Extremophiles (2012) 16:829–839
DOI 10.1007/s00792-012-0478-4

ORIGINAL PAPER

Screening of anaerobic activities in sediments of an acidic environment: Tinto River

Irene Sánchez-Andrea · Patricia Rojas-Ojeda · Ricardo Amils · José Luis Sanz

Received: 27 February 2012 / Accepted: 16 August 2012 / Published online: 7 September 2012

Abstract The Tinto River (Huelva, Spain) is a natural acidic rock drainage environment produced by the bio-oxidation of metallic sulfides from the Iberian Pyritic Belt. A geomicrobiological model of the different microbial cycles operating in the sediments was recently developed through molecular biological methods, suggesting the presence of iron reducers, methanogens, nitrate reducers and hydrogen producers. In this study, we used a combination of molecular biological methods and targeted enrichment incubations to validate this model and prove the existence of those potential anaerobic activities in the acidic sediments of Tinto River. Methanogenic, sulfate-reducing, denitrifying and hydrogen-producing enrichments were all positive at pH between 5 and 7. Methanogenic enrichments revealed the presence of methanogenic archaea belonging to the genera *Methanosarcina* and *Methanobrevibacter*. Enrichments for sulfate-reducing microorganisms were dominated by *Desulfotomaculum* spp. Denitrifying enrichments showed a broad diversity of bacteria belonging to the genera *Paenibacillus*, *Bacillus*, *Sedimentibacter*, *Lysinibacillus*, *Delftia*, *Alcaligenes*, *Clostridium* and *Desulfitobacterium*. Hydrogen-producing enrichments were dominated by *Clostridium* spp. These enrichments confirm the presence of anaerobic activities in the acidic sediments of the Tinto River that are normally assumed to take place exclusively at neutral pH.

Keywords Acidophile · Anaerobes · Biodiversity · Biotechnology · Metabolism · Methanogens · Molecular phylogeny · Molecular biology

Communicated by A. Driessen.

I. Sánchez-Andrea (✉) · P. Rojas-Ojeda · J. L. Sanz
Departamento de Biología Molecular, Universidad Autónoma de Madrid, c/Darwin 2, C-014/021, Campus de Cantoblanco, Crtra. de Colmenar, Km. 15, 28049 Madrid, Spain
e-mail: irenesanchezandrea@hotmail.com

I. Sánchez-Andrea · R. Amils
Centro de Biología Molecular Severo Ochoa, UAM-CSIC, 28049 Madrid, Spain

R. Amils
Centro de Astrobiología, CSIC-INTA, 28850 Torrejón de Ardoz, Spain

Introduction

The exploration of extreme environments has led to the discovery of microbial activities in habitats that only a few years ago were considered uninhabitable. As a consequence, interest in their ecology has grown. The Tinto River is a natural acidic rock drainage (ARD) environment located in the Iberian Pyritic Belt (Huelva, South-Western Spain). The bio-oxidation of metal-sulfides in this system results in waters of low pH (mean pH 2.3), high concentrations of sulfate (6 g/L), ferric iron (2.3 g/L), and other heavy metals (Cu 0.1 g/L, Zn 0.23 g/L, etc.). Apart from the ecological interest of extremophiles, the Tinto River is also of interest in the areas of biogeochemistry (exploring the metabolic activities of the microbial communities associated with ARD), biotechnology (bioleaching processes), microbiology (acidic environments offer an almost unique opportunity to reveal biological habitat complexity) and Mars-analog studies (common mineralogy).

Recent geomicrobiological studies of the Tinto River have shown a high level of microbial diversity in the water column, mainly eukaryotic. Eighty percent of the prokaryotic biomass in the water column is composed of three bacterial genera: *Leptospirillum*, *Acidithiobacillus* and

Acidiphilium (Gonzalez-Toril et al. 2003), all of which involved in the iron cycle. Macroscopic brownish-filamentous structures (streamers) are present along the river, composed mostly of those three bacterial genera enmeshed in a matrix of exopolysaccharides and mineral particles (García-Moyano et al. 2007). A wide variety of eukaryotes, which include algae, ciliates, flagellates, amoebae, fungi and rotifers have been reported. Eukaryotic algae, which contribute to over 60 % of the river biomass (Lopez-Archilla et al. 2001), are mainly chlorophytes but also filamentous algae forming green-phototrophic filaments have been detected (Aguilera et al. 2006, 2007a, b). Heterotrophic protists—mixotrophic flagellates, ciliates or amoebas—are also widely distributed along the river. Among decomposers, fungi are the most abundant and both unicellular and filamentous forms are present (Lopez-Archilla et al. 2001, 2005). The only animal found in the river is a species of bdelloid rotifer related to the genus *Rotifera* (Zettler et al. 2002).

Most of the interest in these ecosystems has focused on the aerobic iron- and sulfur-oxidizing microorganisms due to their bio-mining applications. Despite their ecological interest, the characterization of the anoxic sediments from acidic environments, such as those of the Tinto River, had been neglected, with few exceptions (Blothe et al. 2008; Hao et al. 2007). Our group has recently started an in-depth study of the Tinto basin sediments to gain insight into how the element cycles work under anaerobic conditions and to find potential biotechnological applications for the microorganisms thriving in them. First, an extensive survey of the Tinto River sediment microbiota based on comparative sequence analyses of 16S rRNA genes was performed at two contrasting sampling sites (*SN* and *JL* dams) using two culture-independent approaches: denaturing gel gradient electrophoresis and 16S rRNA genes cloning (Sánchez-Andrea et al. 2011). Microorganisms involved in the iron (*At. ferrooxidans*, *Sulfobacillus* spp., *Ferroplasma* spp., etc.), sulfur (*Desulfurella* spp., *Desulfosporosinus* spp., *Thermodesulfobium* spp., etc.) and carbon (*Acidiphilium* spp., *Bacillus* spp., *Clostridium* spp., *Acidobacterium* spp., etc.) cycles were identified and their distribution correlated with physicochemical parameters of the sediments. Hybridization with domain-specific probes showed that *Bacteria* (>98 %) dominated over *Archaea* (<2 %) (Sánchez-Andrea et al. 2012a). Specific probes showed large differences between both sampling sites. At *SN*, where the pH and redox potential are similar to those of the water column (pH 2.5 and +300 mV), the most abundant microorganisms were identified as iron reducers: *Acidithiobacillus* spp. and *Acidiphilium* spp., given the high solubility of iron at low pH. At *JL* dam, characterized by a higher pH (between 4.2 and 6.2), more reducing redox potential (from +50 to −210 mV) and a lower iron solubility, members of sulfate-reducing genera, *Syntrophobacter, Desulfurella* and *Desulfosporosinus*, dominated.

With these previous molecular ecology studies, the prokaryotes thriving in the sediments have been identified, quantified and their corresponding metabolism inferred. A model of the iron, sulfur and carbon cycles operating in the sediments was proposed, suggesting the presence of iron reducers, methanogens, nitrate reducers, hydrogen producers and sulfate-reducing bacteria (SRB). However, we felt that a culture-dependent approach was needed to prove the existence of microbes performing those potential anaerobic activities and to identify them. Iron reducers are common at low pH and have been well studied in ARD sediments (Blothe et al. 2008), but the other detected activities, unusual at low pH, have received less attention. Therefore, the objectives of this study were, firstly, to investigate the viability of these activities in ARD-related sediments throughout the Tinto River, to identify the responsible microorganisms and finally, if possible, to isolate those with potential biotechnological applications.

