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Comparative microbial ecology of the water column of an extreme acidic pit lake, Nuestra Señora del Carmen, and the Río Tinto basin (Iberian Pyrite Belt)

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Summary. The Iberian Pyrite Belt, located in Southwestern Spain, represents one of the world's largest accumulations of mine wastes and acid mine drainages. This study reports the comparative microbial ecology of the water column of Nuestra Señora del Carmen acid pit lake with the extreme acidic Río Tinto basin. The canonical correspondence analysis identified members of the *Leptospirillum*, *Acidiphilium*, *Metallibacterium, Acidithiobacillus*, *Ferrimicrobium* and *Acidisphaera* genera as the most representative microorganisms of both ecosystems. The presence of archaeal members is scarce in both systems. Only sequences clustering with the *Thermoplasmata* have been retrieved in the bottom layer of Nuestra Señora del Carmen and one station of Río Tinto. Although the photosynthetically active radiation values measured in this lake upper layer were low, they were sufficient to activate photosynthesis in acidophilic microorganisms. All identified photosynthetic microorganisms in Nuestra Señora del Carmen (members of the *Chlamydomonas, Zygnemopsis* and *Klebsormidium* genera) are major members of the photosynthetic eukaryotic community characterized in Río Tinto basin. This study demonstrates a close relationship between the microbial diversity of Nuestra Señora del Carmen pit lake and the diversity detected in the Río Tinto basin, which underlain the influence of the shared mineral substrates in the microbial ecology of these ecosystems. [**Int Microbiol** 2014; 17(4):225-233]

Keywords: iron cycle · acidic pit lakes · acidophilic microorganisms · Río Tinto · Iberian Pyrite Belt

Introduction

Extreme acidic environments are rather scarce and mainly associated with volcanic and metal mining activities [16,24]. The peculiar ecology and physiology of acidophilic microor-

ganisms have interested microbiologists since their discovery [12] because the extreme acidic conditions in which they develop are the product of microbial metabolism. In mining areas oxidation of metal sulfides by acidophilic chemolithotrophic microorganisms leads to the formation of highly acidic, metal-laden waters [34] known as acid mine drainage (AMD).

The Iberian Pyrite Belt (IPB) represents one of the world's largest accumulations of mine wastes and AMDs [40]. Currently, there are more than 25 pit lakes between the provinces of Huelva and Seville (SW Spain) [31]. IPB pit lakes are nor-

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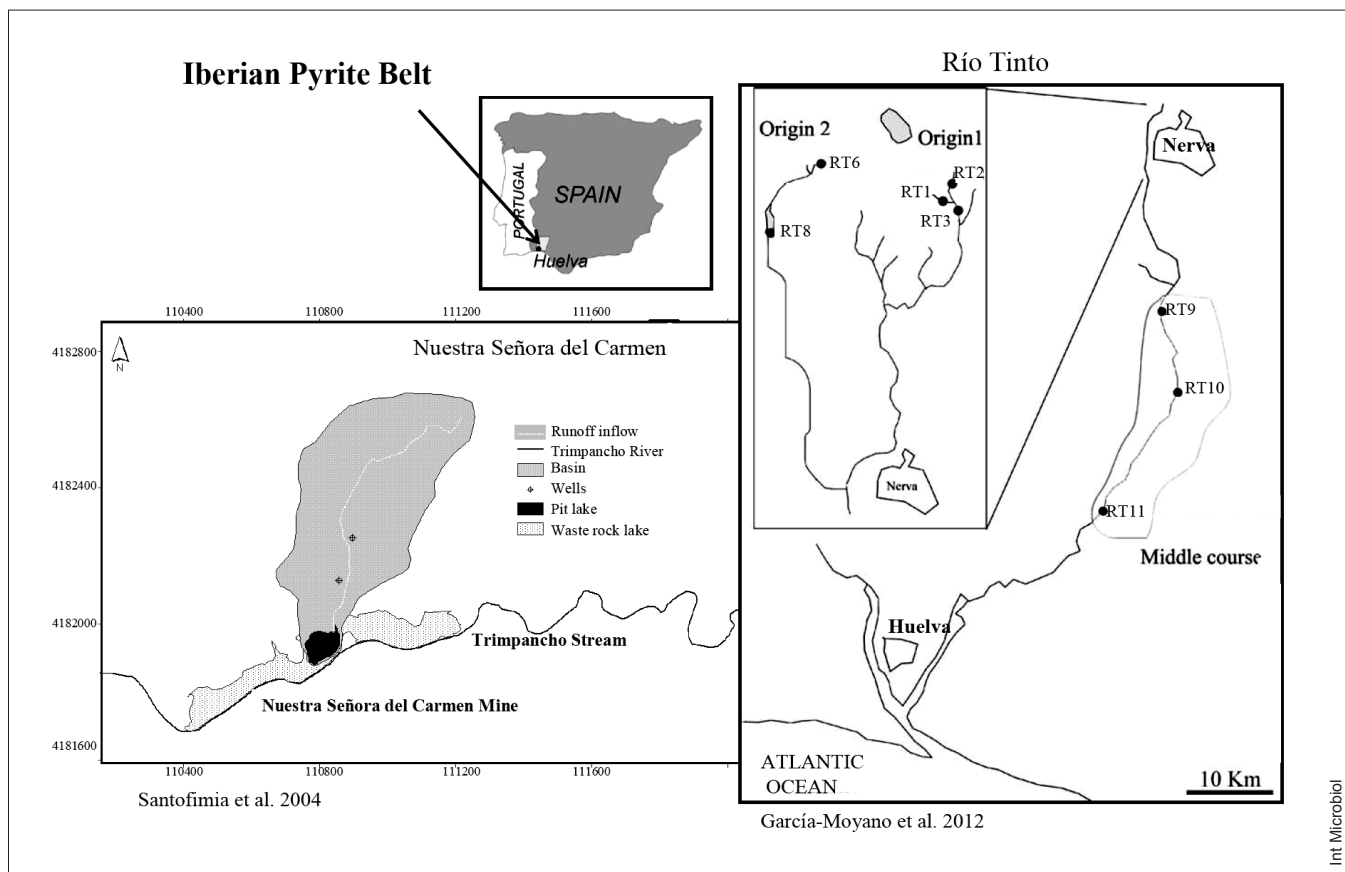E-mail: gonzalezte@cab.inta-csic.es

