environmental frontier species occurred in organic acid water with 1.1-2.0 in pH value, Diatom 10 (1995) 9-11.

[46] S. Woelf, B. Zippel, G. Packroff, In: Planktongesellschafen der Mitteldeutschen Tagebaurestseen. Dt. Ges. F. Limnol. Erw. Zus. D. Jahrestagung Frankfurt/M, 1998, pp. 376-380.

Table 1. Means and standard errors of water physical parameters at each sampling site during the year. Tª- Temperature in ºC. Cond.- Conductivity in mS cm^{-1}, Redox.- redox potential in mV, O_2.- Dissolved oxygen in ppm. Stations with an average pH below 2 are in grey, and the most extreme values for each parameter in bold.

Site	GPS Coordenates	pH	Tª	Cond	Redox	O_2
AG	37º43'29"N / 6º33'3"W	2.6±0.28	19.8±7.5	5.30±1.6	413±44.5	5.8±2.9
3.2	37º43'20"N / 6º33'48"W	2.4±0.23	18.8±5.9	5.50±7.6	562±86.4	**1.0**±2.4
Iz-Iz	37º43'15"N / 6º33'3"W	1.8±0.25	18.6±4.1	25.7±2.3	**569**±22.0	3.4±1.5
FE	37º43'15"N / 6º33'3"W	2.1±0.35	18.1±6.0	5.20±3.8	488±33.6	7.7±2.7
ANG	37º43'15"N / 6º33'10"W	1.7±0.21	24.3±2.4	30.8±3.4	471±16.9	1.7±0.7
NUR	37º43'22"N / 6º33'25"W	2.0±0.26	19.5±2.0	9.40±1.0	515±17.4	1.6±0.7
UMA	37º43'13"N / 6º33'23"W	1.7±0.36	15.6±6.7	**40.2**±8.3	473±10.9	4.5±2.2
RI	37º43'14"N / 6º33'15"W	**1.2**±0.35	15.8±3.6	38.9±16.9	460±3.50	2.4±0.5
CEM	37º42'8"N / 6º33'31"W	2.5±0.12	17.5±5.1	11.4±1.3	446±30.5	2.8±2.3
STB	37º42'6"N / 6º33'31"W	2.3±0.14	15.0±2.4	4.30±2.6	560±14.8	1.5±0.1
EST	37º41'24"N / 6º33'37"W	2.5±0.29	23.1±8.5	10.2±0.9	444±17.7	3.5±0.1
BRR	37º35'36"N / 6º33'4"W	2.5±0.32	**25.5**±10.4	10.2±5.2	460±27.3	1.2±0.1
LPC	37º25'25"N / 6º36'36"W	2.5±0.33	24.7±8.6	3.70±1.1	548±70.6	5.9±0.9

Table 2. Means and standard errors of ion concentrations (mg l^{-1}) at each sampling site during the year. Stations with an average pH below 2 are in grey, and the most extreme values for each parameter in bold.

Sampling site	Fe	S	Co	Cu	Zn	Ni	As	Cd	Cr
AG	1000.30±351.0	1970.10±231	2.30±0.8	11.2±4.3	78.50±46	0.7±0.1	2.40±0.3	3.8±0.4	0.50±0.1
3.2	1288.30±480.0	2069.50±123	2.90±0.6	13.7±6.6	68.40±44	0.4±0.1	1.40±0.4	5.7±0.6	0.70±0.2
Iz-Iz	16969.7±4129	25252.7±658	30.7±8.6	12.4±3.6	14.40±3.1	2.3±1.1	15.7±4.2	**42.8**±16	8.60±1.4
FE	8715.70±2784	7557.60±111	20.3±9.6	6.90±2.1	21.46±7.8	1.3±0.2	6.70±0.7	0.4±0.1	4.80±1.1
ANG	15983.4±3064	24379.8±973	31.9±9.9	132.5±43	**161.9**±52	2.4±0.4	23.5±3.8	30.3±12	8.50±2.7
NUR	3664.50±564.0	6968.70±125	8.10±1.5	36.5±2.7	73.30±7.7	0.8±0.1	5.50±1.7	2.2±0.4	1.60±0.7
UMA	18284.7±7369	24020.5±769	33.6±7.3	85.7±36	118.4±49	2.5±0.7	32.1±5.2	40.2±18	10.7±2.7
RI	**22231.2**±5468	**29007.4**±981	**61.9**±8.2	100.2±36	94.10±31	3.8±0.4	**47.8**±7.4	33.7±11	**13.1**±3.1
CEM	1610.90±466	5224.60±113	6.10±1.3	**278.3**±39	149.0±6.9	**7.9**±1.6	5.70±1.6	4.1±0.3	0.30±0.1
STB	549.2±12.5	4311.50±187	1.40±0.2	5.17±1.1	31.60±9.8	0.2±0.1	0.30±0.1	0.6±0.1	0.20±0.1
EST	1889.7±116.4	4883.50±112	5.02±0.5	194.6±23	112.2±11	4.1±1.3	4.60±1.2	3.8±1.1	1.02±0.2
BRR	2289.5±211.5	4655.75±209	4.12±1.1	100.7±11	317.2±67	1.4±0.3	5.40±1.1	4.9±1.0	4.9±0.2
LPC	237.50±119.0	811.50±129	0.80±0.1	19.1±7.1	50.1±10.3	0.35±0.1	0.20±0.1	0.7±0.1	0.10±0.01