Materials and methods

Sampling sites

Samples were collected from eight sampling sites (Fig. 1): *JL* (37.691207 N, 6.560587 W), *SN* (37.72173 N, 6.557465 W), *GAL* (37.725362 N, 6.561853 W), *BER* (37.601346 N, 6.549933 W), *SAL* (37.669622 N, 6.550212 W), *2.9* (37.7242 N, 6.56207 W), *EST* (37.68832 N, 6.563773 W), and *IZQ* (37.72891 N, 6.550255 W) along the Tinto River (Huelva, Spain) in June 2009 and May 2010. In general, the water layer is thinner than 20 cm and the bed of Tinto River is rocky, therefore samples were taken where there was sufficient sediment accumulation (~10 cm) directly with a sterile 50-mL tube. In two cases, *JL* and *SN*, samples were taken at two dams where there was enough sediment accumulation with 1 m water layer covering the sediment. In both sites, cores (7 cm inner diameter and 45 cm length) sediment samples were taken with a tubular sampler (Eijkelkamp Agrisearch equipment, Giesbeek, The Netherlands). The redox potential (*E*) and pH of the drill core samples were measured in situ with E and pH probes, connected to a Thermo Orion 290A potentiometer placed into the fresh sediment just after extraction. Data of the sampling sites are summarized in Table 1.

Enrichment cultures

Enrichments were prepared anaerobically and aseptically using an anaerobic chamber. Approximately 4 g of sediment was added to 50-mL glass serum bottles with 20 mL

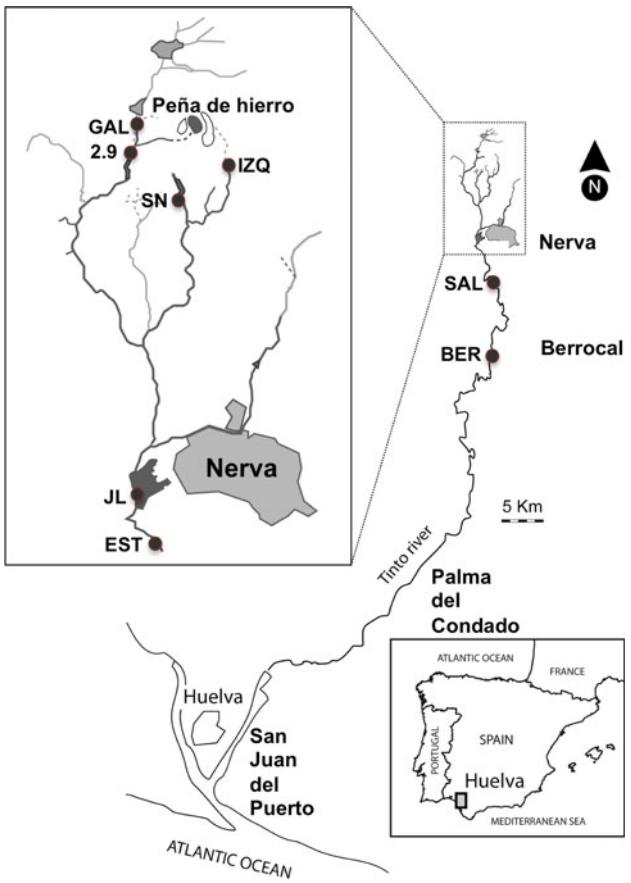

Fig. 1 Map of Tinto River. The river rises in Peña de Hierro and reaches the Atlantic Ocean at Huelva (80-km long). The sampling sites of this study are all located in the upper zone of the river, where the physical–chemical characteristics are more extreme. The *magnification square* shows the sampling points located in the streams near to the source. Most of the sampling sites are on sand/stone bed with the exception of *SN* and *JL,* which are small dams

of media and capped with butyl rubber stoppers. The headspace was then degassed with a N_2/CO_2 (80:20) mixture. Enrichments were incubated at 30 °C in the dark. The growth media used were selective for various microbial activities. For methanogenesis, the basal medium previously described (Sanz et al. 1997) was used at pH 6. To stimulate specific methanogenic groups, six different carbon sources were used: (a) $H_2 + CO_2$ (80:20); (b) volatile fatty acids (VFAs) (0.5 g/L of each sodium salts of acetate, propionate and butyrate); (c) mixture (M) (g/L) (0.7 sodium formate, 0.5 sodium acetate, 0.3 sodium propionate, 0.3 sodium butyrate, 0.5 methanol, 2 sucrose and 0.7 sodium lactate); (d) 2 g/L sodium acetate; (e) 1.5 g/L sodium formate; and (f) 1 g/L methanol. For sulfate reduction, the medium contained (g/L): 2 sodium lactate, 2 $KHCO_3$, 2 yeast extract, 2 $MgSO_47H_2O$, 1.5 Na_2SO_4, 0.5 K_2HPO_4, 0.1 $CaCl_2$, 0.48 $(NH4)_2Fe(SO_4)_2{\cdot}6H_2O$, and 1 mL of micronutrients solution (Sanz et al. 1997); enrichments for SRB were conducted at pH 5 and 7. Both heterotrophic (Manaia and Costa 1991) and autotrophic (Sánchez et al. 2008) denitrifying enrichments were conducted preparing media as described previously. In both media, final pH 5 and 7 were assayed. The medium used for hydrogen production was (g/L): 0.3 NH_4Cl, 0.3 K_2HPO_4, 0.1 $MgSO_4$, 0.5 $NaHCO_3$, 0.2 sucrose, 0.1 meat extract, 0.5 yeast extract, and 1 mL of micronutrients solution (Sanz et al. 1997) at a initial pH of 6. Cysteine was used as reducing agent (0.05 %) in all media.

Analytical measurement

Activity in methanogenic and denitrifying enrichments was determined by monitoring CH_4 and N_2O production in serum vial headspaces. For CH_4 determinations, 100 µL subsamples of headspace were injected into a Varian Star 3400CX gas chromatograph equipped with a split/splitness injector and a flame ionization detector (FID). The GC was fitted with a SPB1000 (25 m × 0.32 mm × 0.25 µm) column manufactured by Supelco with Carbowax 20 M polyethilenglicol as stationary phase. The temperatures for the column, injector and detector were 60, 200 and 250 °C, respectively. Nitrogen was used as carrier gas. The production of N_2O was measured using a HP 5890 Series Chromatograph equipped with an on-column injector and an electron capture detector (ECD). A Porapak 80-100 (1 m × 1/8″ mm × 2 mm) column manufactured by Sugerlabor was used. The temperatures for the column, injector and detector were 40, 300 and 300 °C, respectively. Nitrogen was used as carrier gas. Measurements were made by injecting 50 µL of headspace into the GC. Denitrifying activity was also determined measuring nitrate and nitrite concentrations by ion chromatography, using an Omega Metrohm 790 Personal IC, with a Metrosep A Supp 5 250/4.0 (Omega) column. Sulfate-reducing activity was determined visually by the formation of black precipitates from the reaction of H_2S produced by sulfate reduction (SR) and Fe^{2+} added in the media. The hydrogen production was measured periodically using a hydrogen detector (MDA Scientific Midas Gas Detector, Honeywell).