Fig. 1. Río Tinto basin and Nuestra Señora del Carmen (NSC) pit lake locations in the IPB.

mally acidic and present a wide range of sizes, depths, and physico-chemical characteristics. Their low pH and high concentrations of toxic heavy metals have limited the biological diversity of these lakes [42]. Nuestra Señora del Carmen (NSC) is a 110-m long and 80-m wide pit lake from the IPB, with a depth of 34 m and a volume of ca. 79,500 m^3 [44], an acidic pH (between 2.1 and 2.5), and high concentrations of sulfate and toxic heavy metals (Fig. 1 and Table 1).

The geomicrobiology of the IPB is mainly known from studies of the Río Tinto basin [2,3,4,19,20,21,33,47] or Río Odiel basin [30]. However, our knowledge of microbial diversity in pit lakes from this area is far from complete since very few studies have been reported [17,42,48]. In addition, most studies of the geomicrobiology of the IPB have focused on the biological oxidation of iron and sulfur, due to their importance in biohydrometallurgical processes, whereas little attention has been paid to the biological reduction of these elements under the anoxic conditions existing at the bottom of these lakes. To improve the efficiency of biohydrometallurgical operations, especially those in which control of environ-

mental conditions is difficult such as heap leaching, it is necessary to gain information on the microbial ecology operating in these ecosystems.

In this work we compared the microbial ecology of the water column of an extreme acidic pit lake, NSC, with the diversity detected along the course of Río Tinto, a well-established model of AMD of the Iberian Pyrite Belt (Fig. 1).

Materials and methods

Sampling. Field measurements and water and sediments sampling in the Río Tinto basin were carried out in May 2005 [20] and those in the NSC pit lake were carried out in May 2009 [42]. The Río Tinto environmental conditions were those described by García-Moyano et al. [20]. Environmental conditions for the water column of the pit lake were measured with a Hydrolab Datasonde S5 probe (Hach, USA). Water samples were collected from different depths using an opaque, 2.2-l PVC bottle (Beta Plus Wildlife Supply). All samples were filtered on site with 0.45-μm membrane filters from Millipore, stored in 125-ml polyethylene bottles, acidified with HNO_3 (1 ml), and kept at 4°C during transport. One-liter water samples for DNA extraction were collected and immediately filtered in situ through a Millex-GS Millipore filter (pore size, 0.22 μm; diameter, 50 mm). Filters were stored at –20°C

Table 1. Water chemical composition and physicochemical parameters measured at Río Tinto and Nuestra Señora del Carmen. The table shows the average values for pH, redox potential (ORP), electric conductivity (EC), dissolved oxygen (DO) and major elements of Río Tinto basin and NSC pit lake. (b.d.l.): below detection limit of the technique.

	Nuestra Señora del Carmen				Río Tinto								
	NSC 0.1 m	NSC 5 m	NSC 15 m	NSC 25 m	RT1	RT2	RT3	RT5	RT6	RT8	RT9	RT10	RT11
pH	2.4	2.1	2.1	2.1	2.0	3.7	2.7	2.7	2.6	2.8	2.7	2.7	3.2
ORP (mV)	534	375	375	375	465	329	637	413	413	490	387	530	519
EC (mS/Cm)	8.11	9.09	9.10	9.10	30.50	8.68	19.40	11.10	5.16	6.55	7.80	6.32	2.53
DO (mg/l)	8.12	0.09	0.09	0.09	3.70	6.10	5.00	5.32	7.40	7.82	4.00	6.40	6.70
SO_4^{2-} (g/l)	6.36	6.40	6.52	6.19	15.04	1.65	0.79	2.77	0.79	1.79	1.69	1.39	0.36
Fe(II) (mg/l)	0.90	338	338	353	12837	176	1286	1527	431	1505	572	768	164
Fe(III) (mg/l)	596	368	388	350	2369	750	4194	891	306	273	867	33	16
Cu (mg/l)	27.4	28.6	29.6	28.8	20.4	b.d.l.	6.7	2.1	8.3	17	104.8	93.5	19
Co (mg/l)	0.65	0.67	0.67	0.67	164.30	6.70	8.01	13.81	2.22	8.80	7.80	6.00	0.70
Zn (mg/l)	8.20	8.99	8.90	8.84	20.40	31.51	29.70	28.00	43.32	111.41	118.63	103.71	20.40
Mn (mg/l)	77.8	82.2	84.1	83.5	49.8	179.8	151.2	143.8	30.6	55.8	42.0	34.4	7.7
Ni (mg/l)	0.78	0.77	0.76	0.74	11.31	1.51	3.31	1.42	0.41	1.00	1.81	1.30	0.30

until further processing. Sediment samples were obtained as described in García-Moyano et al. [20]. Sediment samples were collected at 1 cm deep. Samples for microscopic observation were collected in sterile tubes, fixed with 2% of formaldehyde and kept at 4°C until further analysis.

Analytical procedures. Water samples were analyzed by atomic absorption spectrometry (AAS, Varian SpectrAA 220FS), inductively coupled plasma-atomic emission spectrometry (ICP-AES, Varian Vista MPX), and inductively coupled plasma-mass-spectrometry (ICP-MS, Leco Renaissance). The accuracy of the analytical methods was verified against certified water references (TM-27.3 and TMDA-51.3 from the National Water Research Institute). Fe(II) concentration was measured by reflectance photometry with a Merck RQflex10 reflectometer and Reflectoquant analytical strips. Dissolved organic carbon (DOC) was analyzed by a Shimadzu TOC-V CPH analyzer and nitrogen and phosphorus by absorption UV-Vis spectrophotometry with an Alliance Integral Plus continuous flow autoanalyzer.