Table 3. Species present in the river. LM= Light Microscopy, DG= DGGE, 18S= 18Sr RNA cloning.

Order	Family	Genus	ID technique
Volvocales	Chlamydomonadaceae	Chlamydomonas	LM/DG/18S
Volvocales	Dunaliellaceae	Dunaliella	LM/DG/18S
Chlorellales	Chlorellaceae	Chlorella	LM/DG/18S
Zygnematales	Mesotaeniaceae	Mesotaenium	LM/DG
Zygnematales	Zygnemataceae	Zygnemopsis	LM/DG/18S
Ulotrichales	Ulotrichaceae	Stichococcus	LM/18S
Klebsormidiales	Klebsormidiaceae	Klebsormidium	LM/18S
Naviculales	Pinnulariaceae	Pinnularia	LM/DG/18S
Euglenales	Euglenophyceae	Euglena	LM
Porphyridiales	Porphyridiaceae	Cyanidium	LM
Schizopyrenida	Vahlkampfiidae	Vahlkampfia	LM
Schizopyrenida	Vahlkampfiidae	Naegleria	LM
Actinophryida	Actinophyridae	Actinophrys	LM
Kinetoplastida	Bodonidae	Bodo	LM
Ebriida	Cercomonadidae	Cercomonas	LM
Ochomonadales	Ochromonadaceae	Ochromonas	LM
Labyrinthulida	Labyrinthulidae	Labyrinthula	LM/18S
Bdelloidea	Philodinidae	Rotaria	LM
Stichotrichida	Oxythrichidae	Oxytricha	LM/DG/18S
Hymenostomatida	Turaniellidae	Colpidium	LM

Table 4. Sequence identity of excised DGGE bands.

Sample	Most closely related	% Similarity	Taxonomic group	Bands
AG	*Chlorella* sp.	98%	Chlorophyta	2
	Chlamydomonas pitschmanii	98%	Chlorophyta	2
	Pinnularia cf. *interrupta*	96%	Bacillariophyta	1
	Hobsonia santesonii	89%	Fungi	1
Iz-Iz	*Dunaliella parva*	97%	Chlorophyta	2
FE	*Oxytricha granulifera*	98 %	Ciliate	1
	Chlorella sp.	99 %	Chlorophyta	2
	Mesotaenium kramstai	97%	Chlorophyta	2
ANG	*Oxytricha granulifera*	98%	Ciliate	1
	Chlorella sp.	99%	Chlorophyta	3
	Pinnularia cf. *interrupta*	96%	Bacillariophyta	1
NUR	*Zygnemopsis circumcarinata*	94 %	Streptophyta	3
	Pinnularia cf. *interrupta*	98%	Bacillariophyta	2
RI	*Dunaliella parva*	97 %	Chlorophyta	1
	Chlorella sp.	97%	Chlorophyta	1
CEM	*Chlorella* sp.	99%	Chlorophyta	1
	Chlamydomonas pitschmanii	98%	Chlorophyta	3
	Oxytricha granulifera	98%	Ciliate	1
	Dunaliella parva	97 %	Chlorophyta	1
STB	*Chlorella* sp.	99%	Chlorophyta	3
	Pinnularia cf. *interrupta*	96%	Bacillariophyta	1
EST	*Chlamydomonas pitschmanii*	98%	Chlorophyta	4
	Hobsonia santesonii	89%	Fungi	2
	Chlorella sp.	99%	Chlorophyta	3
BRR	*Hobsonia santesonii*	89%	Fungi	2
LPC	*Oxytricha granulifera*	98%	Ciliate	1
	Dunaliella parva	97 %	Chlorophyta	2
	Chlamydomonas pitschmanii	98%	Chlorophyta	3

The identity, % of similarity of the closest relative found in GenBank database is indicated. Bands column indicates the number of bands per sample that yielded the same identity. The most closely related species corresponded to the first known relative.

Figure 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 more detailed map of the headwaters.