Isolation

Hydrogen producers and sulfate reducers, both of biotechnological interest, were isolated from enrichments. When activity was detected, sequential transfers were made into fresh medium. Those producing significant amount of H_2 or those with FeS precipitates were isolated by inoculation on agar plates and incubation at 30 °C in anaerobic jars. The solid media were the same as those mentioned above plus 1.5 % bacteriological agar. Single colonies

Table 1 Summary of anaerobic activities and sampling sites

Site			Methanogenesis	Sulfate reduction		Denitrification				Hydrogen production
Name	pH	E_h (mV)	pH 6	pH 5	pH 7	Autotroph pH 5	Autotroph pH 7	Heterotroph pH 5	Heterotroph pH 7	pH 6
JL[a]	3.8/5.4	+168/−71	−	+	+	+	+	+	+	+
SN[a]	3.9/4.0	+200/−13	−	+	+	−	+	+	+	+
GAL[a]	3.5/4.5[b] 5.9[c]	+200/+ 400[b] −205[c]	+ (VFA, mixture M)	+	+	−	−	−	−	+
BER[d]	2.4	+482.8	+ (H_2/CO_2, VFA, sodium acetate, sodium formate, methanol)	−	+	+	+	+	+	+
SAL[d]	2.3	+418.2	+ (mixture M)	+	+	ND	ND	ND	ND	ND
2.9[d]	2.9/3.7	+390	+ (methanol)	−	−	ND	ND	ND	ND	ND
EST[d]	2.2	+456.9	−	−	−	ND	ND	ND	ND	ND
IZQ[d]	3.9/4.0	+180/−20	+ (sodium formate, methanol)	−	−	ND	ND	ND	ND	ND

ND not determined

+/− presence/absence in the conditions tested

[a] Sediment

[b] Bulk

[c] Macroniches

[d] Water column

were picked and inoculated into 20-mL serum bottles with the selective media.

DNA extraction/16S rRNA gene amplification

Total DNA was extracted from enrichments and isolates with the FastDNA® SPIN® Kit (for soil) (Qbiogene, USA) following the manufacturer's instructions. The 16S rRNA genes were amplified using universal bacterial primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplification reaction was performed according to the Taq DNA polymerase protocol (Promega, USA). The PCR conditions were as follows: 10 min of initial denaturation at 94 °C; 30 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 3 min; followed by 10 min final incubation at 72 °C. The PCR products were purified with Jetquick PCR Product Purification Spin Kit (Genomed, Germany), according to the manufacturer's instructions.

Denaturing gradient gel electrophoresis (DGGE)

The V3 to V5 variable regions of the 16S rRNA gene were amplified with the primers set 341F(GC)-907R (T_a = 52 °C) for *Bacteria* and 622F(GC)-1100R (T_a = 42 °C) for *Archaea*. Primers 341F(GC) and 622F(GC) included a GC clamp (Muyzer and Ramsing 1995). The amplification reaction was performed according to the Taq DNA polymerase protocol (Promega, USA). The PCR conditions were as follows: 5 min of initial denaturation at 94 °C; 30 cycles at 94 °C for 1 min, 52 or 42 °C for 1 min and 72 °C for 2 min; followed by 10 min final incubation at 72 °C. DGGE analysis was carried out using a D-Code Universal Detection System instrument (Bio-Rad) according to the manufacturer's instructions (Bio-Rad). Polyacrylamide (6 %; 37.5:1 acrylamide–bisacrylamide) gels with a 30–60 % urea-formamide denaturant gradient (100 % urea-formamide contains 7 M urea and 40 % deionized formamide) were used in 1× TAE (Tris–acetate–EDTA) buffer, pH 7.4, at 200 V for 4 h at 60 °C. Gels were stained with ethidium bromide and visualized under UV illumination. The bands were cut from the gel with a sterile blade and placed in vials with 100 µL of Milli-Q water. DNA was allowed to diffuse into the water at 4 °C overnight. Five microliters of the eluate was used as a DNA template in a 50-µL PCR with the primers 341F/907R. The PCR products were purified with Jetquick PCR Product Purification Spin Kit (Genomed, Germany), according to the manufacturer's instructions.

Phylogenetic analysis

Samples were sequenced using a Big-Dye sequencing kit (Applied Biosystems). Sequences were checked with the program Finch TV (Geospiza, USA). Complete 16S rRNA

sequences were assembled using DNA Baser program. Comparative analysis was done using BLAST from NCBI (http://www.ncbi.nlm.nih.gov/Blast) and Sequence Match tool from Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/).

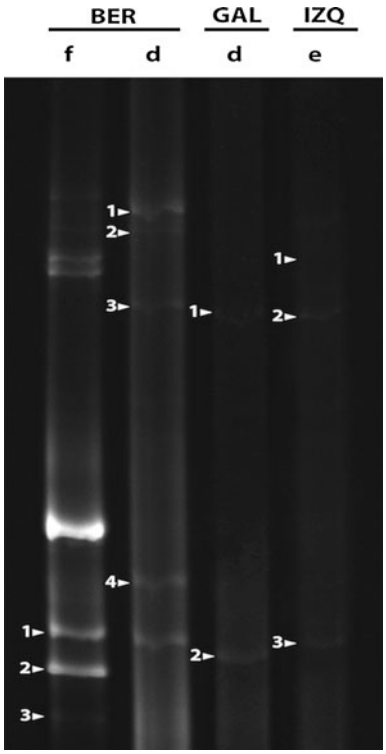

Fig. 2 DGGE fingerprints of 16S rRNA genes obtained with domain-specific primers for *Archaea* from methanogenic enrichments. Sampling locations are displayed on the *top of the lanes* followed by the electron donor nomenclature used in the sequences: methanol (*f*), acetate (*d*) and formate (*e*). *Numbers within lanes* correspond to the sequence nomenclature shown in Table 2

Nucleotide sequence accession numbers

The 16S rRNA genes sequences determined in this study have been deposited in the GenBank database under accession numbers JQ271537-JQ271598.

Results and discussion

Methanogenic and sulfate-reducing activities were assayed at all sampling sites. Denitrification and hydrogen production were studied in four of them (*SN*, *JL*, *BER* and *GAL*) according to previous results. Table 1 shows a qualitative summary of the measured activities at the different sampling sites.

Methanogenesis

A screening of 48 enrichments was performed, analyzing 8 sampling sites and 6 different carbon sources. The results are summarized in Table 1. Eleven enrichments showed methane production (nmol CH_4 per gram wet sediment and day): *SAL* with mixture M (32.9), 2.9 with methanol (24.5), *GAL* with VFA (2.8) and mixture M (29.4), *BER* with $H_2 + CO_2$ (2.5), VFA (1.2), sodium acetate (43.1), sodium formate (25.4) and methanol (30.8), and *IZQ* with sodium formate (37.4) and methanol (5.3). The positive enrichments were transferred once, total DNA was extracted and DGGE was run (Fig. 2). Twenty-two bands were excised from the gel; twelve were successfully amplified and sequenced (Table 2). *SAL* (M) and 2.9 (methanol) samples did not yield amplifiable bands.