Microscopy and morphotype identification. Identification of algae and heterotrophic protists was carried out down to the lowest possible taxonomic level by direct microscopic observation of different morphological features based on previous studies of the eukaryotic communities in acidic environments [2–4]. A Zeiss Axioscope 2 microscope equipped with phase-contrast was used in this work.

DNA extraction, PCR amplification and sequencing. Fast DNA Spin kit for soil (MP Biomedicals, CA, USA) was used for DNA extraction according to the manufacturer's instructions. Samples were washed five times with TE buffer (10mM TrisHCl, 1mM EDTA, pH 8.0) prior to DNA extraction. DNA was purified by passage through a GeneClean Turbo column (MP Biomedicals, CA, USA). The 16S and 18S rRNA genes were amplified according to previously described methodologies [2,21] using the universal *Bacteria*-specific primers 27f and 1492r [29], *Archaea*-specific primers 21f

and 1492r [1,14], and *Eukarya*-specific 20f and 1800r primers [2]. PCR amplified genes were purified by GeneClean Turbo Column (MP Biomedicals, CA, USA) and cloned using the Topo TA Cloning Kit (Invitrogen, CA, USA). M13f and M13r primers were used for sequencing. PCR products were directly sequenced using a Big-Dye sequencing kit (Applied Biosystem) according to manufacturer's instructions.

Phylogenetic analysis. Sequences were analyzed using BLAST at the NCBI database (http://ncbi.nlim.nih.gov/BLAST) and added together with the most important BLAST hits to create a database of over 50,000 homologous prokaryotic 16S rRNA primary sequences by using the ARB software package aligning tool [32]. Phylogenetic trees were generated using parsimony and neighbor-joining with a subset of 100 nearly full-length sequences (>1,400 bp). Filters which excluded highly variable positions were used.

An ARB-generated distance matrix was used as the input file to DOTUR program [45] and sequences were clustered into operational taxonomic units (OTUs) based on 100% and 97% sequence of similarity. Rarefaction analysis and the Chao1 non-parametric diversity estimator [9] were applied to the clone library in order to estimate how completely the library had been sampled and to extrapolate to total sequence diversity. Community diversity was studied by estimating the similarity between communities based on members and structure [45].

Sequences used in this study can be found in the EMBL sequence database under accession numbers JF737859–JF737929, JF807634–JF807641 and KC619546–KC619624 [19,42].

Multivariate analysis. Data were analyzed using a combination of constrained and unconstrained multivariate statistical methods in order to account for both total variation in the data and variations explainable by environmental data. Of the analyzed elements, we retained 12 environment variables that had no missing values, 8 chemical elements (SO_4^{2-}, Fe(II), Fe(III), Cu, Co, Zn, Mn and Ni) and pH, redox potential, electric conductivity, and

dissolved oxygen values (Table 1). Environmental data were transformed using ln(x+0.1) and normalized to zero mean and unit variance. Canonical correspondence analysis (CCA) was used to relate the microbial community data with environmental data. It was conducted using OTUs of 16S rRNA genes acquired at different sampling stations (18 samples from 9 different stations for the river) and 2 depths for the pit lake. Samples from the river were collected from sediments (S) or water column (W) for every station. The analyzed pit lake samples were from the surface (NSC01) and from 15-meter depth (NSC15). The significances of the first CCA axis and of all CCA axes combined were tested using Monte Carlo permutation tests. CCA tests were performed by using the multivariate data analysis software CANOCO 4.5 [7]. The program CANODRAW 4.0 in the Canoco package was used for graphical presentation of ordination results [7].

Results and Discussion

Nuestra Señora del Carmen (NSC) was an open cast exploitation abandoned in 1976 [26]. The NSC pit lake is acidic with high concentrations of sulfate and metals (Mg, Fe, Al, Mn and Cu) (Table 1). It is usually a meromictic lake, showing chemical stratification [43,44]. However, at the beginning of some dry winters, a period of mixing and total homogenization could be observed, temporarily transforming it into a holomictic lake. This process ended after intense episodes of rain