Figure 2. Sampling locations and microbial eukaryotic biofilms found in the river. Scale bar = 5 cm.

a) View of the origin of the Río Tinto.
b) Panoramic view of the sampling station BRR.
c) View of LPC.
d) Green filaments formed mainly by *Zygnemopsis* and *Klebsormidium* located at the origin.
e) *Cyanidium* biofilm at RI.
f) Biofilm of diatoms at ANG.
g) Euglenas and diatoms are main generators of biofilms located at NUR.
h) Biofilm formed by *Cyanidium* and diatoms located at CEM.
i) Biofilms of *Euglena*, *Chlamydomonas* and fungi at EST.

Figure 3. The percentage seasonal distribution of each eukaryotic taxa at each sampling site as determined from microscope counts.

Figure 4. Total cell abundances of eukaryotic taxa at each sampling site as determined from microscope counts.

Figure 5. Eukaryotic community profiles based on specific amplification and DGGE separation of 18s rRNA gene sequences from the different sampling stations.

Figure 6. Phylogenetic tree based on 18S rRNA sequences. The tree was inferred by neighbor-joining analysis of approximately 500 homologous positions of 18S rDNA sequence. New isolates are indicated in bold. Published sequences from organisms isolated in Río Tinto are indicated in bold and italics.

Figure 7. Two dimensional plot of the Principal Component Analysis (PCA) performed for the whole dataset including physicochemical and biological data.

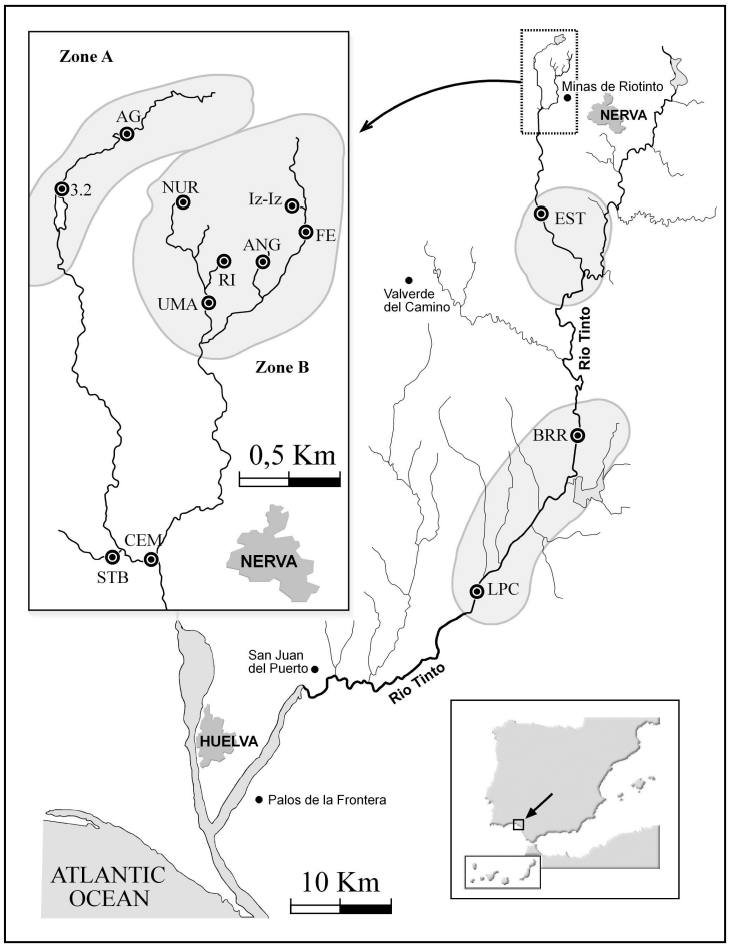
Zone A
AG
3.2
NUR
Iz-Iz
FE
ANG
RI
UMA
Zone B
0,5 Km
CEM
STB
NERVA
Minas de Riotinto
NERVA
EST
Valverde
del Camino
Rio Tinto
BRR
LPC
San Juan
del Puerto
Rio Tinto
HUELVA
Palos de la Frontera
ATLANTIC
OCEAN
10 Km