Sequences were affiliated to both archaeon described phyla: *Crenarchaeota* and *Euryarchaeota*. Regarding the *Crenarchaeota,* some sequences retrieved from *GAL* and

Table 2 Phylogenetic affiliation of archaeal DGGE sequences from methanogen enrichments

DGGE band	Accession no.	Isolation medium	Phylogenetic assignment Class, family, genus species; % similarity
BER (1–3)_CF	JQ271588	Methanol	*Thermoprotei, Thermofiliaceae, Uncultured archaeon* ArcIV_cloneF06 <95 %
	JQ271589		
	JQ271590		
BER (1, 2, 4)_CD	JQ271591	Acetate	*Methanomicrobia, Metanosarcinaceae, Methanosarcina barkeri* strain Sar; 99–100 %
	JQ271592		
	JQ271594		
BER3_CD	JQ271593	Acetate	*Methanomicrobia, Metanosarcinaceae, Uncultured methanosarcina* sp. clone Soil113B_10; 94 %; *Methanosarcina barkeri* strain Sar; 94 %
IZQ1_CE	JQ271595	Formate	*Methanobacteria, Methanobacteriaceae, Methanobrevibacter arboriphilus* strain AZ; 100 %
IZQ2_CE	JQ271596	Formate	*Methanobacteria, Methanobacteriaceae, Methanobrevibacter arboriphilus* strain AZ; 96 %
GAL (1, 2)_CB	JQ271597	VGA	*Thermoprotei, Thermofiliaceae, Uncultured archaeon* clone ArcIV_cloneF06; 99 %
	JQ271598		

BER sites were affiliated to the family *Thermofiliaceae*, which comprise known neutrophilic and hyperthermophilic archaea (Burggraf et al. 1997), although recently uncultured relatives of our *Thermofiliaceae*-related sequences have been detected in acidic sediments (FN870267) (Lu et al. 2010). Related sequences have also appeared linked to methanogenic habitats (AJ576215) (Huang et al. 2003), (AB243802) (Sakai et al. 2007) and (EU155990) (Cadillo-Quiroz et al. 2008).

It was possible to identify phylogenetically down to the genus level some of the *Euryarchaeota* sequences. The nearest cultivated relatives to the DGGE bands at the sampling site *IZQ* incubated with formate was *Methanobrevibacter arboriphilus*, being formate a common *M. arboriphilus* substrate (Asakawa et al. 1993; Rea et al. 2007). This is to our knowledge the first report of this neutrophilic methanogen in enrichments with inoculum from an ARD-related environment. On the other hand, the nearest cultivated relatives to sequences of sampling site *BER* incubated with acetate was *Methanosarcina barkeri*. The presence of *Methanosarcina* and the absence of *Methanosaeta*, the other methanogenic acetoclastic genus, can be attributed to: (1) the high acetate concentration used in the enrichment medium, which is much higher than the *K*s of *Methanosarcina* for acetate, which outcompete *Methanosaeta* (*K*s and μmax 5×10^{-3} mol/L and 2.5×10^{-2}/h for *Methanosarcina* versus 0.5×10^{-3} mol/L and 3×10^{-3} /h for *Methanosaeta*) (2) the ability of *Methanosarcina* to tolerate mildly low pH conditions (Maestrojuán and Boone 1991), consistent with previous studies (Horn et al. 2003; Staley et al. 2011; Florencio et al. 1993). Remarkably, *Ms. barkeri* was identified in a methanol-spiked microcosm inoculated with Tinto River sediments in a previous study (Sanz et al. 2011).

Methanogenesis is usually believed to be inhibited at low pH, however, several studies have shown methanogenesis taking place at mildly acidic conditions, even at pH as low as 4.5 (Florencio et al. 1993; Williams and Crawford 1985; Bräuer et al. 2004; Goodwin and Zeikus 1987; Dunfield et al. 1993; Koschorreck et al. 2008). In fact, while the Gibbs energy of the hydrogenoclastic methanogenesis remains constant at different pH, the acetoclastic methanogenesis is energetically more favorable at pH below 4.5 (Dolfing et al. 2010), which indicates that once the methanogens are able to support a low pH environment, the process is thermodynamically possible and favorable.

In our enrichments, it was observed an increase of pH and a decrease of redox potential value, in accordance with previous studies performed using acidic sediments from Tinto River (Sanz et al. 2011). In this case it was suggested that although methanogens could prefer neutral pH, they can thrive in extremely acidic and oxidizing environment by creating microniches with higher pH. Our results confirm the existence of methanogens in this environment and their activity in enrichments at mildly acidic conditions.

Sulfate reduction

A screening was performed with samples from 8 sites incubated at pH 5 and 7 with lactate as an electron donor. In this set of 16 samples, five showed sulfate-reducing activity (Table 1) at pH 7, as shown by the occurrence of FeS black precipitates. Activity was also detected for enrichments at pH 5 from 4 different sites and during growth pH increased till 6.8.

Enrichments were inoculated on agar plates (SR media at pH 7 and 5). Positive results were only obtained in samples grown on agar at pH 7. Black colonies were picked and inoculated in specific media. Isolates were analyzed by 16S rRNA gene amplification and sequenced to determine their identity. Retrieved sequences were affiliated within the *Clostridium* and *Desulfotomaculum* genera (phylum *Firmicutes*, order *Clostridia*) (Table 3). *Desulfotomaculum* is a dissimilatory SRB able to form terminal and subterminal spores (Campbell and Postgate 1965). In permanently anoxic habitats, the non-sporeformers are dominant; but when the habitats are variable in oxygen conditions the spore-forming sulfate reducers dominate (Widdel 1992). *Desulfotomaculum* is able to survive dryness and oxic conditions for many months, even years. Interestingly enough, the closest relatives to our sequences were found in metalliferous organic soils (DQ479411), coal mines (HQ827821) and polluted estuaries (DQ677019) (Lin et al. 2007). The sequences had a high similarity with *Desulfotomaculum guttoideum* and *Desulfosporosinus orientis*, a SRB (Stahl and Amann 1991) previously detected at Tinto River (García-Moyano et al. 2007; Sánchez-Andrea et al. 2011).

Previous studies in the extreme environment of Tinto River had suggested the presence and abundance of a sulfate-reducing microbial community in the anaerobic zones of the Tinto River (Sánchez-Andrea et al. 2011, 2012a). This is interesting from a biotechnological point of view, as such activities can be used in bioremediation of acidic waters with a high heavy metals content (Sánchez-Andrea et al. 2012b; Johnson and Hallberg 2005; Bijmans et al. 2010; Church et al. 2007) and from an ecological point of view, due to the fact that SRB were supposed to prefer a neutral environment. In recent years, considerable evidences have revealed that SR at pH values below 5 is possible (Koschorreck 2008). Our results confirm the effective adaptation of SRB at low pHs by showing that active sulfate-reducers thrive in Tinto River sediments and are able to grow in enrichment cultures at pH as low as 5. In this work, it has been observed that they started to grow