[44]. In May 2009, the pit lake showed a two-layer chemical and thermal stratification: a 2-meter thin upper layer and a denser homogeneous bottom layer (from 3 m to 35 m depth) (Fig 2). High redox potentials values corresponding to oxygen-saturated conditions were measured in the upper layer, where Fe was predominantly oxidized (Table 1). The bottom layers of acidic meromictic lakes are usually anoxic, which favour the microbial reduction of iron, generating low redox potentials [6,8,15,22,36,37,49]. By contrast, during this study the bottom layer of the NSC pit lake was in a slightly reducing condition with similar concentrations of reduced and oxidized iron (Table 1). This peculiar distribution of the iron redox pair has been previously detected in other acidic pit lakes [23,37]. Preliminary studies in the NSC pit lake [43,44] revealed variable Fe(II)/Fe(III) ratios in the bottom layer as a consequence of the lake dynamics. During the mixing period, dissolved oxygen increases in the bottom layers promoting biological iron oxidation processes, thus reducing the Fe(II)/Fe(III) ratio. When stratification was restored, an increase in the Fe(II)/Fe(III) ratio due to microbial anaerobic respiration was detected [43,44]. Photosynthetically active radiation (PAR) was 4000 μmol/s.m² in the surface and practically zero from 2 meters deep up to the bottom (Fig. 2).
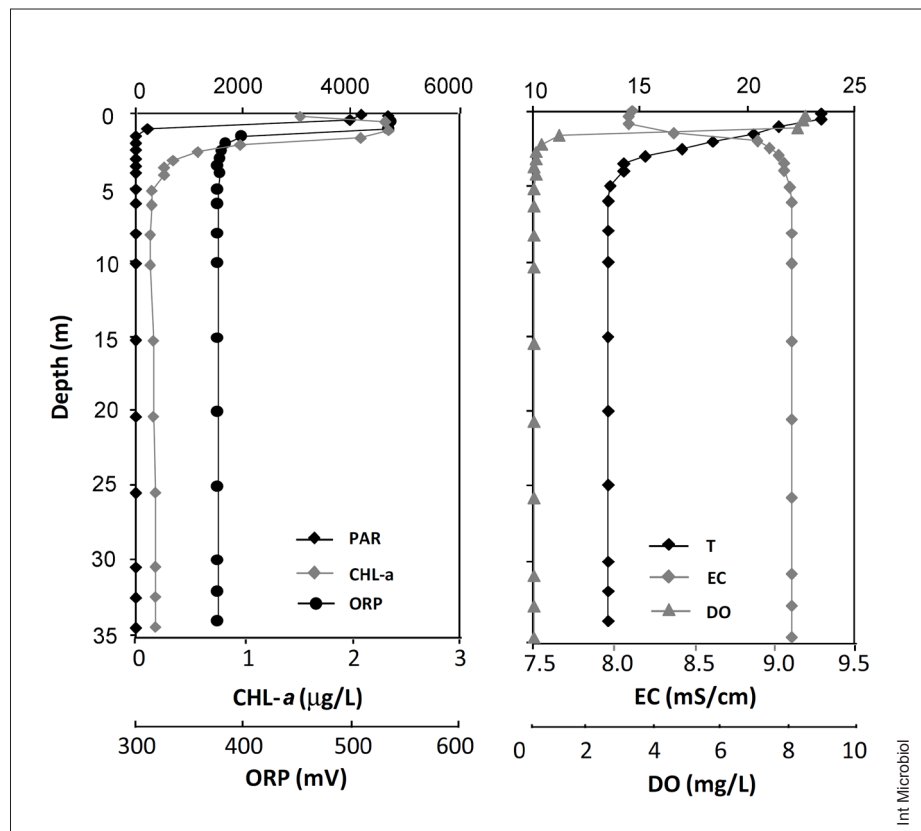
Fig. 2. Physicochemical variables of the NSC pit lake. Temperature (T), electric conductivity (EC), dissolved oxygen (DO), redox potential (ORP), chlorophyll-*a* (CHL-*a*) and photosynthetically active radiation (PAR).
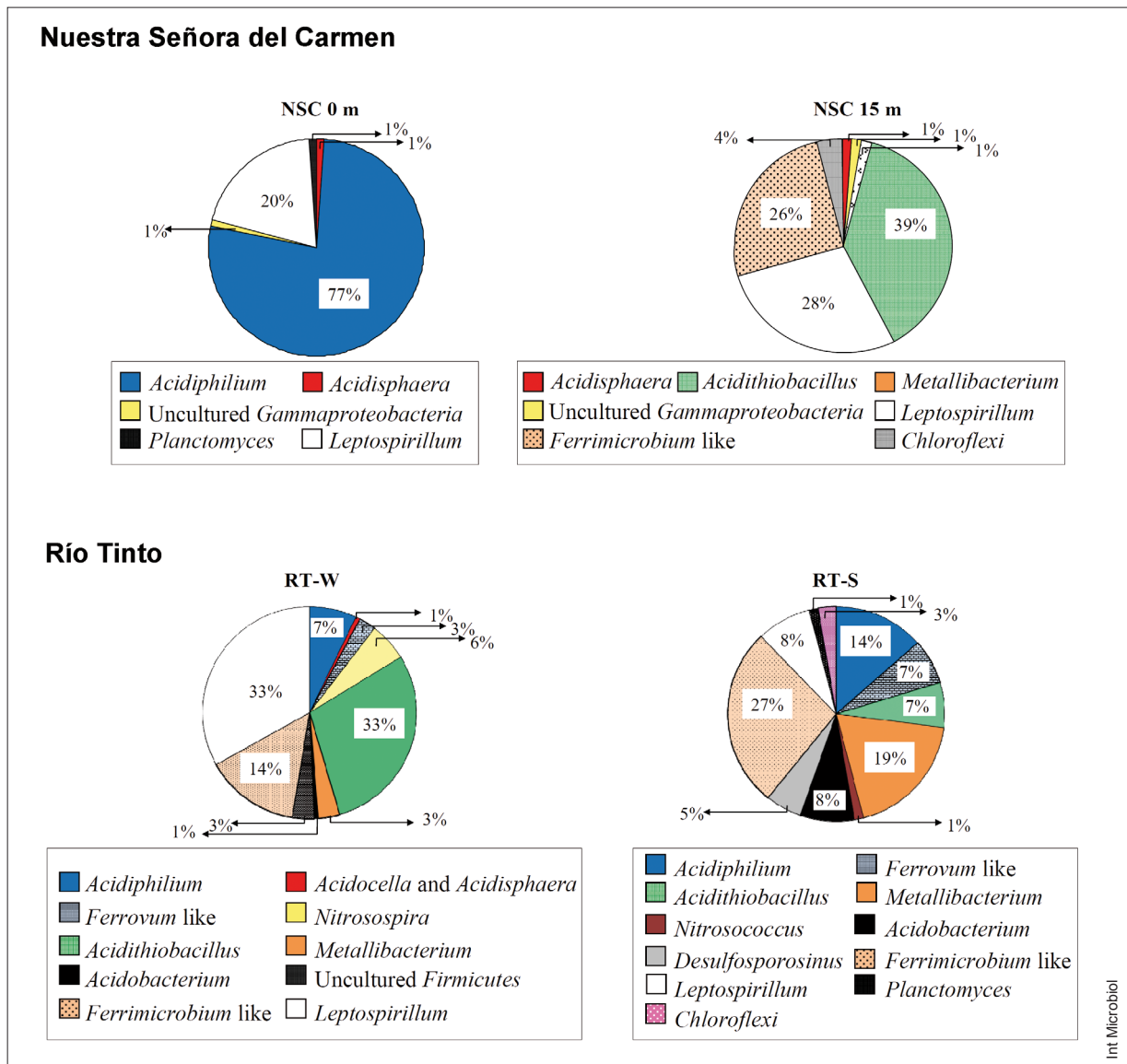
Fig. 3. Bacterial abundance comparison between NSC pit lake and Río Tinto. Relative abundance of OTUs in bacterial genera (in percent) is shown for each depth (0m and 15m) for the NSC pit lake and for the water column (RT-W) and sediments (RT-S) for the Río Tinto basin.