Figure 1

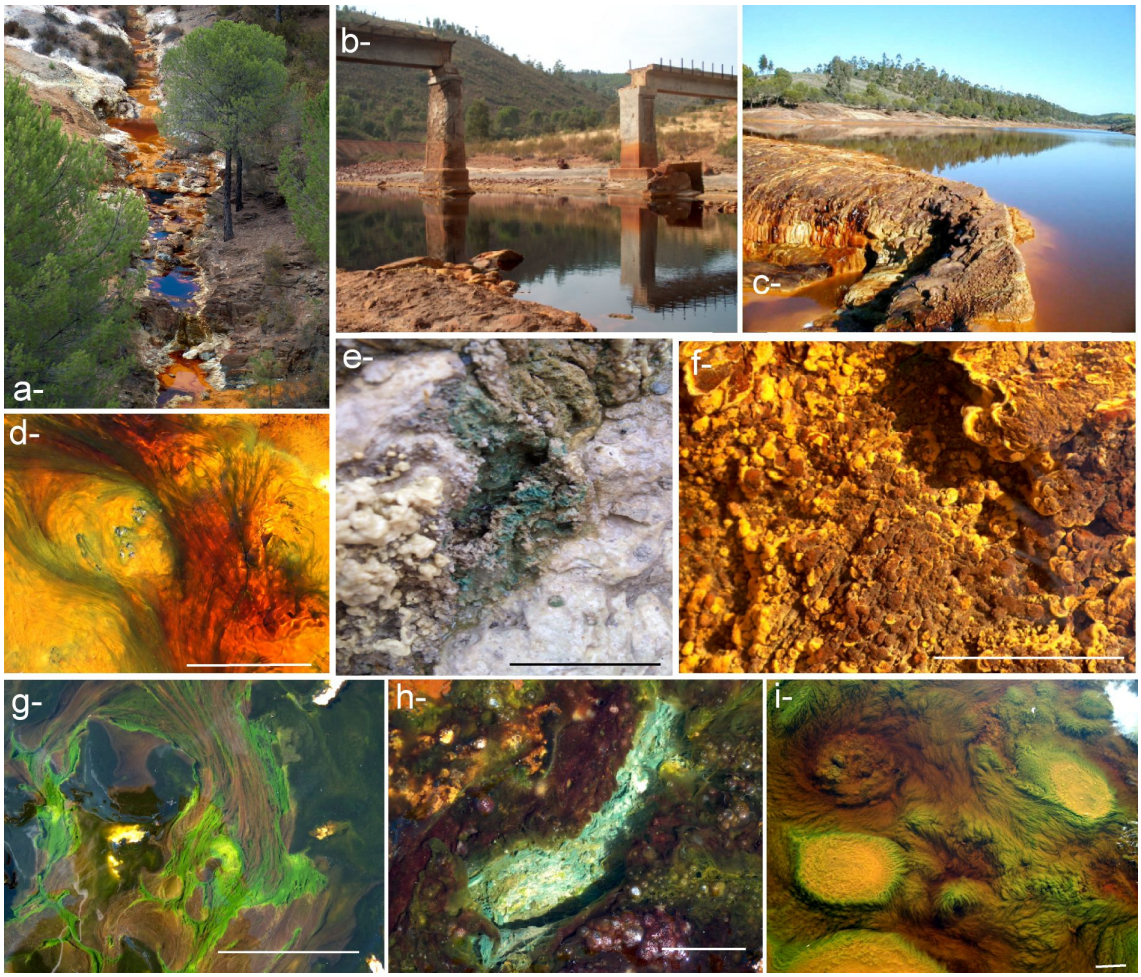

Figure 2

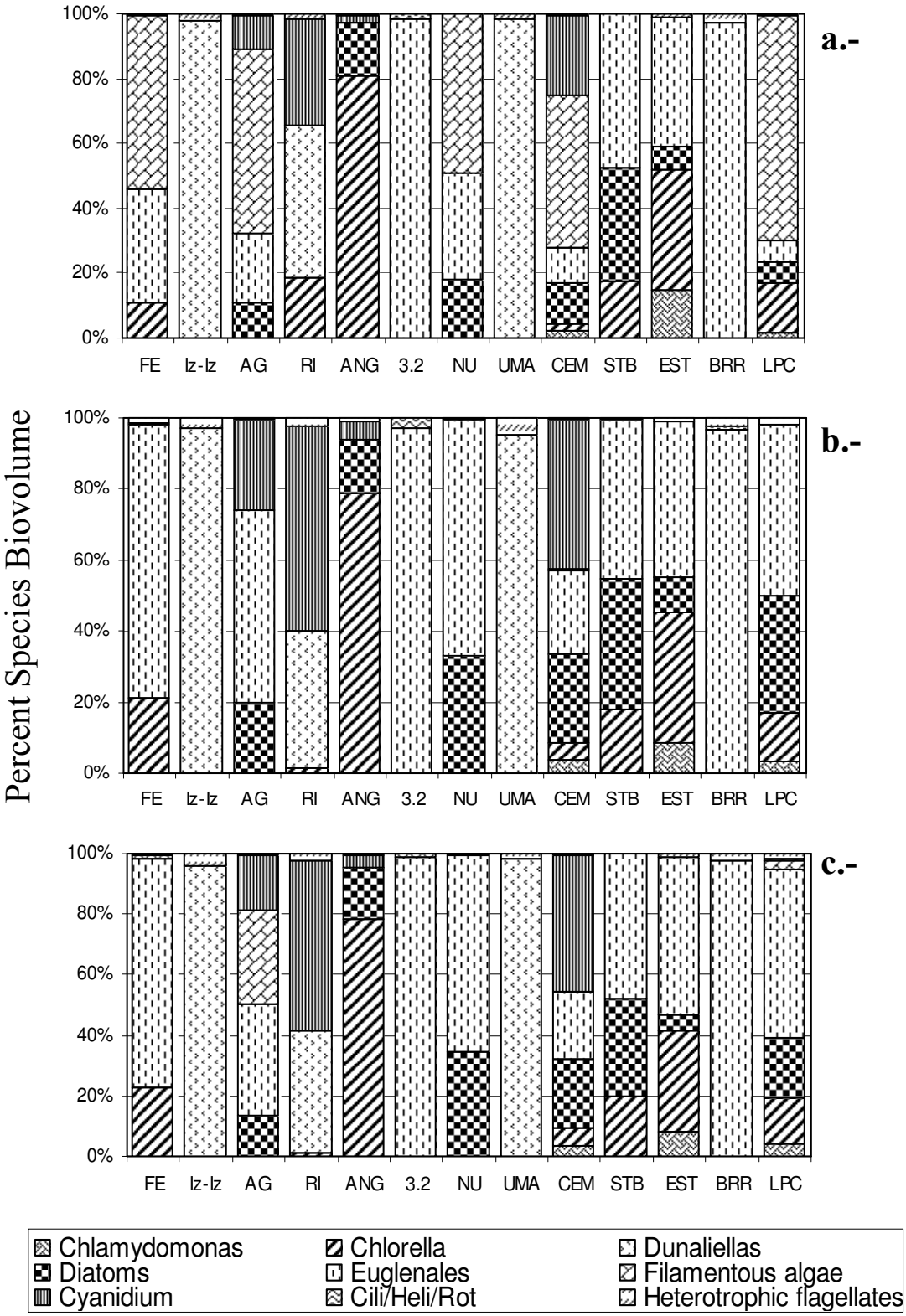
Percent Species Biovolume
100%
80%
60%
40%
20%
0%
FE
Iz-Iz
AG
RI
ANG
3.2
NU
UMA
CEM
STB
EST
BRR
LPC
a.-
b.-
c.-
Chlamydomonas
Chlorella