Table 3 Phylogenetic affiliation of sequences from sulfate-reducer isolates

Isolate	Accession no.	Isolation medium	Phylogenetic assignment Class, family, genus species; % similarity
JL (1, 2, 7)_S	JQ271537	SO_4^{2-}/lactate	*Clostridia, Clostridiaceae, Clostridium* sp. OkiF101; 98 %
	JQ271538		
	JQ271543		
JL3_S	JQ251539	SO_4^{2-}/lactate	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain DPH-1; 99 %
JL4_S	JQ271541	SO_4^{2-}/lactate	*Betaproteobacteria, Alcaligenaceae;* <90 %
JL5_S	JQ271542	SO_4^{2-}/lactate	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain DPH-1; 93 %
SAL (1–3)_S	JQ271544	SO_4^{2-}/lactate	*Clostridia, Peptococcaceae, Desulfotomaculum* sp. Iso-W2; 99 %; *Clostridium celerescens* clone IrT-JG1-12; 99 %
	JQ271546		
GAL (1, 2, 3, 5)_S	JQ271547	SO_4^{2-}/lactate	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain DPH-1; 99–100 %
	JQ271548		
	JQ271550		
	JQ271552		
GAL4_S	JQ271551	SO_4^{2-}/lactate	*Clostridia, Peptococcaceae, Desulfotomaculum* sp. Iso-W2; 99 %; *Clostridia, Clostridiaceae, Clostridium celerescens* clone IrT-JG1-12; 99 %

at low pH and promote their optimal growth conditions increasing the pH and reducing the redox potential.

The failure in the isolation of SRB at pH 5 could be due to the combination of two potential toxic reagents at low pH: the agar and the lactic acid, which probably caused an inhibition of the SR in the agar plates. Subsequent studies have been performed with others electron donors different from lactic acids (neutral donors) with good results at pH as low as pH 4 (data not shown).

Denitrification

Biological denitrification, both assimilative and dissimilative, is the biological process for removing nitrate. Typically in most environments, nitrate is used as an electron acceptor by facultative heterotrophic bacteria comprising many diverse genera (*Pseudomonas*, *Alcaligenes*, *Bacillus*, etc.) under anoxic conditions, where organic matter provides electron donors to reduce nitrate to dinitrogen gas (Mateju et al. 1992). In contrast to the great diversity of heterotrophic denitrifiers, there are just a few autotrophic denitrifiers, able to use hydrogen, ferrous iron, or reduced sulfur compounds as electron donors.

As previously mentioned, autotrophic and heterotrophic denitrification at pH 5 and 7 were assayed at selected sampling sites (*SN, JL, BER* and *GAL*). In this set of 16 enrichments, denitrification activity was followed by nitrate (NO_3^-) reduction and nitrite (NO_2^-) and nitrous oxide (N_2O) production. Eleven of the enrichments showed activity with the exception of all *GAL* enrichments and *SN* enrichments at pH 5 in autotrophic medium (Table 1). Positive activities correlated with the appearance of green color in the bottles, probably due to the presence of polysulfides generated during the oxidation of sulfide coupled to the denitrification (Banda et al. 1989). An increment in the pH was detected in the enrichments with

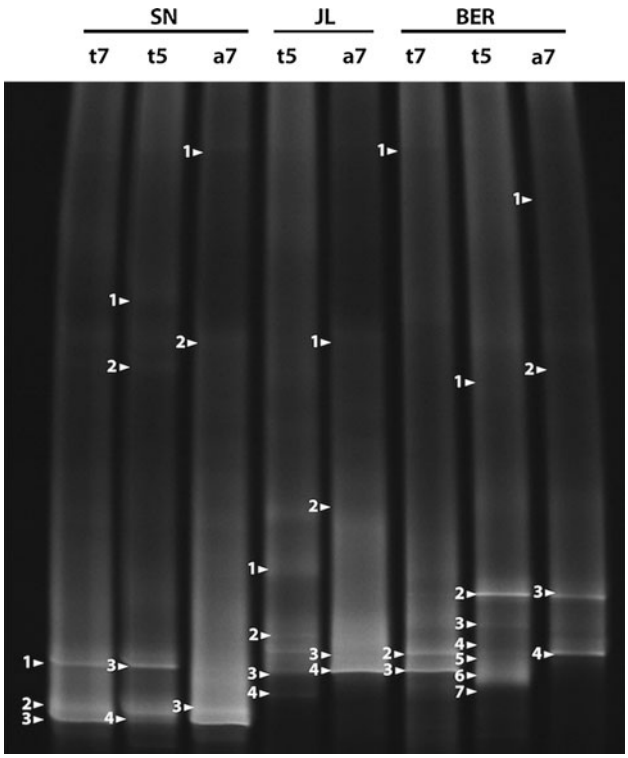

Fig. 3 DGGE fingerprints of 16S rRNA genes obtained with domain-specific primers for *Bacteria* from denitrifying enrichments. Sampling locations are displayed on the *top of the lanes* followed by medium nomenclature: *t* (heterotrophic medium), *a* (autotrophic), *5* (pH 5) and *7* (pH 7) *Numbers within lanes* correspond to the sequence nomenclature shown in Table 4

Table 4 Phylogenetic affiliation of bacterial DGGE sequences from denitrifying enrichments

Band	Accession no.	Isolation medium	Phylogenetic assignment Class, family, genus species; % similarity
JL1_Na5	JQ271565	NO_3^-/ $S_2O_3^{2-}$	*Bacilli, Paenibacillaceae, Paenibacillus* sp. enrichment culture clone 9; 99 %
JL (1–4)_Na7	JQ271566 JQ271569	NO_3^-/ $S_2O_3^{2-}$	*Bacilli, Bacillaceae, Bacillus* sp. cp64; 100 %
JL1_Nt5	JQ271570	NO_3^-/Y.E.	*Clostridia, Clostridiaceae, Sedimentibacter* sp. enrichment culture clone B4120; 96 %
JL2_Nt5	JQ271571	NO_3^-/Y.E.	*Bacilli, Bacillaceae, Lysinibacillus fusiformis* strain B116; 98 %
JL3_Nt5	JQ271572	NO_3^-/Y.E.	*Bacilli, Paenibacillaceae, Ammoniphilus* sp. CC-RT-E; 98 %
JL1_Nt7	JQ271573	NO_3^-/Y.E.	*Bacilli, Bacillaceae, Bacillus* sp. HZ-B; 99 %
SN (1–3)_Na7	JQ271574-76	NO_3^-/ $S_2O_3^{2-}$	*Bacilli, Bacillaceae, Bacillus* sp. HZ-B; 99 %
SN1_Nt5	JQ271577	NO_3^-/Y.E.	*Betaproteobacteria, Comamonadaceae, Delftia tsuruhatensis strain* WYLW2-1; 91 %; *Betaproteobacteria, Alcaligenaceae, Alcaligenes* sp.; 91 %
SN1_Nt7	JQ271578	NO_3^-/Y.E.	*Bacilli, Bacillaceae, Bacillus* sp. *HZ-B;* 99 %
BER (1–4)_Na7	JQ271553 JQ271556	NO_3^-/ $S_2O_3^{2-}$	*Clostridia, Clostridiaceae, Clostridium jejuense* strain *HY-35-12*; 96 %
BER1_Nt5	JQ271557	NO_3^-/Y.E.	*Clostridia, Clostridiaceae, Clostridium jejuense strain HY-35-12;* 97 %
BER (2 ,3, 4)_Nt5	JQ271558 JQ271560, JQ271561	NO_3^-/Y.E.	*Clostridia, Peptococcaceae, Desulfitobacterium* sp. enrichment culture clone CEB3; 97 %
BER (1–3)_Nt7	JQ271562 JQ271564	NO_3^-/Y.E.	*Bacilli, Paenibacillaceae, Paenibacillus* sp. enrichment culture clone S16; 88 %

an average of 0.02 units/day. Nitrate was consumed during the assay with an average of 1 mmol per mg bacterial wet weight and day. Nitrite was detected, but in a 7-fold lower concentration in the autotrophic samples in comparison with the heterotrophic ones. Nitrous oxide accumulated at 0.02 mmol per mg bacterial wet weight and day. A larger accumulation of nitrous oxide, 5-fold, was detected in the enrichments growing at pH 5 versus those growing at pH 7.