We took advantage of the recent characterization of the microbial diversity analyzed in two layers (surface and 15 m depth) of the NSC pit lake [42] for its comparison with the diversity reported for different sampling stations along the Río Tinto basin [20]. In the upper layer of the NSC pit lake, almost 80% of the identified sequences belonged to the *Alphaproteobacteria* class, being *Acidiphilium* the most abundant phylotype. Only 20% of the sequences detected at the surface were related to the *Nitrospirae* phylum, all clustering within the genus *Leptospirillum*. Additionally, sequences belonging to the *Planctomycetes* phylum (2%), the *Gammaproteobacteria* class (2%) and the *Acidisphaera* genus (2%)

were detected in this layer (Fig. 3). No sequences belonging to the archaeal domain could be retrieved from the surface sample.

By contrast, at 15 m depth, 39% of the sequences all related to the Gammaproteobacteria class, clustered within the species *Acidithiobacillus ferrooxidans*. A 28% of the sequences were related to the *Nitrospirae* phylum, with *Leptospirillum* being the most abundant genus and 26% of the sequences clustered within the *Actinobacteria* class. Sequences related to the *Chloroflexi* (4%) and *Acidisphaera* (1%) genera were also detected (Fig. 3). Sequences belonging to the archaeal domain, all of them related to the *Euryarchaeota*

phylum, specifically to the *Thermoplasmata* class, were identified in this layer.

As expected, Fe(II) concentrations in the upper layer of the NSC pit lake and the water column of Río Tinto were extremely low (Table 1). Even though at the surface *Acidiphilium* spp. together with photoreduction of iron could generate Fe(II), the reduced form of iron does not accumulate because is rapidly oxidized by iron oxidizing bacteria, like *Leptospirillum* [5,11,18,41,42].

When compared to the diversity reported for Rio Tinto's water column the most important difference was the absence of the iron oxidizing bacteria *A. ferrooxidans* in the upper oxidized layer of the pit lake (Fig. 3). Considering the physicochemical characteristics of both systems (Table 1 and Fig. 2) and the fact that *A. ferrooxidans* is present at the 15-m deep layer, a possible interpretation is that *A. ferrooxidans* is mainly involved in the reduction of Fe rather than its oxidation in this ecosystem.

The bottom layer of the NSC pit lake showed a higher level of diversity, which correlates with the higher diversity observed in the sediments of the Río Tinto basin [20]. In this case, the detected *Acidithiobacillus* spp. and members of the *Acidimicrobiaceae* family, both facultative iron reducing bacteria under anoxic conditions [13], could be responsible of the increase in the Fe(II)/Fe(III) ratio in this layer when the pit lake was chemically stratified, while *Leptospirillum* and some *Acidimicrobiaceae* members could oxidize iron when stratification broke down and the mixing introduced oxygen in the bottom layer. A similar situation might be operating in the sediments of Río Tinto, in which iron reducers of the *Acidithiobacillus, Acidiphilium* and *Acidimicrobium* genera can be found together with iron oxidizers of the *Leptospirillum, Ferrovum* like and *Ferrimicrobium* genera, although in this case the influence of an effective compartmentalization facilitated by the semisolid matrix of the sediments, inexistent in the water column, has to be considered.

A sulfur cycle is also operating at the bottom layer of the NSC similarly to the Río Tinto basin. Reduced sulfur compounds are the energy source used by *A. ferrooxidans* during anaerobic respiration [25]. In addition, some strains of *A. ferrooxidans* and *Thermoplasmata* could grow anaerobically using hydrogen as electron donor and sulfate as an electron acceptor [25,35]. Sulfate-reducing bacteria were not detected in the NSC pit lake while microorganisms capable of this anaerobic respiration were identified in the Río Tinto sediments [38,39] (Fig. 3). This is probably due to the environmental compartmentalization that can be generated in the sediments. Although microbial sulfate reduction can proceed in environ-

ments with a low pH, it is well established that inhibition processes are present in AMD systems. Possible inhibitory factors include the metabolites and organic acids, which can be toxic depending on pH. Metal sulfide precipitation and competition with other bacteria, namely iron-reducing bacteria, can inhibit sulfate reduction [28]. Based on this, it is easier to find SRB in Río Tinto sediments than in the NSC water column. Sediment microniches are suitable for the SRB activity, while in the water column the SRB activity is easily inhibited.

Sequences closely related to *Chloroflexi* have been detected in both the NSC pit lake and the water column of one Río Tinto sampling site (RT8) [19,42]. This type of sequences have been retrieved from different mine drainage environments [20,46,50]. Although the role of these bacteria in extreme acidic environments has to be clarified, bacteria belonging to this phylum have been isolated and related with the iron cycle [27].

No archaeal sequences could be retrieved neither in the upper layer of NSC nor in the Río Tinto water column. However, sequences related to the *Euryarchaeota* phylum of *Archaea*, specifically to the *Thermoplasmata* class, were detected in the bottom layer of the pit lake and the sediments of one sampling station from the Río Tinto basin (RT9).