Dunaliellas
Diatoms
Euglenales
Filamentous algae
Cyanidium
Cili/Heli/Rot
Heterotrophic flagellates

Figure 3

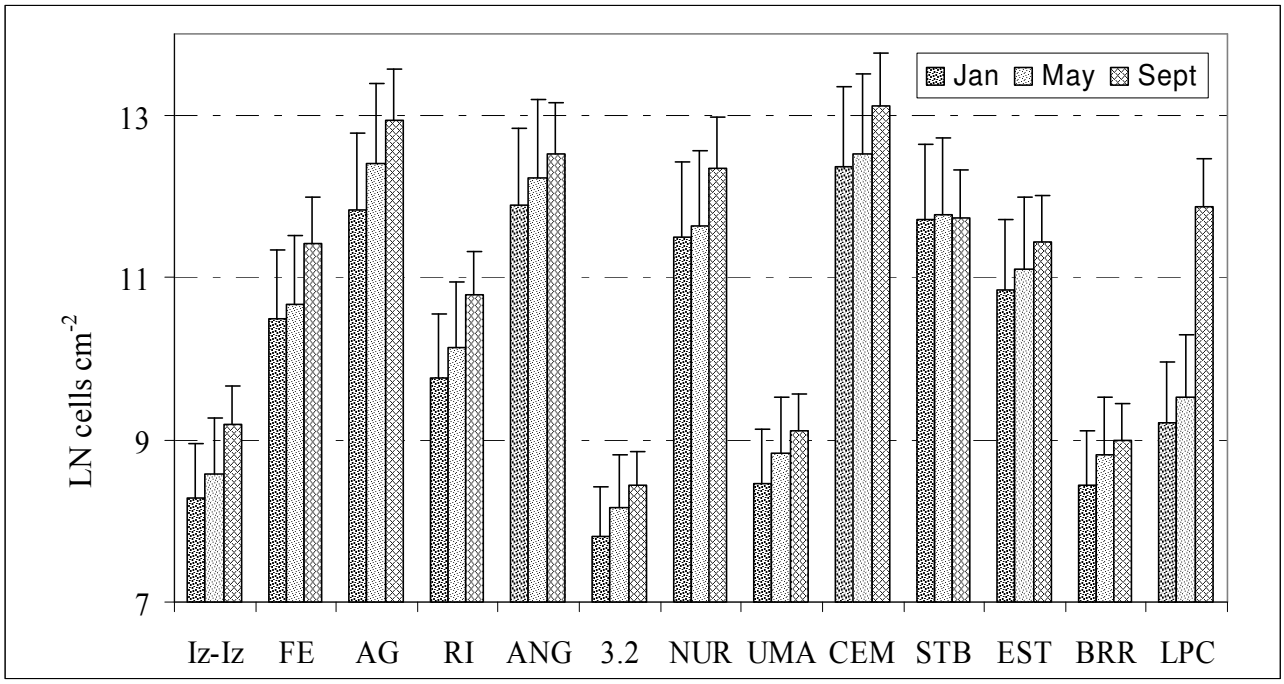
Jan
May
Sept
LN cells cm-2
13
11
9
7
Iz-Iz
FE
AG
RI
ANG
3.2
NUR
UMA
CEM
STB
EST
BRR
LPC