The optimum pH range for complete reduction of nitrate to nitrogen gas is considered to be between 6 and 8. In acid soils, below this optimal pH range, it has been reported that final steps of the denitrification are inhibited. In these environments, the proportion of intermediate products such as nitrite (Blösl and Conrad 1992; Nagele and Conrad 1990) and nitrous oxide (Simek et al. 2002) increase with respect to dinitrogen gas. A study with enrichment cultures suggested that denitrification occurred in ARD streams, and might reduce acidity (Baeseman et al. 2006), as confirmed in this assay.

Microbial populations were analyzed by DGGE to study the diversity and taxonomic position of the responsible microbes. From the eleven positive enrichments, just eight yielded amplifiable DNA (Fig. 3). Forty-nine bands were excised from the gels; thirty-two were successfully amplified and sequenced. As shown in Table 4, most of the sequences were affiliated with the phylum *Firmicutes,* with just one exception related to the *Betaproteobacteria* class. The identified microorganisms belonged to the genera *Bacillus*, *Paenibacillus*, *Lysinibacillus*, *Ammoniphilus*, *Rummelibacillus* (*Bacilli* class), *Clostridium*, *Desulfitobacterium, Sedimentibacter* (*Clostridia* class), and *Alcaligenes* (*Betaproteobacteria* class), previously reported as a denitrifying bacteria (Verbaendert et al. 2011; Villemur et al. 2006). The closest relatives to some of the sequences, such as *Bacillus* spp. (JN082261), *Sedimentibacter* spp. (EF464626) and *Desulfitobacterium* spp. (JF346160), have been identified previously in ARD systems. The most diverse communities appeared in the heterotrophic enrichments at pH 5, in which 9 different bacterial species were identified. In the rest of the enrichments (autotrophic media at both pHs and heterotrophic one at pH 7), only members of *Bacillus* and *Paenibacillus* genera were identified.

In previous studies of the subsurface of the Iberian Pyrite Belt, activities in the enrichments of thiosulfate oxidizers using nitrate as an electron acceptor were detected, but the organisms responsible for these activities were neither identified nor isolated and characterized (Fernández-Remolar et al. 2008). This study is the first to confirm the presence, activity and diversity of a denitrifying community in Tinto River sediments, which also have

Table 5 Phylogenetic affiliation of sequences from hydrogen-producer isolates

Isolates	Accession no.	Isolation medium	Phylogenetic assignment Class, family, genus species; % similarity
BER1_H	JQ271579	Sucrose	*Clostridia, Clostridiaceae, Clostridium* sp. enrichment culture clone *NHT;* 99 %
BER2_H	JQ271580	Sucrose	*Clostridia, Clostridiaceae*, *Clostridium* sp. enrichment culture clone *NHT*; 100 %
BERGAL_H	JQ271581	Sucrose	*Clostridia, Clostridiaceae, Clostridium beijerinckii* strain RZF1108; 99 %
GAL1_H	JQ271582	Sucrose	*Clostridia, Clostridiaceae, Clostridium bifermentans* strain: JCM 1386.; 100 %
GAL2_H	JQ271583	Sucrose	*Bacilli, Bacillates incertae sedis, Rummeliibacillus pycnus* strain NBRC 101231; 98 %
SN1_H	JQ271584	Sucrose	*Clostridia, Clostridiaceae, Clostridium celerecrescens* clone IrT-JG1-12; 99 %*; Clostridia, Peptococcaceae, Desulfotomaculum* sp. Iso-W2; 99 %
SN2_H	JQ271587	Sucrose	*Clostridia, Clostridiaceae, Clostridium* sp. FGH; 99 %
JL1_H	JQ271585	Sucrose	*Clostridia, Clostridiaceae, Clostridium beijerinckii* strain: HU-2; 99 %
JL2_H	JQ271586	Sucrose	*Clostridia, Clostridiaceae*, *Clostridium beijerinckii* strain: HU-2; 100 %

implications, highlighting the presence and importance of the nitrogen cycle in ARD-related sediments.

Hydrogen production

Because hydrogen is released as a consequence of different anaerobic metabolisms, four of the selected sampling sites (*JL*, *SN*, *BER* and *GAL*) were assayed for hydrogen production. All of the enrichments showed significant production of H_2 (300–400 mL/g chemical oxygen demand consumed). After plating, twelve colonies per sample were isolated from agar plates and analyzed by 16S rRNA gene amplification (Table 5). The 48 sequences, grouped into 9 OTUs, clustered in the *Firmicutes* phylum. Except one isolate belonging to the *Rummelibacillus* genus (*Bacilli/Bacillales incertae sedis*), the rest of the isolates were affiliated to the *Clostridium* genus (*Clostridia/Clostridiaceae*).

Hydrogen can be produced by anaerobic bacteria, including obligate, facultative and photosynthetic strains (Nandi and Sengupta 1998). This is often done through dark or photo fermentation. Dark fermentation, also called acid fermentation is the conversion of organic substrates to H_2 by anaerobic microorganisms, such as *Clostridium*, *Syntrophobacter* or *Enterobacter*. *Clostridium* species are the most well-known microorganisms involved in H_2 production by acid fermentation (Ueno et al. 2001; Hu and Chen 2007; Wang et al. 2007). They usually ferment sugars or proteins to acids (acetate, propionate, butyrate, lactate), alcohols (ethanol), carbon dioxide and molecular hydrogen.

In previous studies of the boreholes in the Tinto River origin (Amils et al. 2008), the fluid extracted from those underground habitats contained dissolved gases, such as H_2, N_2O, and CH_4. The chemical origin of H_2 was proposed to be derived from a water–rock interaction, but the identification of hydrogen-producing bacteria, such as *Syntrophobacter* spp. and *Clostridium* spp., in the previous molecular ecology study of Tinto River sediments (Sánchez-Andrea et al. 2011) suggested the possibility of a biological production of this gas. This study confirms the bioproduction of hydrogen through culture-dependent techniques, showing that *Clostridium* spp. are able to survive and grow in the tested conditions. Further characterization of the hydrogen producer isolates showed growth at pH 4.5 (Rajhi et al. 2012). The hydrogen produced by these organisms is a valuable substrate, serving as electron donors for numerous activities such as denitrification, sulfate reduction, and methanogenesis while avoiding the toxicity associated with organic acids at low pHs (Ghose and Wikén 1955).