The eukaryotic 18S rRNA gene clone library for NSC only showed positive results for the surface layer. In this layer, measured PAR values were low (Fig. 2), but sufficient to activate photosynthesis in acidophilic species [10]. All identified sequences were related to the genus *Chlamydomonas,* although microscopic observations also showed the presence of filamentous algae related to the *Zygnemopsis* and *Klebsormidium* genera as well as diatoms belonging to the genus *Pinnularia*, which could not be detected by molecular techniques [2]. *Chlamydomonas* is one of the most abundant algae detected in the Río Tinto basin, followed by the filamentous algae belonging to the *Zygnemopsis* and *Klebsormidium* genera [3]. In the river, diatoms have been detected mainly associated to the most extreme conditions [3]. Although the level of eukaryotic diversity detected in the Río Tinto basin is much higher than in the NSC pite lake due to its size (92 km) and the existence of very productive biofilms covering the rocks along the entire course of the river [2,3,4] the similarities existing between both ecosystems are noteworthy.

Canonical correspondence analysis (CCA) was used to correlate microbial community data with environmental data in both ecosystems. Fig. 4 shows the results of this analysis as a three biplots, to facilitate its interpretation. The CCA was conducted using OTUs of 16S rRNA genes acquired at different depths for the NSC pit lake and different sampling stati-
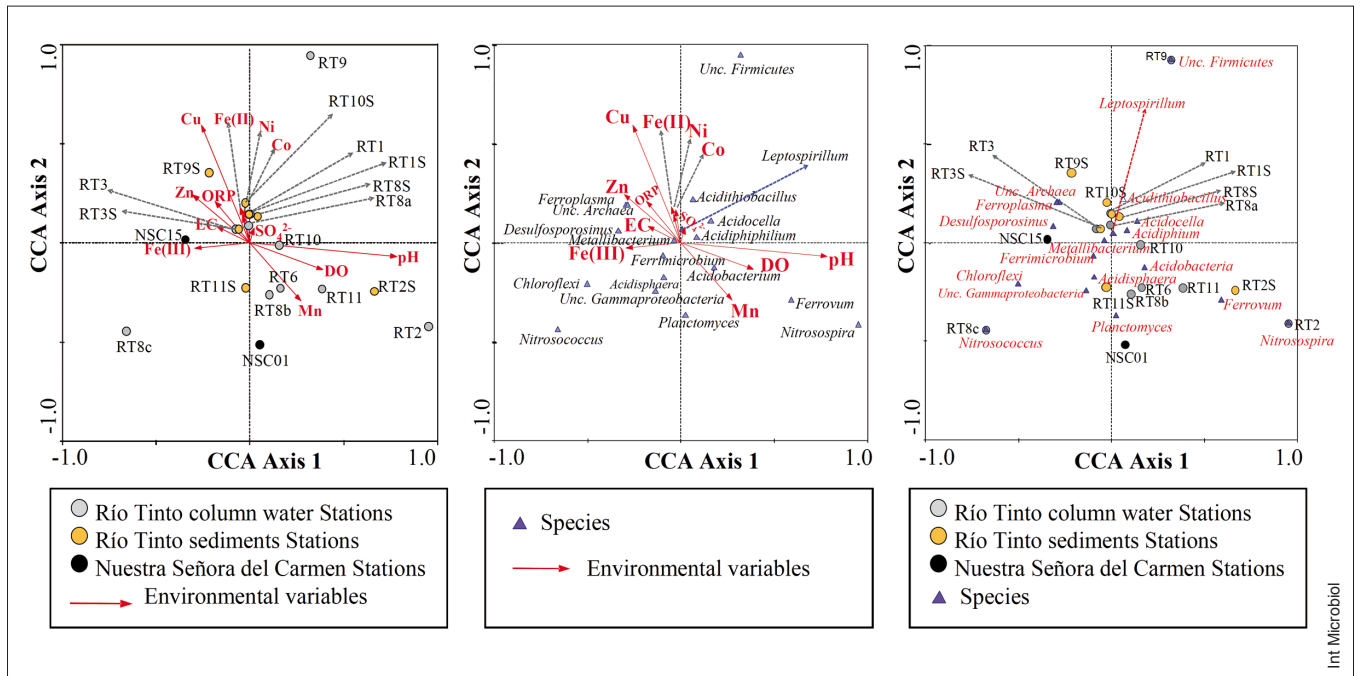
Fig. 4. Canonical correspondence analysis based on variance of species (OTUs) with respect to environmental data. Results were graphed in three biplots. The eigenvalues for each axis generated by CCA indicate how much of the variation seen in species data can be explained by that canonical axis. A 51% of the correlation between OTUs, sample sites and environmental data was explained by two axes. The presence or absence of data was used for bacterial and archaeal OTUs. Different OTUs are represented by genera names and triangles. Environmental variables used in the analysis are shown by arrows. Sampling sites are indicated by dots and the station name (NSC01, NSC15, RT1w, RT1s, RT2w, RT2s, RT3w, RT3s, RT5w, RT5s, RT6w, RT6s, RT8w, RT8s, RT9w, RT9s, RT10w, RT10s, RT11w and RT11s). Samples from the river were from sediments (S) or water column (W). Pit lake samples analyzed were from the surface (NSC01) and from 15-m depth (NSC15).Units of the elements were mg/l. Units of sulfate was g/l. Units of environmental data were mV for redox potential (ORP), mS/cm for electric conductivity (EC) and mg/l for dissolved oxygen (DO).

ons for the Río Tinto basin. Samples are plotted in different areas of the diagram depending on their environmental characteristics. The CCA generates an ordinate diagram in which axes are created by a combination of environmental variables [7] The eigenvalues for each axis generated by CCA indicate how much of the variation seen in the species data can be explained by that canonical axis. In this case, 51% of the correlation between OTUs sampling sites and environmental data could be explained by two axes (p-value < 0.05).