Figure 4

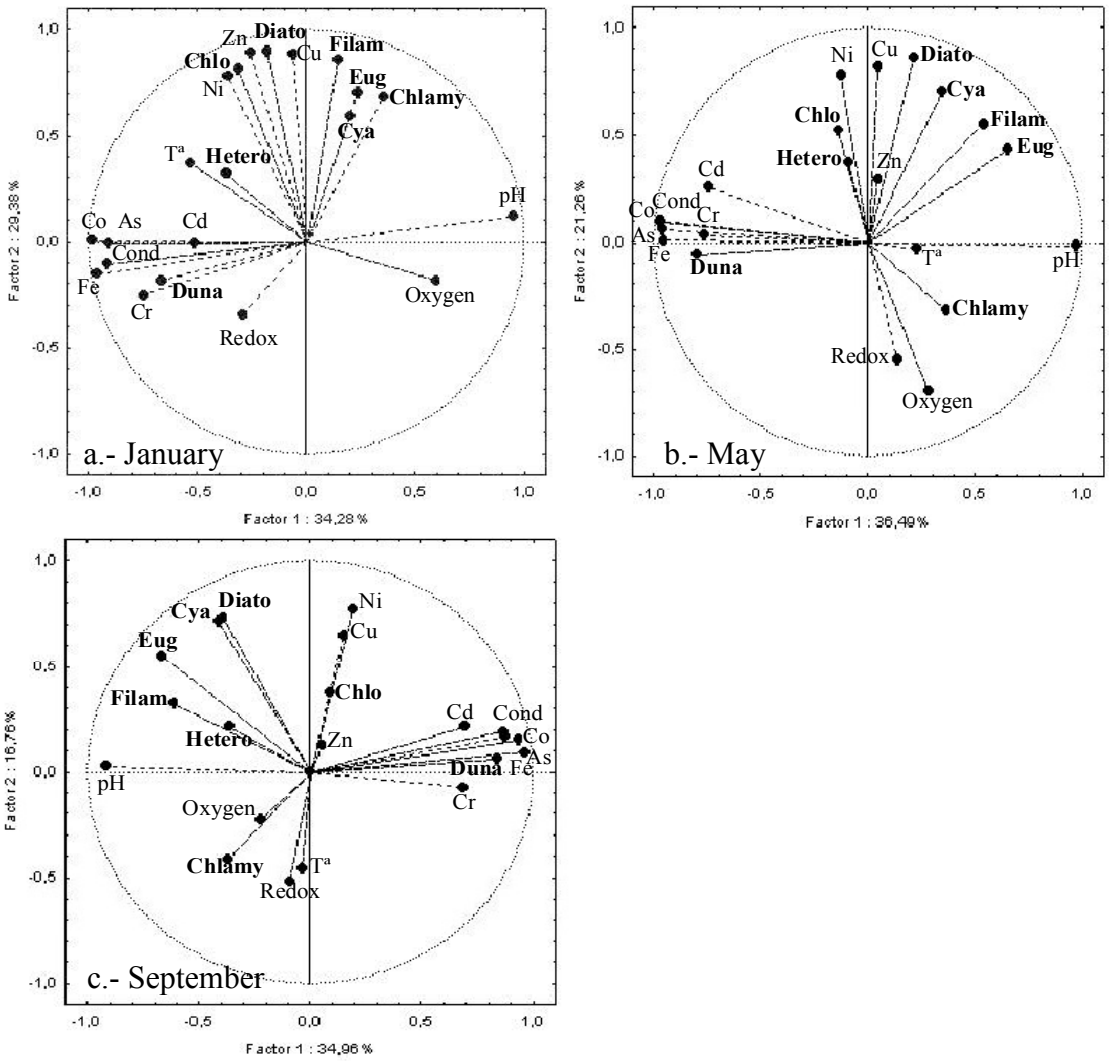
a.- January
Factor 1 : 34,28 %
Factor 2 : 29,38 %
Zn
Diato
Chlo
Cu
Filam
Ni
Eug
Chlamy
Cya
Ta
Hetero
pH
Co
As
Cd
Cond
Fe
Duna
Cr
Oxygen
Redox
b.- May
Factor 1 : 36,49 %
Factor 2 : 21,26 %
Ni
Cu
Diato
Cya
Chlo
Filam
Eug
Hetero
Zn
Cd
Cond
Co
Cr
As
Fe
Duna
Ta
pH
Chlamy
Redox
Oxygen
c.- September
Factor 1 : 34,96 %
Factor 2 : 16,76 %
Cya
Diato
Ni
Eug
Cu
Chlo
Filam
Cd
Cond
Hetero
Zn
Co
As
pH
Duna
Fe
Cr
Oxygen
Chlamy
Ta
Redox