Environmental and biotechnological implications

The main goal of this study was to prove the existence of functional anaerobic activities previously inferred by molecular ecology methods (Sánchez-Andrea et al. 2011) and to identify the responsible microorganisms. We detected growth of methanogens, hydrogen producers, denitrifiers and sulfate reducers in enrichments using Tinto River sediments as inocula. Additionally, this study confirms the bioproduction of H_2, CH_4 and N_2O, some of the gases detected in a borehole of a drill performed in the sulfidic deposits of the Iberian Pyrite Belt, the source of the extreme conditions detected in the Tinto River (Amils et al. 2008).

In several studies performed with the Tinto River sediments, it has been shown that the extreme acidic and oxidant conditions of the water column are not detected in the sediments, which appear forming layers with different environmental conditions, probably as a result of microbial activities (Sánchez-Andrea et al. 2011; Sánchez-Andrea et al. 2012a, b), or in microniches (Sanz et al. 2011). Independent of the initial pH at which the different

activities were tested, an increase in the pH and a reduction in the redox potential were observed during incubation. It seems that even surrounded by extreme conditions, microorganisms related with these activities are able to start growing while creating their own niches to promote their optimal growth conditions.

Together with the ecological implications of the results shown in this study, many potential biotechnological applications arise. Methanogenesis is an important process in wastewater treatment, and methanogens with a tolerance for mildly acidic or metalliferous conditions could be very useful in remediating acidic metal-polluted waste streams. Biological processes using renewable sources for hydrogen production are more economical and environmental friendly than the physical–chemical processes presently used (Manish and Banerjee 2008; Show et al. 2011), so interest in biological hydrogen production is increasing. Additionally, SRB can be used for remediation of acidic water with high heavy metals content or radionuclides (Johnson and Hallberg 2005; Muyzer and Stams 2008; Valls and Lorenzo 2002; Klonowska et al. 2008; Sánchez-Andrea et al. 2012b).

Acknowledgments This research was supported by the Spanish "Ministerio de Ciencia e Innovación" Grant CTM2009-10521 to J.L. Sanz and Grant CGL2009-11059 to R. Amils. Irene Sánchez-Andrea is a pre-doctoral fellow supported by the same Ministerio.

References

Aguilera A, Manrubia SC, Gomez F, Rodriguez N, Amils R (2006) Eukaryotic community distribution and its relationship to water physicochemical parameters in an extreme acidic environment, Rio Tinto (Southwestern Spain). Appl Environ Microbiol 72:5325–5330

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

Aguilera A, Zettler E, Gómez F, Amaral-Zettler L, Rodríguez N, Amils R (2007b) Distribution and seasonal variability in the benthic eukaryotic community of Río Tinto (SW, Spain), an acidic, high metal extreme environment. Syst Appl Microbiol 30:531–546

Amils R, Fernández-Remolar D, Gómez F, González-Toril E, Rodríguez N, Briones C, Prieto-Ballesteros O, Sanz JL, Díaz E, Stevens TO (2008) Subsurface geomicrobiology of the Iberian Pyritic Belt. In: Dion P, Nautiyal CS (eds) Soil biology: microbiology of extreme soils, vol 13. Springer, Berlin, pp 205–223

Asakawa S, Morii H, Akagawa-Matsushita M, Koga Y, Hayano K (1993) Characterization of *Methanobrevibacter arboriphilicus* SA isolated from a paddy field soil and DNA-DNA hybridization among *M. arboriphilicus* strains. Int J Syst Bacteriol 43:683–686

Baeseman JL, Smith RL, Silverstein J (2006) Denitrification potential in stream sediments impacted by acid mine drainage: effects of pH, various electron donors, and iron. Microb Ecol 51:232–241

Banda RMH, Dance IG, Bailey TD, Craig DC, Scudder ML (1989) Cadmium polysulfide complexes, [Cd (Sx)(Sy)] 2-: syntheses, crystal and molecular structures, and cadmium-113 NMR studies. Inorg Chem 28:1862–1871

Bijmans MFM, de Vries E, Yang CH, Buisman CJN, Lens PNL, Dopson M (2010) Sulfate reduction at pH 4.0 for treatment of process and wastewaters. Biotechnol Prog 26:1029–1039

Blösl M, Conrad R (1992) Influence of an increased pH on the composition of the nitrate-reducing microbial populations in an anaerobically incubated acidic forest soil. Syst Appl Microbiol 15:624–627

Blothe M, Akob DM, Kostka JE, Goschel K, Drake HL, Kusel K (2008) pH gradient-induced heterogeneity of Fe(III)-reducing microorganisms in coal mining-associated lake sediments. Appl Environ Microbiol 74:1019–1029

Bräuer SL, Yavitt JB, Zinder SH (2004) Methanogenesis in McLean Bog, an acidic peat bog in upstate New York: stimulation by H_2/CO_2 in the presence of rifampicin, or by low concentrations of acetate. Geomicrobiol J 21:433–443

Burggraf S, Huber H, Stetter K (1997) Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. Int J Syst Bacteriol 47:657–660

Cadillo-Quiroz H, Yashiro E, Yavitt JB, Zinder SH (2008) Characterization of the archaeal community in a minerotrophic fen and terminal restriction fragment length polymorphism-directed isolation of a novel hydrogenotrophic methanogen. Appl Environ Microbiol 74:2059–2068

Campbell LL, Postgate JR (1965) Classification of the spore-forming sulfate-reducing bacteria. Microbiol Mol Biol Rev 29:359–362

Church CD, Wilkin RT, Alpers CN, Rye RO, McCleskey RB (2007) Microbial sulfate reduction and metal attenuation in pH 4 acid mine water. Geochem Trans 8:10

Dolfing J, Xu A, Head IM (2010) Anomalous energy yields in thermodynamic calculations: importance of accounting for pH-dependent organic acid speciation. The ISME Journal 4:463–464

Dunfield P, Dumont R, Moore TR (1993) Methane production and consumption in temperate and subarctic peat soils: response to temperature and pH. Soil Biol Biochem 25:321–326

Fernández-Remolar DC, Prieto-Ballesteros O, Rodríguez N, Gómez F, Amils R, Gómez-Elvira J, Stoker CR (2008) Underground habitats in the Río Tinto Basin: a model for subsurface life habitats on Mars. Astrobiology 8:1023–1047

Florencio L, Nozhevnikova A, Van Langerak A, Stams AJM, Field JA, Lettinga G (1993) Acidophilic degradation of methanol by a methanogenic enrichment culture. FEMS Microbiol Lett 109:1–6

García-Moyano A, González-Toril E, Aguilera A, Amils R (2007) Prokaryotic community composition and ecology of floating macroscopic filaments from an extreme acidic environment, Río Tinto (SW, Spain). Syst Appl Microbiol 30:601–614

Ghose TK, Wikén T (1955) Inhibition of bacterial sulphate-reduction in presence of short chain fatty acids. Physiol Plantarum 8:116–135

Gonzalez-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

Goodwin S, Zeikus JG (1987) Ecophysiological adaptations of anaerobic bacteria to low pH: analysis of anaerobic digestion in acidic bog sediments. Appl Environ Microbiol 53:57–64

Hao C, Zhang H, Haas R, Bai Z, Zhang B (2007) A novel community of acidophiles in an acid mine drainage sediment. World J Microbiol Biotechnol 23:15–21

Horn MA, Matthies C, Kusel K, Schramm A, Drake HL (2003) Hydrogenotrophic methanogenesis by moderately acid-tolerant methanogens of a methane-emitting acidic peat. Appl Environ Microbiol 69:74–83