Metals (with the exception of Mn), sulfate, redox potential (ORP) and electric conductivity (EC) correlate inversely with pH and dissolved oxygen. The most important conclusion is that there is an important homogeneity in most of the samples, both at the physico-chemical and microbiological level. This is the reason why so many data occupy the centre of the graph, and the size of the arrows are rather small. The microorganisms appearing in most sampling stations were *Leptospirillum*, followed by *Acidiphilium*, *Metallibacterium*, *Acidithiobacillus*, *Ferrimicrobium* and *Acidisphaera*, all of them present in NSC and Río Tinto samples (Fig. 4). Río Tinto sam-

pling stations RT1, RT3, and RT8 appear at the centre of the graphs. They are very similar, so they can be considered the most representative samples of the river from an environmental and microbiological point of view (Fig. 4). These samples show the highest concentrations on sulfate, Ni and Co. These stations are the closest to the deep layer of the NSC pit lake. The dissolved oxygen concentration is the only difference.

From this analysis, it is clear that the NSC acidic pit lake has many common geomicrobiological features with the Río Tinto basin that underlie the influence of the shared substrates of the IPB in the microbial ecology of both ecosystems.

This study demonstrates a close relationship between the microbial diversity of NSC acidic pit lake and the diversity detected in the Río Tinto basin. The canonical correspondence analysis identified members of the *Leptospirillum*, *Acidiphilium*, *Metallibacterium*, *Acidithiobacillus*, *Ferrimicrobium* and *Acidisphaera* as the most representative microorganisms of both ecosystems. The presence of archaeal members was scarce in both systems. Only sequences clustering with the *Thermoplasmata* were retrieved in the bottom layer of NSC

and one sampling station of Río Tinto. Although the measured PAR values were very low in the upper NSC layer, they were sufficient to activate photosynthesis in acidophilic microorganisms. All identified photosynthetic microorganisms in the NSC (members of the *Chlamydomonas, Zygnemopsis* and *Klebsormidium* genera) were important members of the characterized photosynthetic eukaryotic community identified in the Río Tinto basin, which underlines the commonality between both ecosystems also at the eukaryotic level.

Competing interests. None declared.

References

1. Achenbach L, Woese C (1995) 16S and 23S rRNA-like primers. In: Sower KR, Schreier HJ (eds) Archaea: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, pp 521-523

2. Aguilera A, Gómez F, Lospitao E, Amils R (2006) 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 Appl Microbiol 29:593-605

3. Aguilera A, Manrubia SC, Gómez F, Rodriguez N, Amils R (2006) Eukaryotic community distribution and their relationship to water physicochemical parameters in an extreme acidic environment, Río Tinto (SW Spain). Appl Environ Microbiol 72:5325-5330

4. Aguilera A, Souza-Egipsy V, Gómez F, Amils R (2007) Development and structure of eukaryotic biofilms in an extreme acidic environment, Río Tinto (SW, Spain). Microb Ecol 53:294-305

5. Blodau C (2006) A review of acidity generation and consumption in acidic coal mine lakes and their watersheds. Sci Total Environ 369:307-332

6. Boehrer B, Schultze M (2008) Stratification of lakes. Rev Geophys 46:1-27

7. Braak CJF, Šmilauer P (2002) CANOCO reference manual and CanoDraw for Window's user's guide: software for canonical community ordination (version 4.5). Microcomputer Power ed., Ithaca, NY

8. Brown JF, Jones DS, Mills DB, Macalady JL, Burgos WD (2011) Application of a depositional facies model to an acid mine drainage site. Appl Environ Microbiol 77:545-554

9. Chao A (1984) Non-parametric estimation of the number of classes in a population. Scand J Stat 11:265-270

10. Cid C, García-Descalzo L, Casado-Lafuente V, Amils R, Aguilera A (2010) Proteomic analysis of the response of an acidophilic strain of *Chlamydomonas* sp. (Chlorophyta) to natural metal-rich water. Proteomics 10:2026-2036

11. Collienne RH (1983) Photoreduction of iron in the epilimnion of acidic lakes. Limnol Oceanogr 28:83-100

12. Colmer AR, Temple KL, Hinkle HE (1950) An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. J Bacteriol 99:317-328

13. Coupland K, Johnson DB (2008) Evidence that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria. FEMS Microbiol Lett 279:30-35

14. DeLong EF (1992) *Archaea* in coastal marine environments. Proc Natl Acad Sci USA 89:5685-5689

15. Díez-Ercilla M, López-Pamo E, Sánchez-España J (2009) Photoreduction of Fe(III) in the acidic mine pit lake of San Telmo (Iberian Pyrite Belt): field and experimental work. Aquat Geochem 15:391-419

16. Ehrlich HL, Newman DK (2009) Geomicrobiology. Fifth ed., CRC Press Taylor and Francis Group, Boca Raton, Florida

17. Falagán C, Sánchez-España J, Johnson DB (2014) New insights into the biogeochemistry of extremely acidic environments revealed by a combined cultivation-based and culture-independent study of two stratified pit lakes. FEMS Microbiol Ecol 87:231-243

18. Gammons CH, Nimick DA, Parker SR, Snyder DM, McCleskey RB, Amils R, Poulson SR (2008) Photoreduction fuels biogeochemical cycling of iron in Sapin's acid rivers. Chemical Geology 252:202-213

19. García-Moyano A, González-Toril E, Aguilera A, Amils R (2012) Comparative microbial ecology study of the sediments and the water column of Río Tinto, an extreme acidic environment. FEMS Microbiol Ecol 81:303-314

20. García-Moyano A, González-Toril E, Moreno-Paz M, Parro V, Amils R (2008) Evaluation of *Leptospirillum* spp. in Rio Tinto, a model of interest to biohydrometallurgy. Hydrometallurgy 94:155-161

21. González-Toril E, Llobet-Brossa E, Casamayor EO, Amann R, Amils R (2003) Microbial ecology of an extreme acidic environment, the Tinto River. Appl Environ Microbiol 69:4853-4865