Figure 5

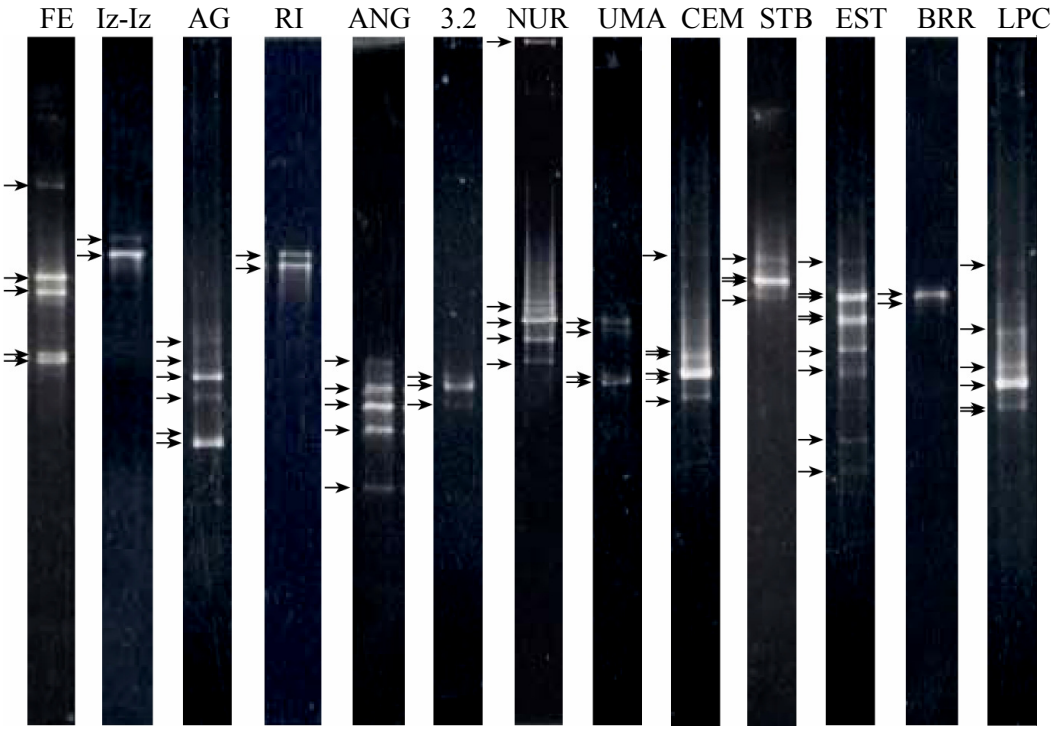

Figure 6

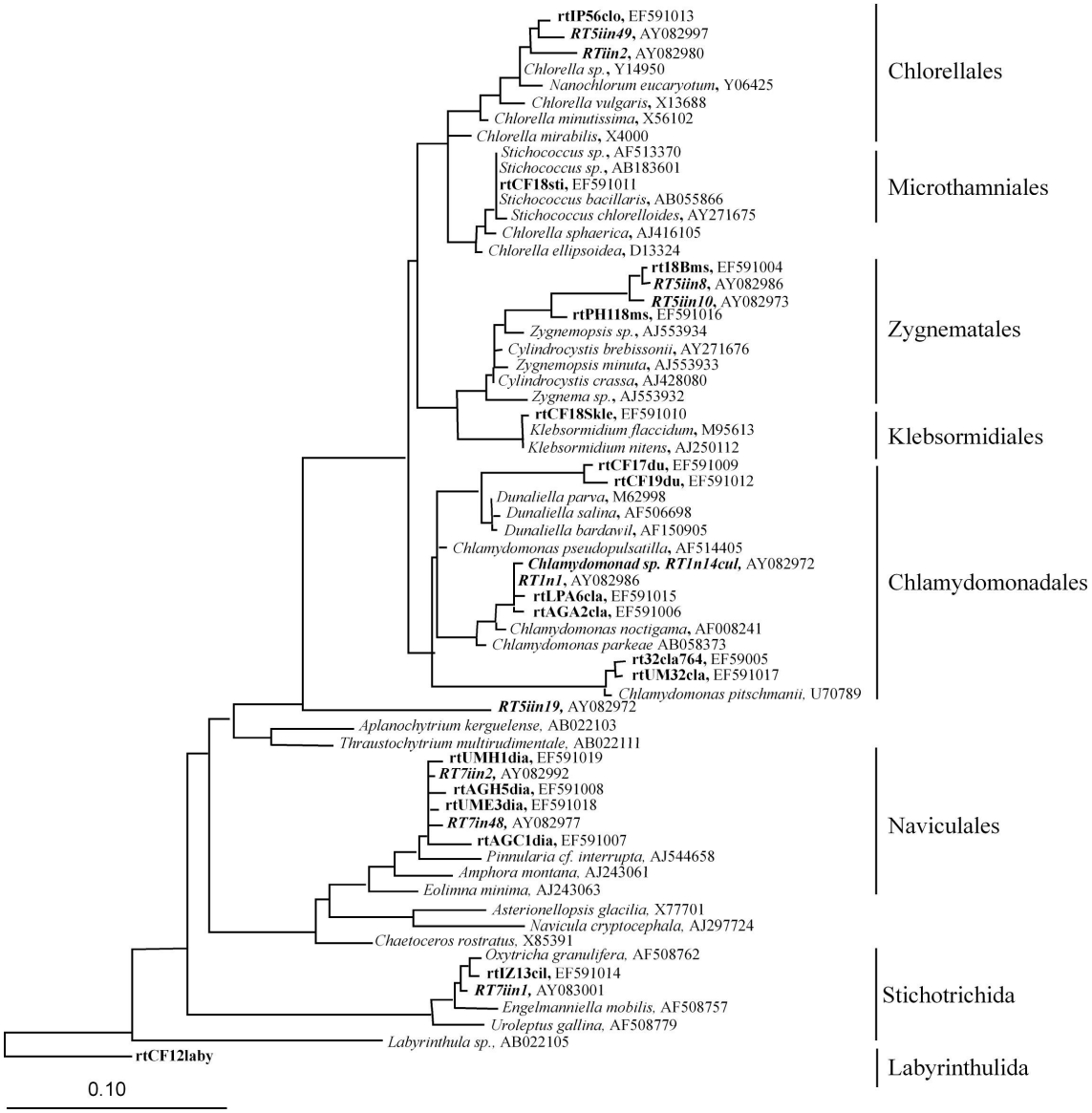
rtIP56clo, EF591013
RT5iin49, AY082997
RTiin2, AY082980
Chlorella sp., Y14950
Nanochlorum eucaryotum, Y06425
Chlorella vulgaris, X13688
Chlorella minutissima, X56102
Chlorella mirabilis, X4000
Stichococcus sp., AF513370
Stichococcus sp., AB183601
rtCF18sti, EF591011
Stichococcus bacillaris, AB055866
Stichococcus chlorelloides, AY271675
Chlorella sphaerica, AJ416105
Chlorella ellipsoidea, D13324
rt18Bms, EF591004
RT5iin8, AY082986
RT5iin10, AY082973
rtPH118ms, EF591016
Zygnemopsis sp., AJ553934
Cylindrocystis brebissonii, AY271676
Zygnemopsis minuta, AJ553933
Cylindrocystis crassa, AJ428080
Zygnema sp., AJ553932
rtCF18Skle, EF591010
Klebsormidium flaccidum, M95613
Klebsormidium nitens, AJ250112
rtCF17du, EF591009
rtCF19du, EF591012
Dunaliella parva, M62998
Dunaliella salina, AF506698
Dunaliella bardawil, AF150905
Chlamydomonas pseudopulsatilla, AF514405