Hu B, Chen S (2007) Pretreatment of methanogenic granules for immobilized hydrogen fermentation. Int J Hydrogen Energy 32:3266–3273

Huang LN, Chen YQ, Zhou H, Luo S, Lan CY, Qu LH (2003) Characterization of methanogenic Archaea in the leachate of a closed municipal solid waste landfill. FEMS Microbiol Ecol 46:171–177

Johnson DB, Hallberg KB (2005) Acid mine drainage remediation options: a review. Sci Total Environ 338:3–14

Klonowska A, Clark M, Thieman S, Giles B, Wall J, Fields M (2008) Hexavalent chromium reduction in *Desulfovibrio vulgaris Hildenborough* causes transitory inhibition of sulfate reduction and cell growth. Appl Microbiol Biotechnol 78:1007–1016

Koschorreck M (2008) Microbial sulphate reduction at a low pH. FEMS Microbiol Ecol 64:329–342

Koschorreck M, Wendt-Potthoff K, Scharf B, Richnow HH (2008) Methanogenesis in the sediment of the acidic Lake Caviahue in Argentina. J Volcanol Geotherm Res 178:197–204

Lin B, Hyacinthe C, Bonneville S, Braster M, Van Cappellen P, Röling WFM (2007) Phylogenetic and physiological diversity of dissimilatory ferric iron reducers in sediments of the polluted Scheldt estuary, Northwest Europe. Environ Microbiol 9:1956–1968

Lopez-Archilla AI, Marin I, Amils R (2001) Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. Microb Ecol 41:20–35

López-Archilla AI, González AE, Terrón MC, Amils R (2005) Diversity and ecological relationships of the fungal populations of an acidic river of Southwestern Spain: the Tinto River. Can J Microbiol 50:923–934

Lu S, Gischkat S, Reiche M, Akob DM, Hallberg KB, Kusel K (2010) Ecophysiology of Fe-cycling bacteria in acidic sediments. Appl Environ Microbiol 76:8174–8183

Maestrojuán GM, Boone DR (1991) Characterization of *Methanosarcina barkeri* MST and 227, *Methanosarcina mazei* S-6T, and *Methanosarcina vacuolata* Z-761T. Int J Syst Bacteriol 41:267–274

Manaia CM, Costa MS (1991) Characterization of halotolerant *Thermus* isolates from shallow marine hot springs on S. Miguel, Azores. J Gen Microbiol 137:2643–2648

Manish S, Banerjee R (2008) Comparison of biohydrogen production processes. Int J Hydrogen Energy 33:279–286

Mateju V, Cizinska S, Krejci J, Janoch T (1992) Biological water denitrification—a review. Enzyme Microb Technol 14:170–183

Muyzer G, Ramsing NB (1995) Molecular methods to study the organization of microbial communities. Water Sci Technol 32:1–9

Muyzer G, Stams AJM (2008) The ecology and biotechnology of sulphate-reducing bacteria. Nat Rev Microbiol 6:441–454

Nagele W, Conrad R (1990) Influence of soil pH on the nitrate-reducing microbial populations and their potential to reduce nitrate to NO and N_2O. FEMS Microbiol Lett 74:49–57

Nandi R, Sengupta S (1998) Microbial production of hydrogen: an overview. Crit Rev Microbiol 24:61–84

Rajhi H, Conthe M, Puyol D, Díaz E, Sanz JL (2012) Dark fermentation: Isolation and characterization of hydrogen-producing strains from different sludges. Bioresource Technol (submitted)

Rea S, Bowman JP, Popovski S, Pimm C, Wright ADG (2007) *Methanobrevibacter millerae* sp. nov. and *Methanobrevibacter olleyae* sp. nov., methanogens from the ovine and bovine rumen that can utilize formate for growth. Int J Syst Evol Microbiol 57:450–456

Sakai S, Imachi H, Sekiguchi Y, Ohashi A, Harada H, Kamagata Y (2007) Isolation of key methanogens for global methane emission from rice paddy fields: a novel isolate affiliated with the clone cluster rice cluster I. Appl Environ Microbiol 73:4326–4331

Sánchez I, Fernández N, Amils R, Sanz JL (2008) Assessment of the addition of "*Thiobacillus denitrificans*" and "*Thiomicrospira denitrificans*" to chemolithoautotrophic denitrifying bioreactors. Int Microbiol 11:179–184

Sánchez-Andrea I, Rodriguez N, Amils R, Sanz JL (2011) Microbial diversity in anaerobic sediments at Rio Tinto, a naturally acidic environment with a high heavy metal content. Appl Environ Microbiol 77:6085–6093

Sánchez-Andrea I, Knittel K, Amann R, Amils R, Sanz JL (2012a) Quantification of Tinto River sediment microbial communities: the importance of sulfate-reducing bacteria and their role in attenuating acid mine drainage. Appl Environ Microbiol 78(13):4638–4645

Sánchez-Andrea I, Triana D, Sanz JL (2012b) Bioremediation of acid mine drainage coupled with domestic wastewater treatment. Water Sci Technol (in press)

Sanz JL, Rodriguez N, Amils R (1997) Effect of chlorinated aliphatic hydrocarbons on the acetoclastic methanogenic activity of granular sludge. Appl Microbiol Biotechnol 47:324–328

Sanz JL, Rodríguez N, Díaz EE, Amils R (2011) Methanogenesis in the sediments of Rio Tinto, an extreme acidic river. Environ Microbiol 13:2336–2341

Show K, Lee D, Chang J (2011) Bioreactor and process design for biohydrogen production. Biores Technol 102:8524–8533

Simek M, Jisova L, Hopkins DW (2002) What is the so-called optimum pH for denitrification in soil. Soil Biol Biochem 34:1227–1234

Stahl DA, Amann R (1991) Development and application of nucleic acid probes. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 205–228

Staley BF, de los Reyes FL III, Barlaz MA (2011) Effect of spatial differences in microbial activity, pH, and substrate levels on methanogenesis initiation in refuse. Appl Environ Microbiol 77:2381–2391

Ueno Y, Haruta S, Ishii M, Igarashi Y (2001) Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbiol Biotechnol 57:555–562

Valls M, Lorenzo V (2002) Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol Rev 26:327–338

Verbaendert I, De Vos P, Boon N, Heylen K (2011) Denitrification in Gram-positive bacteria: an underexplored trait. Biochem Soc Trans 39:254–258

Villemur R, Lanthier M, Beaudet R, Lépine F (2006) The *Desulfitobacterium* genus. FEMS Microbiol Rev 30:706–733

Wang X, Hoefel D, Saint CP, Monis PT, Jin B (2007) The isolation and microbial community analysis of hydrogen producing bacteria from activated sludge. J Appl Microbiol 103:1415–1423

Widdel F (1992) The genus Desulfotomaculum. The prokaryotes 2:1792–1799

Williams RT, Crawford RL (1985) Methanogenic bacteria, including an acid-tolerant strain, from peatlands. Appl Environ Microbiol 50:1542–1544

Zettler LAA, Gómez F, Zettler E, Keenan BG, Amils R, Sogin ML (2002) Microbiology: eukaryotic diversity in Spain's River of Fire. Nature 417:137