22. Hedrich S, Schlömann M, Johnson DB (2011) The iron-oxidizing proteobacteria. Microbiology 57:1551-1564

23. Herzsprung K, Friese P, Packroff G, Schimmele M, Wendt-Potthoff K, Winkler M (1998) Vertical and annual distribution of ferric and ferrous iron in acidic mining lakes. Acta Hydrochim Hydrobiol 26:253-262

24. Johnson BD, Hallberg KB (2003) The microbiology of acidic mine waters. Res Microbiol 154:466-473

25. Johnson BD, Hallberg KB (2009) Carbon, iron and sulfur metabolism in acidophilic micro-organisms. Adv Microb Physiol 54:201-255

26. Junta de Andalucía (1986) La minería andaluza. In: Consejería de Economía y Fomento, Dirección General de Industria, Energía y Minas (Eds) Libro blanco, Empresa Nacional de ADARO de Investigaciones Mineras

27. Kawaichi S, Ito N, Kamikawa R, Sugawara T, Yoshida T, Sako Y(2013) *Ardenticatena maritima* gen. nov., sp. nov., a ferric iron- and nitrate-reducing bacterium of the phylum *'Chloroflexi'* isolated from an iron-rich coastal hydrothermal field, and description of *Ardenticatenia* classis nov. Int J Syst Evol Microbiol 63:2992-3002

28. Koschorreck M (2008) Microbial sulphate reduction at a low pH. FEMS Microbiol Ecol. 64:329-42

29. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, and Goodfellow M, (Eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichiester, pp. 115-175

30. López-Archilla AI, Amils R (1999) A comparative ecological study of two acidic rivers in southwestern Spain. Microb Ecol 38:146-156

31. López-Pamo E, Sánchez-España J, Díez M, Santofimia E, Reyes J (2009) Cortas mineras inundadas de la Faja Pirítica: inventario e hidroquímica. Instituto Geológico y Minero de España, Serie Medio Ambiente, Madrid, Número 13

32. Ludwig W, Strunk O, Westram R, Richter L, et al. (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32:1363-1371

33. Malki M, González-Toril E, Sanz JL, Gómez F, Rodríguez N, Amils R (2006) Importance of the iron cycle in biohydrometallurgy. Hydrometallurgy 83: 223–228

34. Nordstrom DK, Alpers CN (1999) Geochemistry of acid mine waters. In: Plumlee GS, Logsdon MJ (eds) The environmental geochemistry of mineral deposits. Reviews in Economic Geology, Littleton, CO., pp. 133-156

35. Ohmura N, Sasaki K, Matsumoto N, Saiki H (2002) Anaerobic respiration using Fe^{3+}, S^0, and H_2 in the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans*. J Bacteriol 184:2081-2087

36. Pellicori DA, Gammons CH, Poulson SR (2005) Geochemistry and stable isotope composition of the Berkeley pit lake and surrounding mine waters, Butte, Montana. Appl Geochem 20:2116-2137

37. Ramstedt M, Carlsson E, Lovgren L (2003) Aqueous geochemistry in the Udden pit lake, northern Sweden. Appl Geochem 18:97-108

38. Sánchez-Andrea I, Knittel K, Amann R, Amils R, Sanz JL (2012) Quantification of Tinto River sediments microbial communities: the importance of Sulfate-Reducing Bacteria and their role in attenuating acid mine drainage. Appl Environ Microbiol 78:4638-4645

39. Sánchez-Andrea I, Stams A, Amils R, Sanz JL (2013) Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments. Environ Microbiol Rep 5:672-678

40. Sánchez-España J, López-Pamo E, Santofimia E, Adurive O, Reyes-Andrés J, Barettino D (2005) Acid mine drainage in the Iberian Pyrite Belt (Odiel river watershed, Huelva, SW Spain): Geochemistry, mineralogy and environmental implications. Appl Geochem 20:1320-1356

41. Sánchez-España J, López-Pamo E, Santofimia E, Díez-Ercilla M (2008) The acidic mine pit lakes of Iberian Pyrite Belt: An approach to their physical limnology and hydrogeochemistry. Appl Geochem 23:1260-1287

42. Santofimia E, González-Toril E, López-Pamo E, Gomariz M, Amils R, Aguilera A (2013) Microbial diversity and its relationship to physicochemical characteristics of the water in two extreme acidic pit lakes from the Iberian Pyrite Belt (SW Spain). PLoS ONE 8:e66746

43. Santofimia E, López-Pamo E, González-Toril E, Aguilera A (2011) Changes in the Fe(II)/Fe(III) ratio by bacterial activity according to dynamics of an acid pit lake. Mineral Magazine V 75:1796

44. Santofimia E, López-Pamo E, Reyes M, Andrés J (2012) Changes in stratification and iron redox cycle of an acidic pit lake in relation with climatic factors and physical processes. J Geochem Expl 116-117:40-50

45. Schloss PD, Handelsman J (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501-1506

46. Senko JM, Wanjugi P, Lucas M, Bruns MA, Burgos WD (2008) Characterization of Fe(II) oxidizing bacterial activities and communities at two acidic Appalachian coalmine drainage-impacted sites. ISME J 2:1134-1145

47. Souza-Egipsy V, González-Toril E, Zettler E, Amaral-Zettler L, Aguilera A, Amils R (2008) Prokaryotic community structure in algal photosynthetic biofilms from extreme acidic streams in Río Tinto (Huelva, Spain). Int Microbiol 11: 251-60

48. Wendt-Potthoff W, Koschorreck M, Díez-Ercilla M, Sánchez-España J (2012) Microbial activity and biogeochemical cycling in a nutrient-rich meromictic acid pit lake. Limnologica 42:175-188

49. Wetzel RG (2001) Limnology: lake and river ecosystems. Third ed., Academic Press, San Diego

50. Zhang HB, Shi W, Yang MX, Sha T, Zhao ZW (2007) Bacterial diversity at different depths in lead-zinc mine tailings as revealed by 16S rRNA gene libraries. J Microbiol 45:479-84