Chlamydomonad sp. RT1n14cul, AY082972
RT1n1, AY082986
rtLPA6cla, EF591015
rtAGA2cla, EF591006
Chlamydomonas noctigama, AF008241
Chlamydomonas parkeae AB058373
rt32cla764, EF59005
rtUM32cla, EF591017
Chlamydomonas pitschmanii, U70789
RT5iin19, AY082972
Aplanochytrium kerguelense, AB022103
Thraustochytrium multirudimentale, AB022111
rtUMH1dia, EF591019
RT7iin2, AY082992
rtAGH5dia, EF591008
rtUME3dia, EF591018
RT7in48, AY082977
rtAGC1dia, EF591007
Pinnularia cf. interrupta, AJ544658
Amphora montana, AJ243061
Eolimna minima, AJ243063
Asterionellopsis glacilia, X77701
Navicula cryptocephala, AJ297724
Chaetoceros rostratus, X85391
Oxytricha granulifera, AF508762
rtIZ13cil, EF591014
RT7iin1, AY083001
Engelmanniella mobilis, AF508757
Uroleptus gallina, AF508779
Labyrinthula sp., AB022105
rtCF12laby
0.10
Chlorellales
Microthamniales
Zygnematales
Klebsormidiales
Chlamydomonadales
Naviculales
Stichotrichida
Labyrinthulida

Figure 7